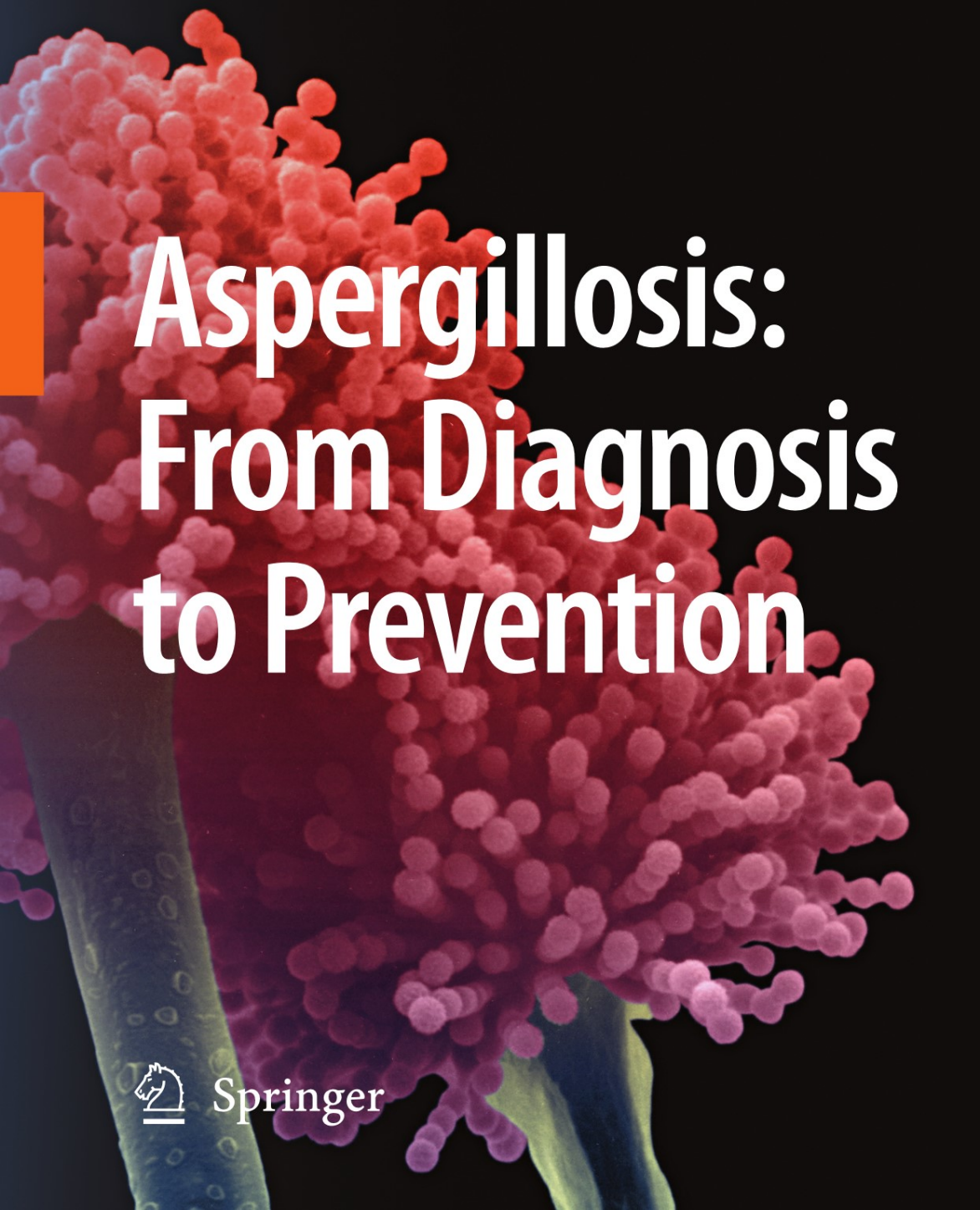
Alessandro C. Pasqualotto
Editor

Aspergillosis: From Diagnosis to Prevention



Springer

Aspergillosis: From Diagnosis to Prevention

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Editor
Prof. Alessandro C. Pasqualotto, MD PhD
Santa Casa – Complexo Hospitalar and Universidade Federal do Rio Grande do Sul (UFRGS)
Molecular Diagnostics Laboratory
Hospital Dom Vicente Scherer, 8th floor
Av Independência 155
Porto Alegre, 90035-075, Brazil
acpasqualotto@hotmail.com; pasqualotto@santacasa.tche.br

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Cover illustration: Front cover image shows *Aspergillus* conidiophores (fruiting bodies) and conidia (asexual spores). Front cover image copyright Dennis Kunkel Microscopy, Inc.

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Preface

The number of publications in recent years showing the importance of *Aspergillus* species as emergent pathogens is massive. In fact, aspergillosis has become so significant that even conferences and books are now dedicated to this subject. But the story was not always like that. Aspergillosis has only been recognised in the last ~220 years and no *Aspergillus*-related disease was known before that. Which means that *Aspergillus* and humans were apparently coexisting in peace (at least from the human perspective) for about 2 million years. So what has changed?

The answer probably relies on advanced medical care, with has resulted in prolonged survival for previously devastating diseases at the cost of increased immunossupression. Absolute avoidance of exposure to these airborne fungi is not something realistic, since *Aspergillus* conidia are widely dispersed in the environment and can easily reach human lungs and/or paranasal sinuses. Despite that, most humans will never get aspergillosis, and the interaction between fungi and host defence cells is an area of intense research.

This is not a book about a fungus. Instead, this project aims to reveal what lies beneath this big umbrella named "aspergillosis", a generic term that groups extraordinary diverse clinical conditions. For instance, invasive aspergillosis typically affects severely immunocompromised patients such as those with prolonged neutropenia or receiving therapy with steroids. On the other pole of the disease spectrum sits allergic forms of aspergillosis, with exuberant clinical syndromes that mostly affect patients with asthma and cystic fibrosis. Slowly destructive lung diseases may also occur in the apparently immunocompentent host, especially in the context of previous damage to the lung architecture. These are difficult-to-diagnose infections, and diagnostic tests perform differently depending on the specific clinical scenario.

This book summarizes the current knowledge about aspergillosis, covering important aspects of its epidemiology, pathogenesis, clinical manifestations, diagnosis, treatment and prevention. Editing this book was not an easy task and I would like to thank Max Haring at Springer for giving me this duty – his expertise was of great importance during the whole process. I believe some of the existing overlap amongst chapters was nearly impossible to avoid, since most *Aspergillus*-related syndromes do overlap. Retrospectively looking I have realised that many more topics could have been added to the book but at some stage the project had to be finalised. I am

glad to announce that we have succeeded in obtaining collaboration from authors from all over the globe, including most world-leaders in the *Aspergillus* field. If this project deserves any merit, that should be given to authors, who did their best to expressed their knowledge in written words. Thank you for believing in this book and for investing your precious time on this.

A few additional acknowledgements are needed. I am in debt with Luiz Carlos Severo who supervised my PhD thesis on *Candida* bloodstream infections – he first opened my eyes to the fantastic fungal world. Severo also convinced me that I should go to England and work with David Denning, a brilliant and enthusiastic scientist who masters *Aspergillus* diseases. David has introduced me to aspergillosis and its many faces, and also to many scientists working on the field. He has given continuous support to my work and I guess I am very lucky for that. David is an example on how to join people for a common purpose. And finally to Daniela, my beloved wife and best friend who just gave birth to our little daughter Clara – I could not do this work without their support and affection.

We all hope that the information here presented adds for a better understanding of aspergillosis, ultimately reducing the elevated morbidity and mortality associated with these complex diseases.

Porto Alegre, Brazil Alessandro C. Pasqualotto

Contents

Contributors

Ritesh Agarwal Department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India, riteshpgi@gmail.com

Kazuo Akiyama Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan, k-akiyama@sagamihara-hosp.gr.jp

Elias Anaissie University of Arkansas for Medical Sciences, Little Rock, AR, USA, AnaissieEliasJ@uams.edu

Javier Araiza Dermatology Service & Mycology Department, General Hospital of México DO, Mexico, Mexico, javier_arai2a5@yahoo.com.mx

Gerard Babatasi Department of Cardiothoracic Surgery University Hospital, Caen Cote de Nacre, France, babatasi-g@chu-caen.fr

S. Arunmozhi Balajee Mycotic Diseases Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, fir3@cdc.gov

Rosemary A. Barnes Department of Medical Microbiology, University Hospital of Wales, Cardiff, Wales, UK, barnesra@cardiff.ac.uk

Richard C. Barton Department of Microbiology, Mycology Reference Centre, Leeds Teaching Hospitals Trust, Leeds, UK, Richard.Barton@leedsth.nhs.uk

Ronen Ben-Ami Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA, rbenami@mdanderson.org

Josep Bisbe Internal Medicine Service, Hospital Sant Jaume, Olot, Girona, Spain, jbisbe@hospiolot.com

Alexandro Bonifaz Dermatology Service & Mycology Department, Hospital General de México OD, México, Mexico, a_bonifaz@yahoo.com.mx

Andrew M. Borman Mycology Reference Laboratory, Health Protection Agency, Bristol, UK, andy.borman@UHBristol.nhs.uk

David Bracco Department of Anesthesiology, CHUM Hotel Dieu, Montreal, QC, Canada

Arunaloke Chakrabarti Division of Medical Mycology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, arunaloke@hotmail.com

Shivsekhar Chatterjee Division of Infectious Diseases, Harper University Hospital, Detroit, MI, USA

Rogelio Chavolla-Magaña General Hospital of México DO, Mexico, Mexico, rchavo@hotmail.com

Arnaldo Lopes Colombo Special Mycology Laboratory, Division of Infectious Diseases, Federal University of São Paulo (UNIFESP), São Paulo, Brazil, colomboal@terra.com.br

Oliver A. Cornely Department of Internal Medicine, University of Cologne, Köln 50924, Germany, oliver.cornely@ctuc.de

Reto Crameri Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland, crameri@siaf.uzh.ch

Manuel Cuenca-Estrella Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain, mcuenca-estrella@isciii.es

Eszter Deak Mycotic Diseases Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, gufo@cdc.gov

David W. Denning National Aspergillosis Centre; Education and Research Centre, University Hospital of South Manchester (Wythenshawe Hospital), Manchester, UK, ddenning@manchester.ac.uk

J. Peter Donnelly Department of Haematology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, p.donnelly@usa.net

John Dotis Third Department of Pediatrics, Aristotle University, Thessaloniki, Greece, yandot@med.auth.gr

Philippe Eggimann Department of Intensive Care Medicine, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland, philippe.eggimann@chuv.ch

Martin C. Freund Deptartment of Radiology, Innsbruck Medical University, Innsbruck, Austria, Martin.freund@i-med.ac.at

F. Ghinelli Department Infectious Diseases St. Anna Hospital and University, Ferrara, Italy

Peter Gibson Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, University of Newcastle, New Lambton, NSW, Australia, Gibson@hnehealth.nsw.gov.au

Sandra K. Gilley Pulmonary/Critical Care Fellows, Medical University of South Carolina, Charleston, South Carolina, USA, gilley@musc.edu

A. G. Glaser Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

Patricio Godoy Division of Microbiology, Austral University of Chile, Valdivia, Chile, pgodoymartine2@gmail.com

Luciano Z. Goldani Section of Infectious Diseases, Hospital das Clinicas de Porto Alegre, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil, lgoldani@ufrgs.br

Mark R. Goldblatt Pulmonary/Critical Care Fellows, Medical University of South Carolina, Charleston, South Carolina, USA, goldbla@musc.edu

Andreas H. Groll Infectious Disease Research Program, Center for Bone Marrow Transplantation and Department of Pediatric Hematology/Oncology, University Children's Hospital, Muenster, Germany, grollan@ukmuenster.de

Josep Guarro Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Tarragona, Spain, josep.guarro@urv.cat

Luciana Silva Guazzelli Post-graduation Program in Pulmonary Sciences, Universidade Federal do Rio Grande do Sul (UFRGS), Mycology laboratory, Santa Casa-Complexo Hospitalar, Porto Alegre, Brazil, lucgua22elli@hotmail.com

William W. Hope Manchester Academic Health Science Centre, Regional Mycology Laboratory Manchester University Hospital of South Manchester Education and Research Centre, Southmoor Road, Manchester M23 9PT, Manchester, UK, william.hope@manchester.ac.uk

Susan J. Howard Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK, susan.j.howard@manchester.ac.uk

Jack Hsu Division of Hematology/Oncology, University of Florida College of Medicine, Gainesville, FL, USA, jack.hsu@medicine.ufl.edu

Shahid Husain Transplant Infectious Diseases, Division of Infectious Diseases and Multi-organ Transplantation, University Health Network/University of Toronto, Toronto, ON, Canada, shahid.husain@uhn.on.ca

Elizabeth M. Johnson Mycology Reference Laboratory, Health Protection Agency, Bristol, UK, elizabeth.johnson@UHBristol.nhs.uk

Carol A. Kauffman Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Medical School, Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, MI, USA, ckauff@umich.edu

Corné H.W. Klaassen Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands, c.klaassen@cwz.nl

Dimitrios P. Kontoyiannis Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA, dkontoyi@mdanderson.org

Richard Kradin Infectious Disease Pathology, Departments of Pathology and Pulmonary/Critical Care Medicine, Massachusetts General Hospital, Boston, MA, USA, rkradin@partners.org

Philippe Lagacé-Wiens Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada, rubinste@cc.umanitoba.ca

Katrien Lagrou Department of Medical Diagnostic Sciences, University Hospitals Leuven, Leuven, Belgium, katrien.lagrou@u2.kuleuven.be

Cornelia Lass-Flörl Divison of Hygiene & Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria, cornelia lass-floerl@i-med.ac.at

Russell E. Lewis University of Houston College of Pharmacy and, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA, rlewis@uh.edu

M. Libanore Department Infectious Diseases St. Anna Hospital and University, Ferrara, Italy, m.libanore@ospfe.it

Johan Maertens Department of Haematology, University Hospitals Leuven, Leuven, Belgium, johan.maertens@uz.kuleuven.be

Ana Luiza Maia Endocrinology, Hospital das Clinicas de Porto Alegre, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil, almaia@ufrgs.br

Bradley F. Marple Department of Otolaryngology, University of Texas Southwestern Medical Center, Dallas, TX, USA, Bradley.Marple@UTSouthwestern.edu

Kieren A. Marr School of Medicine, Johns Hopkins University, Baltimore, MD, USA, kmarr@fhcrc.org

Wouter Meersseman Department of General Internal Medicine, Gasthuisberg University Hospital, Leuven, Belgium, wouter.meersseman@uzleuven.be

Jose M. Miro Infectious Diseases Service, Hospital Clinic – IDIBAPS, University of Barcelona, Barcelona, Spain, jmmiro@ub.edu

Johan W. Mouton Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Ziekenhuis Nijmegen, The Netherlands, Mouton@cwz.nl

Hashmatullah Nawabi French Medical Institute for Children, Surgeons of Hope Foundation, Kabul, Afghanistan

Ricardo Negroni Hospital Francisco Javier Muniz, Buenos Aires, Argentinas, ricardonegroni@intramed.net

Nelson P. Nicolasora Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Medical School, Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, MI, USA, Nelson_nicolasora@yahoo.com

Robert Niven Wythenshawe Hospital, Manchester, UK, robert.niver@smuht.nwest.nhs.uk; Robert.Niven@uhsm.nhs.uk

Marcio Nucci University Hospital, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, mnucci@hucff.ufrj.br

Flávio de Mattos Oliveira Mycology laboratory, Santa Casa-Complexo Hospitalar, Porto Alegre, Brazil, flavio@santacasa.tche.br

Alessandro C. Pasqualotto Post-graduation Program in Pulmonary Sciences, Universidade Federal do Rio Grande do Sul (UFRGS), Molecular Diagnostics Laboratory, Santa Casa-Complexo Hospitalar, Porto Alegre, Brazil, acpasqualotto@hotmail.com

Thomas F. Patterson Division of Infectious Diseases, The Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA, patterson@uthscsa.edu

David S. Perlin Public Health Research Institute, UMDNJ-New Jersey Medical School, Newark, NJ, USA, perlinds@umdnj.edu

Didier Pittet Infection Control Program, Department of Internal Medicine, University of Geneva Hospitals and Faculty of Medicine, Geneva, Switzerland, didier.pittet@hcuge.ch

Curig Prys-Picard PACC Division, The University of Texas Medical Branch, Galveston, TX, USA, copryspi@utmb.edu

Bishan D. Radotra Department of Histopathology, Postgraduate Institute of Medical Education & Research, Chandigarh, India

Viviane Reis Special Mycology Laboratory, Division of Infectious Diseases, Federal University of São Paulo (UNIFESP), São Paulo, Brazil, vi2017@yahoo.com

C. Rhyner Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

Juan Luis Rodriguez-Tudela Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain, juanl.rodriguez-tudela@isciii.es

Emmanuel Roilides Third Department of Pediatrics, Aristotle University, Hippokration Hospital, Thessaloniki, Greece, roilides@med.auth.gr

Luigina Romani Microbiology Section, Department of Experimental Medicine and Biochemical, University of Perugia, Via del Giochetto, Perugia, Italy, lromani@unipg.it

Ethan Rubinstein Section of Infectious Diseases Departments of Medicine and Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada, erubins@yahoo.com; rubinste@cc.umanitoba.ca

Maria J.G.T. Rüping Department of Internal Medicine, University of Cologne, Köln 50924, Germany, maria.rueping@ctuc.de

Matthew W. Ryan Department of Otolaryngology, University of Texas Southwestern Medical Center, Dallas, TX, USA, matthew.ryan@utsouthwestern.edu

Helen Sambatakou Second Department of Internal Medicine and Infectious Diseases Unit, Hippokration General Hospital, The University of Athens, 11527, Athens, Greece, helensambatakou@msn.com

Stefan Schwartz Medizinische Klinik III, Universitätsmedizin Berlin, Berlin, Germany, stefan.schwartz@charite.de

Brahm H. Segal Division of Infectious Diseases, Roswell Park Cancer Institute; The State University of New York (SUNY), Buffalo, NY, USA, brahm.segal@roswellpark.org

Luiz Carlos Severo Post-graduation Program in Pulmonary Sciences, Universidade Federal do Rio Grande do Sul (UFRGS), Mycology laboratory, Santa Casa-Complexo Hospitalar, Porto Alegre, Brazil, Brazilsevero@santacasa.tche.br

Ashok Shah Department of Respiratory Medicine, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India, ashokshah99@yahoo.com

L. Sighinolfi Department Infectious Diseases St. Anna Hospital and University, Ferrara, Italy

Fernanda P. Silveira Division of Infectious Diseases, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

G. Sivasubramanian Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, MI, USA, gsivasub@med.wayne.edu

Jack D. Sobel Division of Infectious Diseases, Harper University Hospital, Detroit, MI, USA, jsobel@med.wayne.edu

Mambun Socheat Cardiothoracic Center, Phnom-Penh, Cambodia

Ayman O. Soubani Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Wayne State University School of Medicine, Detroit, MI, USA, asoubani@med.wayne.edu

Koen Theunissen Department of Haematology, Virga Jesse Hospital, Hasselt, Belgium, k-theunissen@virgajesse.be

Philip A. Thomas Department of Microbiology, Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirapalli, India, philipthomas@satyam.net.in

George R. Thompson Division of Infectious Diseases, The Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, USA, thompsong2@uthscsa.edu

Juan-Carles Trullas Internal Medicine Service, Hospital Sant Jaume, Olot Girona, Spain, jctv5153@comg.cat

Jörg J. Vehreschild Department of Internal Medicine, University of Cologne, Köln 50924, Germany, Janne.Vehreschild@ctuc.de

Paul E. Verweij Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; Nijmegen University Centre of Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, P.Verweij@mmb.umcn.nl

M. Vilhelmsson Department of Medicine, Clinical Allergy Research Unit, Karolinska Institutet and Karolinska University Hospital Solna, Stockholm, Sweden

Thomas J. Walsh Immunocompromised Host Section, Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA, walsht@mail.nih.gov

Peter A.B. Wark Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, University of Newcastle, New Lambton, NSW, Australia, peter.wark@hnehealth.nsw.gov.au

P. Lewis White NPHS Microbiology Cardiff, University Hospital of Wales, Cardiff, Wales, UK, lewis.white@nphs.wales.nhs.uk

John R. Wingard Division of Hematology/Oncology, University of Florida College of Medicine, Gainesville, FL, USA, wingajr@medicine.ufl.edu

Melissa Orzechowski Xavier Post-graduation Program in Pulmonary Sciences, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil, melissaxavier@ig.com.br

Minoru Yoshida Fourth Department of Internal Medicine, Teikyo University School of Medicine, Kawasaki city, Japan, myoshida@med.teikyo-u.ac.jp

S. Zeller Department of Medicine, Clinical Allergy Research Unit, Karolinska Institutet and Karolinska University Hospital Solna, Stockholm, Sweden

PART I
INTRODUCTION

Introduction

David W. Denning

The genus *Aspergillus* is extraordinary as exemplified by the diversity of its disease manifestations, almost all termed "aspergillosis". All forms of aspergillosis are sapronoses, that is transmissible form an abiotic environment, and not communicable from person to person, or zoonoses. The spectrum of aspergillosis can be broadly divided into 4 categories:

- Invasive life-threatening infection in immunocompromised patients;
- Subacute or chronic infection in patients with pre-existing pulmonary or sinus disease and probably some subtle defect in innate immunity;
- Allergic or eosinophilic disease which is manifest in many forms including allergic bronchopulmonary aspergillosis, eosinophilic rhinosinusitis and extrinsic allergic alveolitis;
- Locally invasive infection as a result of trauma or surgery, notably keratitis and post-operative infection.

No other infectious agent of man produces this remarkable diversity of infectious and allergic syndromes.

The first case of aspergillosis described was probably that of a fungus ball of the sinuses [1]. The first pulmonary case described was that of chronic cavitary pulmonary aspergillosis with an associated fungus ball [2], the same year as pleural aspergillosis was described [3]. *Aspergillus* otitis and keratitis were described soon thereafter [4, 5]. Various allergic manifestations were described by [6, 7]. At the end of the 19th century *Aspergillus* tracheobronchitis [8] and both maxillary and sphenoid sinusitis caused by *Aspergillus* [9, 10] were described for the first time. The first half of the 20th century saw numerous descriptions of asthma exacerbated by fungi, skin testing for *Aspergillus* and other fungi, occupational associations with allergic manifestations of aspergillosis, detailed histopathological descriptions of cerebral aspergillosis, *Aspergillus* osteomyelitis, granulomatous features, possibly

D.W. Denning (✉)
National Aspergillosis Centre, Education and Research Centre, University Hospital of South Manchester (Wythenshawe Hospital), Manchester M23 9LT, UK
e-mail: ddenning@manchester.ac.uk

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of chronic granulomatous disease before it was formally recognised and native valve endocarditis caused by *Aspergillus*. In 1952, the syndrome of allergic bronchopulmonary aspergillosis was described [11]. It was not until 1953 that the first case of invasive aspergillosis in the immunocompromised host was described, in a man who died with aplastic anaemia caused by chloramphenicol [12]. Numerous additional cases followed in association with the newly introduced corticosteroids or cytotoxic chemotherapy. In the 1960s *Aspergillus* antibody (precipitin) testing was found to be useful for diagnosing both fungus ball in the lung and allergic bronchopulmonary aspergillosis.

While amphotericin B deoxycholate and surgery have be used for treatment since the early 1960s, and 5-flucytosine a little in the 1970s and 1980s, it was not until the 1990s that treatment started to be studied and discussed in any depth. The introduction of itraconazole, the first effective oral therapy azole for aspergillosis, prompted much discussion and study regarding prophylaxis, primary therapy selection, therapeutic drug monitoring, oral switch therapy and definitions of disease. Multicentre and then randomised studies of therapy followed. Unfortunately the 1990s also saw the first reports of azole resistance, currently an increasing problem. The groundwork was laid in the 1990s for the major improvements in drug treatment – notably lipid-associated amphotericin B preparations, voriconazole and posaconazole and the echinocandins, caspofungin and micafungin.

The 1990s also saw the introduction of the *Aspergillus* antigen test, based on galactomannan detection, and the beta 1,3-D-glucan serological test, in Japan. Combined with greater and earlier clinical suspicion, rapid computed tomography scanning of the thorax, bronchoscopy, and more adventurous surgery in some centres, better outcomes started to be reported in leukaemia patients. Only in the 2000s have we seen the impact of *both* better diagnostics and treatments translate into lower mortality rates.

Alongside these highly significant developments, some illumination of the basic biology in the *Aspergillus* genus has been gathering pace. The availability of numerous *Aspergillus* genomes, each with 10,000–14,000 genes, has greatly facilitated improvements in our understanding of pathogenesis. The astonishing genetic diversity in the *Aspergillus* genus is truly remarkable, matching the evolutionary distance between fish and man. *Aspergilli* are capable of surviving, even thriving, in many environments including high temperature compost, high salinity environments such as the Dead Sea and of course the human lung, despite an array of immune defences. This versatility is the result of a life style of sophisticated adaptation, and provides a warning to us about any premature claims of the conquering of aspergillosis.

The improvements we have seen are welcome and necessary. But much remains to be done. Diagnosis of both allergy and invasive infection is still relatively rudimentary and slow; perhaps polymerase chain reaction (PCR)-based methods, and more refined panels of allergens, will see step changes in our diagnostic capabilities. Our understanding of pathogenesis is somewhat patchy – for example, we do not know why *Aspergillus* species disseminate to the brain and other organs. Combination therapy may or may not be useful for treatment. Those with chronic obstructive pulmonary disease and intensive care unit patients represent major challenges,

with current mortality rates of ~90%. Azole resistance is becoming a problem, and when the isolates are cross-resistant as they usually are, leaves us with no oral drugs. Prevention with oral azole prophylaxis is still the subject of much controversy.

This book is a bold attempt to summarise the current state-of-the-art in the prevention, diagnosis, and management of all forms of aspergillosis. The authorship is international, reflecting the global challenge that aspergillosis presents. I commend this book, with the expertise of the authors reflected on each page, to you.

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Differences and Similarities Amongst Pathogenic *Aspergillus* Species

Josep Guarro, Melissa Orzechowski Xavier, and Luiz Carlos Severo

Abstract Aspergillosis is a general term that refers to diseases caused by different *Aspergillus* species. These species all share a similar morphological structure, i.e. long specialized hyphae (conidiophores) that are expanded at the apical end from which the asexual propagules emerge. However, there are important differences amongst *Aspergillus* species regarding extrolites production and the morphology of both asexual (anamorph) and sexual (teleomorph) structures. *Aspergilli* can cause severe invasive infections in immunocompromised patients yet rarely infect immunocompetent people. Although most cases of aspergillosis are caused by *A. fumigatus*, many other species – some of them recently described by the use of molecular techniques – have been implicated. In the section *Fumigati,* recent multilocus sequence studies have shown that different cryptic species exist and that other species previously thought to be only saprobe can also be recovered from clinical specimens. A similar conclusion has been reached through the molecular examination of other *Aspergillus* sections. In general, the pathogenic species of *Aspergillus* do not follow an identical clinical behavioral pattern. Both the significant differences in the type of infections that they cause and their response to antifungal drugs emphasise the importance of accurate identification so as to develop effective therapeutic strategies, given that nowadays different therapeutic options are available.

Keywords Aspergillosis · *Aspergillus* spp · *Aspergillus flavus* · *Aspergillus fumigatus* · *Emericella* · *Neosartorya*

Contents

J. Guarro (✉)
Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Tarragona, Spain
e-mail: josep.guarro@urv.cat

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1 Introduction

The term aspergillosis refers to diseases caused by fungi belonging to the genus *Aspergillus*. *Aspergillus* species have emerged as important causes of life-threatening infections in immunocompromised patients. That may range from localised conditions to fatal disseminated infections. Disease may also result from an allergic reaction to inhaled spores. *Aspergillus* is a large genus of common contaminants containing more than 180 species, a fifth of which can be quite frequently recovered from humans and other animals. Most *Aspergillus* species of clinical importance are anamorphs (asexual states) related to the following teleomorph (sexual states) genera in the Ascomycota phylum: *Emericella, Eurotium, Fennellia, Hemicarpenteles, Neopetromyces, Neosartorya* and *Petromyces* [1]. *Aspergillus fumigatus* causes the majority of cases of aspergillosis, followed by *A. flavus, A. niger, A. terreus* and *A. nidulans*. In addition, other implicated species include *A. aculeatus, A. avenaceus, A. casiellus, A. candidus, A. carneus, A. clavato-nanicus, A. clavatus, A. conicus, A. deflectus, A. granulosus, A. janus, A. japonicus, A. lentulus, A. ochraceus, A. oryzae, A. protuberus, A. restrictus, A. sclerotiorum, A. sydowii, A. tamarii, A. tubingensis, A. unguis, A. calidoustus,* and *A. versicolor* [2, 3]. All the above-mentioned species usually show only the anamorph when they are grown in culture. However, some others such as *Neosartorya fischeri (A. fischeranus), N. pseudofischeri* (*A. thermomutatus), N. hiratsukae (A. hiratsukae), Emericella nidulans (A. nidulans), E. quadrilineata (A. tetrazonus), Fennellia flavipes (A. flavipes), F. nivea (A. niveus), Eurotium chevalieri (A. chevalieri), E. herbariorum (A. glaucus), E. amstelodami (A. hollandicus), E. repens (A. reptans)* and *Petromyces alliaceus (A. alliaceus)* usually develop the anamorph and the teleomorph together in the same culture. Recently, molecular studies have reported the occurrence of species in clinical samples, such as *N. fennelliae (A. fennelliae), N. spinosa (A. spinosus), N. coreana (A. coreanus), A. fumisynnematus, A. viridinutans, A. fumigatiaffinis* and *N. udagawae (A. udagawae)* [4, 5]. Regardless of the species, pathogenesis in the *Aspergillus* genus is multifactorial and rather complex. It depends on the morphological characteristics of the fungus,

as well as physiological and metabolic aspects. The purpose of this chapter is to discuss differences and similarities related to the main pathogenic *Aspergillus* species.

2 Morphology

Most species in the anamorph genus *Aspergillus* have non-melanised structures, although some unusual species may produce blackish conidia. When grown in culture, *Aspergillus* species form colonies that usually develop quickly to display different colours, which are in turn very useful for the phenotypic identification of some species. The hyphae of the vegetative mycelium are hyaline and septate. *Aspergilli* produce two types of propagules: the conidia, formed by mitosis, and the ascospores that are produced by meiosis. Asexual reproduction takes place in specialised structures, called conidiophores. These structures emerge from the vegetative mycelium as erect, aseptate, thick-walled and specialised hyphae that expand apically to form a vesicle (Fig. 1). On this vesicle numerous conidiogenous cells emerge synchronically. These are known as phialides. The phialides are flask-shaped elements whose interior successively produces conidia. The conidia are formed in interconnected chains, are strongly hydrophobic and generally hyaline or pale but in mass may be of different colours that are reproduced in the colonies. The phialides can be composed by a single element or formed by short branches (metulae). When metulae are formed, conidiogenous cells are called biseriate. In the absence of metulae, conidiogenous cells are considered uniseriate (Figs. 1 and 2). When grown in culture,

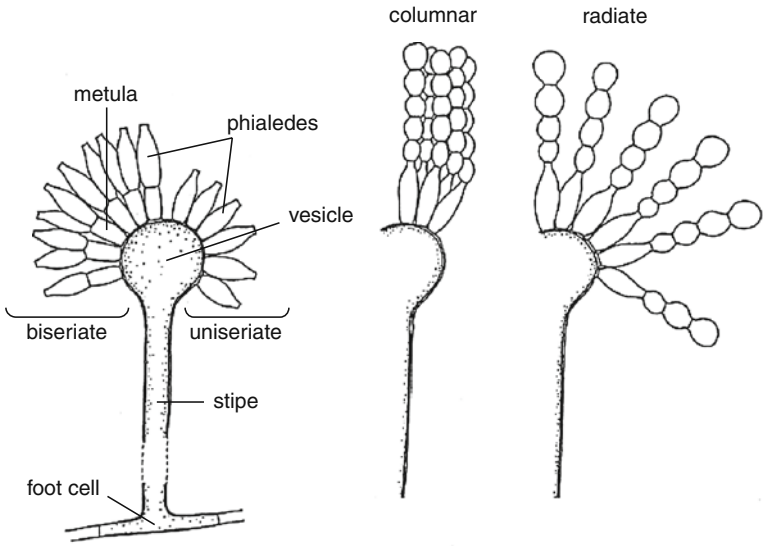

Fig. 1 Diagram of terminology used for the identification of *Aspergillus* species

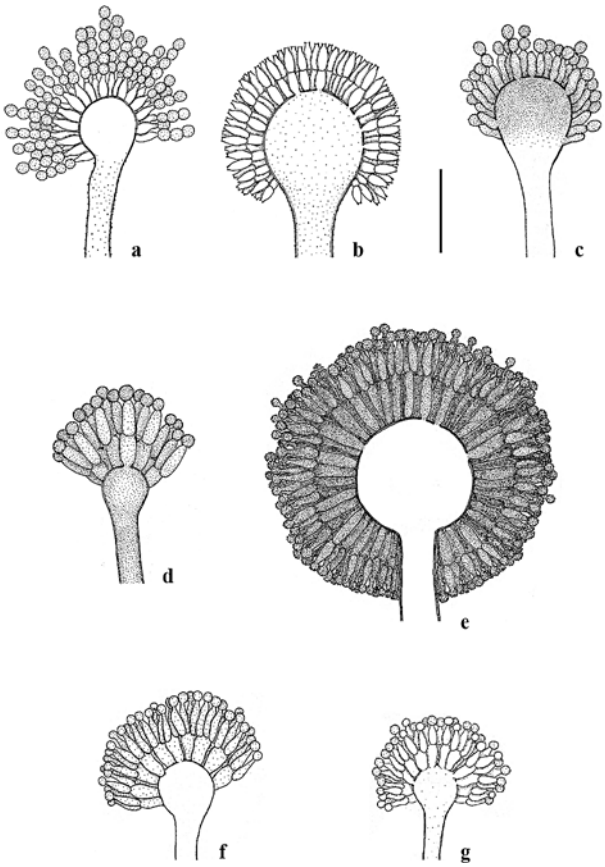

Fig. 2 Upper part of the conidiophores of species of *Aspergillus* of clinical interest. (**a**) *Aspergillug flavus* (conidiophore uniseriate); (**b**) *Aspergillug flavus* (conidiophore biseriate); (**c**) *Aspergillus fumigatus*; (**d**) *Aspergillus nidulans*; (**e**) *Aspergillus niger*; (**f**) *Aspergillus terreus*; (**g**) *Aspergillus ustus*. Bar = 25 μm

the teleomorphs of *Aspergillus* form the typical fruiting bodies of the ascomycetes, called ascomata. Ascomata are spherical structures containing globose asci usually with 8 ascospores. The ascospores are one-celled, subglobose to lenticular and have different types of ornamentation on their surface (Fig. 3). The main features that identify the most prevalent *Aspergillus* species causing human diseases are shown in Table 1 and Figs. 2 and 3. The most distinctive morphological features and useful dichotomic keys for the identification of most of the species of clinical interest and their teleomorphs are provided in the Hoog et al. [2], as well as elsewhere in this book.

The invasive form of aspergillosis is characterised by the presence in tissue of dichotomously branched, septate and hyaline hyphae. However, this pattern is not species-specific, and may be present in many other infections caused by other

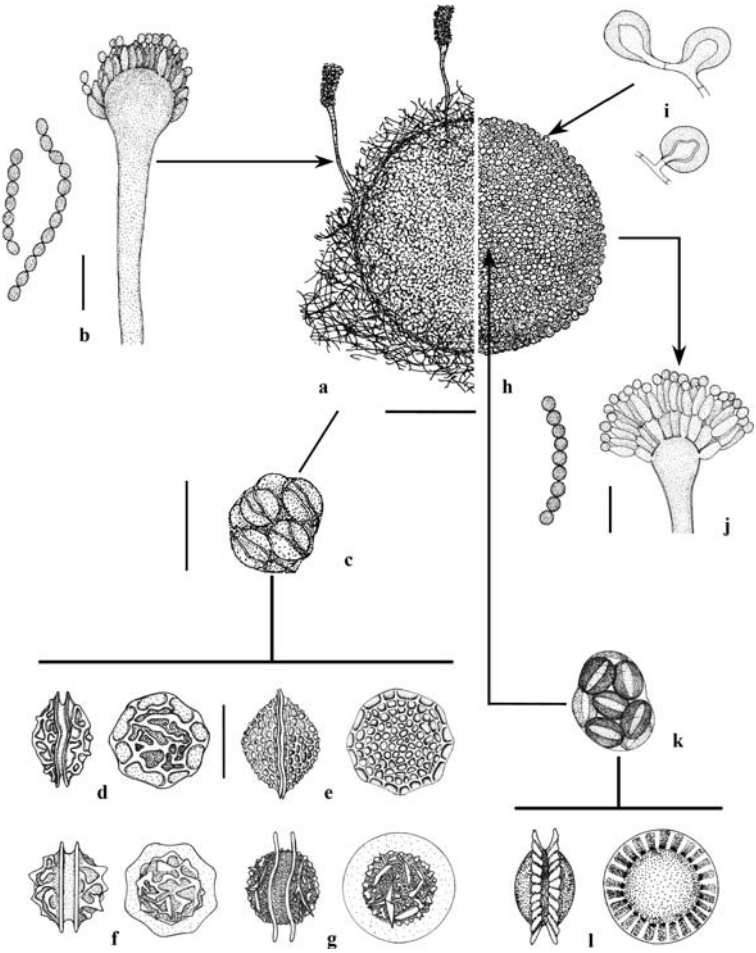

Fig. 3 *Neosartorya* spp. (**a**) part of an ascoma with conidiophores associated; (**b**) conidiophore and conidia; (**c**) ascus; (**d**) ascospore of *N. fischeri*; (**e**) ascospore of *N. hiratsukae*; (**f**) ascospore of *N. pseudofischeri*; (**g**) ascospore of *N. udagawae*; (**h**) *Emericella nidulans* – part of an ascoma; (**i**) Hülle cells; (**j**) conidiophore of *A. nidulans*; (**k**) ascus; (**l**) ascospore. Bars=200 μm (**a**, **h**); 10 μm (**b**, **c**, **j**, and **k**); 5 μm (**d** and **e**)

hyaline fungi. Nevertheless, *A. terreus* shows a very characteristic distinctive feature when grown in vivo; it displays small, globose and solitary conidia either directly attached to the hyphae or with a small pedicel, called aleurioconidia [1]. If these structures are detected on microscopic examination of tissue, on fine needle aspirates or on bronchoscopy specimens, a rapid presumptive identification of *A. terreus* is possible [6]. In one study, the metabolic activity and growth rate at 37°C of a clinical isolate of *A. terreus* were 2-fold higher than those of two soil isolates. In addition, the clinical isolate was >1,000-fold more virulent than the soil isolates in a

Table 1 Key distinctive morphological features of *Aspergillus* species of clinical interest

Species	Key distinctive features
Emericella nidulans (*A. nidulans*)	*Colonies*: greenish with yellow patches *Ascomata*: globose, yellow, surrounded by numerous Hülle cells *Ascospores*: saturn-shaped (with two crests), violet *Conidiophore*: biseriate *Metulae*: over the upper 3/4 of the vesicle *Stipe*: smooth, brown
Neosartorya spp.	*Colonies*: whitish to cream *Ascomata*: globose, white to cream *Ascospores*: lens-shaped to spherical, hyaline, with two equatorial crests, with different types of ornamentation on the valves *Conidiophore*: similar to that of *A. fumigatus*
Aspergillus fumigatus	*Colonies*: blue–green *Conidial chains*: forming compact upright columnar masses *Conidiophore*: uniseriate *Phialides*: over the upper 2/3 of the vesicle *Stipe*: smooth
Aspergillus flavus	*Colonies*: yellow-green to green *Conidial chains*: radial orientation *Conidiophores*: Biseriate and uniseriate *Metulae*: over the whole vesicle *Stipe*: granular wall *Conidial chains*: radiating or massing into few columns
Aspergillus niger	*Colonies:* dirty black *Conidial chains:* radiating or massing into few columns *Conidiophores*: biseriate *Metulae*: over the whole vesicle *Stipe*: smooth
Aspergillus terreus	*Colonies*: sandy brown *Conidial chains*: forming compact upright columnar masses *Conidiophores*: biseriate *Metulae*: over the upper one-half to 2/3 of the vesicle in nearly parallel arrangement *Stipe*: smooth
Aspergillus ustus	*Secondary conidia*: formed on the submerged mycelium, attached to hypha by short pegs *Colonies*: greyish-brown with a yellow-brown reverse *Conidial chains*: radiating or forming short columns *Conidiophores*: biseriate *Metulae*: over the 3/4 of the vesicle *Stipe*: smooth, brown *Hülle cells*: sinuous, occasionally present

murine model [7]. This was associated to the presence of the aleuroconidia produced by *A. terreus* in vivo.

There is general agreement that cultures are fundamental for the definitive diagnosis and species determination in patients with invasive fungal diseases [8]. The different species of *Aspergillus* generally grow well in ordinary mycological media.

The standard medium for species identification is Czapek agar at 25ºC. However, higher temperatures are also often used (30, 37, 45ºC), particularly for inducing teleomorphs [9]. Other strategies include the use of more concentrated culture media, like Czapek or MEA with 20% sucrose, 40% sucrose or 12% sucrose with 25% NaCl. The morphological differentiation of the teleomorphs is detected by observing the ornamentation of the surface of the ascospores (Fig. 3), which requires considerable expertise and the use of electron scanning microscopy.

The morphological characteristics of *Aspergillus* species can be important determinants of the site of infection. For instance, the small alveolar diameter limits the efficacy of *Aspergillus* conidia to cause invasive pulmonary aspergillosis (IPA). In this scenario, most conidia >3 μm will be expelled by the mucocilliary system therefore not reaching the terminal alveoli. Not coincidently, *A. fumigatus* has one of the lowest conidia in the genus (2.0–3.0 μm) and it is the main species causing IPA. *A. terreus* – another species with small conidia (2.0–2.5 μm) – has emerged as an important cause of invasive aspergillosis (IA) in some hospitals [10, 11]. On the contrary, in some cases of cutaneous aspergillosis, rhinosinusitis or chronic cavitary pulmonary aspergillosis, the route of entry may be sufficiently large to allow bigger conidia to cause the infection. In these patients, disease can be caused by *Aspergillus* species producing larger conidia, such as *A. niger* (3.5–4.5 μm) and *A. flavus* (3.0–6.0 μm) [12, 13]. In addition, strong *A. niger* conidia are connected by strong bridges, which keep conidia in chains increasing even more the size of the infecting particle. This phenomenon impairs *A. niger* dissemination by the air and penetration into the distal respiratory tract of the host.

The conidia surface also influences conidia ability to adhere to the host tissues. The external surface of *A. fumigatus, A. niger*, and *A. nidulans* conidia are composed by hydrophobic proteins, and physicochemical properties of *Aspergillus* conidia rely on their outer cell-wall rodlet layer. These proteins also confer resistance to extreme atmospheric conditions and facilitate airborne dispersion of *Aspergillus* conidia. One of these molecules has been identified as RodAp, a 16-KDa protein found on *A. fumigatus* and *A. nidulans* conidia. Since these proteins protect fungal propagules against phagocytosis by alveolar macrophages, they may be involved in virulence [14, 15].

Melanin is a dark brown to black pigment present in the cell wall of many fungi. This high molecular weight pigment is formed by the oxidative polymerization of phenolic or indolic compounds. Melanin synthesis has been linked to virulence in organisms such as *Cryptococcus neoformans* [16] and *Sporothrix schenckii* [17, 18]. This pigment seems to confer protection against environmental UV radiation. In addition, it seems to protect against phagocytosis in vitro and in vivo, by impairing conidia migration in intracellular acidified compartments. Melanin also seems to reduce opsonisation by "camouflaging" binding sites, which for instance may reduce the ability of the C3 component of complement system to bind conidia [19, 20]. Mutant albine strains have shown reduced virulence in comparison to wild type strains in models of experimental aspergillosis. Additionally, albine conidia are more susceptible to the oxidative mechanisms of monocytes and polymorphonuclear leukocytes [19, 21–23].

Differences in melanisation between *A. fumigatus* and *A. nidulans* were demonstrated by exposing these fungi to tricyclazole, a fungicidal inhibitor of the THN-reductase enzyme, which is involved in melanin synthesis via DHN-melanin pathway [19]. Exposure to tricyclazole resulted in inhibition of conidial pigmentation in *A. fumigatus* but not *A. nidulans*, showing that pigmentation involves different pathways in these species. Studying melanisation in *A. fumigatus* strains by immunofluorescence technique, Youngchim et al. [24] showed that anti-melanin antibodies avidly bind to *Aspergillus* conidia. The strength of this binding decreased with conidial germination to become null after hyphae formation. These data suggest that melanin may be more important as a facilitating factor for fungal survival in the external environment than as a virulence factor in the host.

3 Growth Rate and Thermotolerance

The majority of *Aspergillus* species show prompt in vitro growth when proper laboratorial conditions are applied. For instance, a 7-day culture on Czapek agar shows colonies of *A. fumigatus, A. flavus, A. niger, A. terreus* e *A. nidulans* with 45–60 mm, 55–65 mm, 40–62 mm, 30–48 mm and 38–43 mm in diameter, respectively [25]. The ability of *Aspergillus* to grow in host tissues is an important step in the pathogenesis of IA.

Araujo and Rodrigues [26] showed that germination rates at 37°C differed significantly for the most common pathogenic *Aspergillus* species. Using the same inoculum of *Aspergillus* spores in RPMI 1640 medium, *A. fumigatus* germinated faster than *A. flavus*, which in turn germinated faster than *A. niger*. Interesting results were also obtained when germination rate was evaluated at different temperatures. The percentage of germination markedly increased 3- to 10-fold for both *A. fumigatus* and *A. flavus* when temperature was increased from 20 to 30°C, and again 2- to 3-fold from 30 to 37°C. However at 41°C germination of *A. fumigatus* was still enhanced, whilst germination of A. flavus decreased by 45% (as compared with 37°C). The study shows that temperature influences growth of pathogenic *Aspergilli*, with *A. fumigatus* being the species most able to adapt to extreme changes in environmental conditions. Since phenotypic characterization has shown that both *A. fumigatus* and *Neosartorya fischeri* can grow at 42°C – with the latter rarely being identified as a human pathogen – *A. fumigatus* may possess other genetic determinants besides thermotolerance that allow it to establish a successful in vivo infection [27].

Accordingly, other *Aspergillus* species with low pathogenicity demonstrate an optimal temperature for growth of around 30°C, which is lower than the optimal temperature for *A. niger* [28, 29]. However, *A. niger* can show similar germination rates to *A. fumigatus* when exposed to a low pH (e.g., 3.5–4.5) [26]. Although not completely elucidated pH greatly influences the frequency of *A. niger* infections, as illustrated by the occurrence of aspergillosis in patients with metabolic acidosis. *A. niger* has been associated with severe infections in patients with diabetes mellitus, including kidney disease due to glomerular deposition of calcium oxalate crystals.

In the presence of glucose *A. niger* produce oxalic acid, which binds to calcium from the host to form crystals; consequently, the presence of crystals in host tissues suggests *A. niger* as the aetiology of the disease [12].

Conidial germination and hyphae growth are likewise significantly influenced by the presence of albumin. Rodrigues et al. [30] showed that human albumin (2–4%) increased *A. fumigatus* conidia germination by 50%. On the contrary, albumin decreased *A. niger* and *A. flavus* germination by 20 and 25%, respectively. Similar results were obtained using bovine albumin. The study used albumin concentrations that mimicked physiologic human conditions, again suggesting that *A. fumigatus* conidia might be more able to germinate in vivo, in comparison to other *Aspergillus* species.

4 Induction of Cytokines and Chemokines

The host immune response against any infection is mediated by substances produced and liberated by the own cells, which stimulate, inhibit or attract another cells to the site of infection, modulating the response by the stimulation of Th1 or Th2 cells. The pattern of response depends on the offending pathogen. Differently from *A. terreus*, other *Aspergillus* species such as *A. fumigatus*, *A. niger* and *A. candidus* induce an increase in the release of MIP-2 and KC chemokines in the brochoalveolar lavage in experimental models. In addition, *A. fumigatus* also upregulates MIP-1 release, which is responsible for neutrophils chemotaxis, and tumour necrosis factor-alpha (TNF-α), an acute phase reagent involved in the initiation and promotion of the inflammatory process. Regardless of the absence of in vivo studies to corroborate these data, it is possible that different *Aspergillus* species may interact with diverse immune pathways, influencing the pathogenesis of aspergillosis. *A. terreus* infections have been associated with less severe inflammation than those infections caused by *A. fumigatus* [31].

5 Fungal Evasion from the Complement System

Similarly to many other organisms, *Aspergillus* species have the capacity to escape the host complement system. This property has been described for *A. fumigatus*, *A. terreus*, *A. niger* and *A. nidulans*. These may strongly bind to molecules such as factor H – the main inhibitor of the alternative complement pathway – and C4b-binding protein (C4bp) – the main inhibitor of the classical and lectin pathways – promoting a fungal evasion of the immune system that increase the pathogenic potential of the organism [32].

Previous studies revealed that monocytes-derived human macrophages exhibited lower phagocytic capacities against non-*A. fumigatus* aspergilli, in special *A. nidulans* and *A. niger*, when compared with *A. fumigatus*. In addition, polymorphonuclear leukocytes induced significantly less hyphal damage to both *A. flavus* and *A. nidulans* than to *A. fumigatus* [33]. The underlying mechanisms behind this process have not been elucidated and several factors are possibly

involved, including variables related to morphology and metabolism, which are discussed as follows.

6 Metabolic Characteristics

Adhesins are substances that promote the fungal interaction with the host cells. In special, many adherence factors from *A. fumigatus* have affinity for fibrinogen, a plasmatic glycoprotein from de coagulation cascade and from the inflammatory process. Affinity also occurs for laminin, the main structural component of the pulmonary basal membrane, and for the collagen IV [34, 35]. The adherence or binding to the host cells is facilitated by the presence of negatively charged carbohydrates present in the conidia surface. Wasylnka and Moore [36] showed that *A. fumigatus* has better in vitro capacity to adhere to the intact basal lamina from the pulmonary cells than others species such as *A. ornatus* and *A. wentii*. In addition, *A. fumigatus* shows 10–50% more capacity to adhere to fibronectin than *A. flavus*. Based on the fact that a pulmonary lesion may result in a local increase in fibronectin concentrations, abnormalities affecting the lower respiratory tract could lead to an increased susceptibility to *A. fumigatus* infection, due to the better adhesion of the *Aspergillus* conidia to the basal membrane.

6.1 Enzymes

Extracellular enzymes are produce by fungi for survival and for protection in their habitat. In the host, enzymes may increases pathogen resistance to immune cells, allowing the fungus to damage some natural host barriers to provide them with the essential nutrients necessary for their perpetuation.

The production of elastinolic proteases by *Aspergillus* species in vivo contribute in vitro to fungal pathogenicity, since elastin is a major structural component of the lungs. Elastin degradation may facilitate tissue invasion by these organisms. Elastases degrade not only elastin but also collagen, laminin, fibrinogen and other important components of the pulmonary matrix [34, 37]. Accordingly, *Aspergillus* isolates recovered from patients with IA have shown a high capacity of elastase production, with elastase-deficient mutants showing reduced virulence in neutropenic rat models of aspergillosis [37, 38]. In addition, in vivo studies showed that successive inoculations with *Aspergillus* strains resulted in increased elastinolitic activity in comparison to environmental strains, suggesting that the *Aspergilli* have the capacity of host adaptation by producing necessary enzymes for survival [38]. Whilst high elastase production has been seen with *A. fumigatus* strains, 10-times lower concentrations are seen with *A. flavus* [37, 39]. Some intra-species variation however occurred, with one *A. flavus* isolate producing exceptionally high levels of elastolytic activity. Moreover, *A. niger* strains do not seem to produce relevant amounts of elastases [39].

Aspergillus species also produce catalases, antioxidant enzymes that have a relevant role in protecting the fungus against host defenses. Three catalases have been identified in *A. fumigatus* strains, one of which is produced by the conidia and the other two by *Aspergillus* hyphae. The conidial catalase allows the fungus to resist to high temperatures, exposure of heavy metals and detergents. Catalase has shown to protect the fungus against hydrogen peroxide both in vitro and in the environment. However, catalase did not show in vivo activity against alveolar macrophages. Conversely, mycelial catalases protect the fungus against polymorphonuclear cells, with catalase-deficient mutants strains being much more vulnerable in vitro to H_2O_2 damage and to polymorphonuclear cells in lungs of experimental immunosuppressed models [40]. Nevertheless, in vitro these enzymes do not protect fungal cells against the oxidative mechanism of polymorphonuclear cells in immunocompetent hosts, a role played by another important enzyme, superoxide dismutase [40].

Likewise catalase, superoxide dismutases (SOD) have an antioxidant action during the phagocytosis. SOD is an extracellular and intracellular enzyme found in the cytoplasm and in the cellular wall of *A. fumigatus* conidia and hyphae. Holdom et al. [41] showed that *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans* can produce SOD, however the activity of these enzymes differ in accordance with temperature of incubation. Although SOD showed 100% activity for all tested *Aspergillus* species at 20°C, at 37°C (human temperature) only the *A. fumigatus* SOD maintained full activity, whereas SOD activity from *A. flavus* and *A. niger* decreased by 10%, and SOD from *A. terreus* and *A. nidulans* showed 70 and 12% activity, respectively. These differences occurred regardless of a pre-treatment at 70°C for 30 min, showing that all SOD were inactivated with exception of *A. fumigatus* SOD, which maintained 100% activity [41, 42]. The in vivo extrapolation of these results suggests that *A. fumigatus* might demonstrate higher antioxidant capacity, in comparison to other *Aspergillus* species, which may facilitate fungal escaping from the host immune cells.

Another enzymes previously identified in *A. fumigatus* strains can also be involved in fungal pathogenicity. These include the haemolysins associated with erythrocyte lysis and cytotoxic effects on macrophages and endothelial cells in vitro [43], ribonucleases (RNAse) that causes eukaryotic cells rRNA cleavage, and peptidases that promote humoral factors degradation. In addition, phospholypases may be associated with injury to epithelial tissues [35, 44]. Since RNAse production has not been associated with the pathogenic species *A. flavus*, the importance of these enzymes in *Aspergillus* pathogenicity has been questioned [35].

The enzyme histidine-kinase has been associated with response mechanisms to extracellular changes in osmolality, resistance to fungicides such as dicarboximides and regulation and/or building of the cell wall. Its deletion produces a delayed conidiation in comparison with the parent strain [45] and in systemic aspergillosis mouse models the mutant strains show a significantly reduced virulence [46]. It is therefore a proposed virulence factor and is also an interesting target for the development of new antifungal drugs [47].

6.2 Toxins

Fungi belonging to the genus *Aspergillus* are commonly associated with the production of toxins. These substances may vary in terms of chemical composition, mechanism of action and requirements for toxin production. Many of these can cause mycotoxicosis, a condition that may occur after ingestion of contaminated foods. Others can be produced in the host and thus, relate to fungus pathogenesis.

The hyphae of *A. fumigatus* release a number of low molecular weight toxins, particularly gliotoxin (GT), helvolic acid and fumagillin. A high concentration of the last two toxins is necessary to cause some injury in the immune cells or mucocilliary system of the host, which provide doubt about their participation on *Aspergillus* virulence. On the other hand GT that also appears to be detected in some aerial conidia of *A. fumigatus* [48] has been shown to have important toxic effects to host cells. GT is a hydrophobic metabolite produced by distinct *Aspergillus* species that belongs to the class of epipolythiodioxopiperazine (ETP) compounds, characterised by the presence of a quinoid moiety and disulfide bridge across the piperazine ring [49, 50].

Studies have been showed the GT has the capacity of immunossupression induction by injuring the mucocilliary system. That may occur with low concentrations (~200 ng/ml) [51]. In addition, GT may result in functional reduction of macrophages and neutrophils [52–54], and in blocking the lymphocytes activation and the cytokines stimulation and liberation, mainly due to the apoptosis induction of antigen presentation cells, as monocytes and dendritic cells [52, 55]. These immunosuppressive properties of GT had been proved with lower toxin concentrations (20–50 ng/ml) [52, 55] than those detected on tissues (mean, 3,976 $\pm$ 1,662 ng/g of lung tissue) and on blood circulation (mean, 37 $\pm$ 30 ng/ml) in experimental models of aspergillosis [56]. Associating these data with the fact that GT is produced from *Aspergillus* hyphae [57] – the fungal structure responsible for tissue invasion – GT might have an important role in *Aspergillus* pathogenesis in vivo [55, 56].

GT production seems to be more important for *A. fumigatus* and *A. niger* strains, in comparison to other pathogenic *Aspergillus* species such as *A. flavus* and *A. terreus*. In one study, 93 and 75% of *A. fumigatus* and *A. niger* strains, respectively, were shown to produce a mean of 600 ng/ml of GT. Controversially, GT was detected for 25% of *A. terreus* strains (mean GT concentrations of 169 ng/ml), and only 4% of *A. flavus* strains (mean 62 ng/ml – a 10x lower GT concentration in comparison to *A. fumigatus* and *A. niger*) [58]. A recent study also showed that nearly all clinical and environmental isolates of *A. fumigatus* produced gliotoxin (96 and 98%, respectively) [59]. The frequency of gliotoxin production amongst *A. flavus* in this study was very low too (13% only), and the mean concentrations of gliotoxin in culture filtrates were about 80-fold lower than the concentrations observed for *A. fumigatus*. Lack of gliotoxin production in *A. fumigatus* significantly reduced cytotoxicity on macrophage-like P388D1 cells and CD8 T-cells. In contrast, absence of gliotoxin did not influence cytotoxicity in *A. flavus*. After

stratifying patients according to the EORTC/MSG criteria [58], no difference in the frequency or degree of gliotoxin production was observed between patients with definitive IA or *Aspergillus* colonisation. Together, these data suggest a limited role for gliotoxin in the pathogenicity of IA, particularly in infections caused by species other than *A. fumigatus*.

Aflatoxin, another important mycotoxin produced by *A. flavus* and *A. parasiticus*, is associated with hepatotoxicity, carcinogenicity, teratogenicity, and immunosuppressive actions. Amongst these compounds, aflatoxin B1 is particularly important as it is one of the most potent natural carcinogenic ever characterised [60]. Likewise GT, the immunosuppressive action of aflatoxin was demonstrated in experimental rabbits infected with *Aspergillus*, resulting in a significant reduction in conidia phagocytosis by alveolar macrophages, which was attributed to a decreased in serum complement factors responsible for opsonization [61]. Although aflatoxin has been detected in patients with *A. flavus* infections, including cases of fungal balls [62–64], it is believed that this toxin has a limited role in in vivo fungal pathogenesis [13], since non-toxigenic strains are as able to result in aspergillosis as aflatoxigenic-productive strains in experimental models [65, 66].

7 Prevalence

Invasive aspergillosis is the most common filamentous fungal infection in immunocompromised patients. This is a severe infection with a 357% increase in death rates reported in the United States of America from 1980 to 1997 [67]. *A. fumigatus* is by far the most frequent species present in such infections, followed at some distance by *A. flavus, A. niger, A. terreus, A. nidulans* and *A. ustus,* and with a lower percentage *Neosartorya* spp. (Table 2). Significantly, in recent years the proportion of infections caused by non-*fumigatus Aspergillus* species has increased considerably [11, 68]. Marked variation occur amongst centres in *Aspergillus* species causing aspergillosis, with some reporting a high incidence of infections caused by *A. terreus* or *A. flavus* [13]. Climatic factors and use of antifungal drugs may play a central role in the distribution of *Aspergillus* species causing these infections.

Table 2 Percentage of aspergillosis case reports involving immunocompromised patients [1]

Species	Percentage
Aspergillus fumigatus	68.7
Aspergillus flavus	17.0
Aspergillus niger	5.7
Aspergillus terreus	3.9
Aspergillus nidulans	1.1
Aspergillus ustus	1.1
Neosartorya spp.	0.7
Each of the rest of species	≤ 0.5

It is also likely that many aspergillosis cases attributed to *A. fumigatus* are actually produced by other species. Molecular studies have shown that some strains of *A. fumigatus* that grow as whitish, slowly growing, sporulating colonies hitherto attributed to *A. fumigatus* actually belong to the recently identified species *A. lentulus* [69]. Probably the same is true of those strains of *Neosartorya* spp. that do not produce the teleomorph in culture, which is not uncommon in clinical strains. The importance of molecular methods in the identification of *Aspergillus* species is discussed elsewhere in this book. Three species of *Neosartorya* have been reported as pathogenic to humans: *N. pseudofischeri, N. fischeri* and *N. hiratsukae*. The species most commonly cited of this genus is *N. pseudofischeri* (anamorph *A. thermomutatus*), seven cases up to now. However, several cases recorded as former varieties of *N. fischeri* can also probably be attributed to *N. pseudofischeri*. This is probably also the case of many isolates that produce fast-growing, slowly sporulating colonies and which have been attributed to *A. fumigatus* [70]. Recent molecular studies have also shown that, of 33 isolates of clinical origin phenotipically identified as poorly sporulating *A. fumigatus,* 8 actually belonged to *Neosartorya udagawae* a species isolated from Brazilian soil and which had never before been recovered from clinical samples [71].

8 Habitat and Distribution

The different species of *Aspergillus* usually have a very wide distribution and although quite a diversity of species have been associated with human infection, they normally inhabit other ecological niches. *Aspergilli* are typical soil fungi common in warm climates but they can be found in extreme climates, too. Most of them have capabilities which allow them to develop under relatively extreme growth conditions. For example, a number of species of *Eurotium* are osmotolerant and can grow on materials with low water activity and others of the *Fumigati* section are thermotolerant, they have tolerated temperatures of pasteurization. This high temperature tolerance differentiates *A. fumigatus* from the closely related species *A. lentulus*, since the latter is not able to grow at 50ºC. Other *Aspergilli* are capable of cellulose degradation, insect pathogenesis/degradation, and hydrocarbon degradation [1, 60]. There are not important differences amongst the most relevant pathogenic *Aspergilli* in growing substrates and geographical distribution. All of them are commonly found in soil samples and colonising seeds, especially grains, root crops, spices, compost and many different foodstuffs and living plants. The two most common species *A. fumigatus* and *A. flavus* are cosmopolitan fungi and they can be found everywhere. *A. fumigatus* is abundant in the air during biological waste treatment. Nosocomial infections produced by these two species in immunocompromised patients may be due to hygiene problems or construction works. As already discussed in this chapter, a major concern about the association of *A. flavus* and its relatives with plant products (particularly corn, cotton, peanuts, and tree nuts) lies in their ability to produce aflatoxins, polyketide secondary metabolites that are potentially carcinogenic.

9 Pathology

The main source of aspergillosis is via the air through the inhalation of conidia. The *Aspergilli* produce a large number of dry, hydrophobic conidia which are easily inhaled. One of the most typical clinical manifestations of aspergillosis is IPA. However, only a few species, particularly *A. flavus* and *A. fumigatus,* are frequent causative agents of pulmonary aspergillosis, either allergic, invasive or a combination of both. Although in total the figure is high, the proportion of pulmonary infections caused by *A. flavus* is significantly lower than those caused by *A. fumigatus* or *A. terreus,* another species causing common IPA. The size of the conidia is the factor that determines that some species can reach the lower respiratory tract and penetrate deeply into the lungs more easily than others (Table 3) as we discussed previously.

A. fumigatus is the most common species of opportunistic pathogen moulds and the main agent of aspergillosis in patients with impaired natural immunity. It causes opportunistic disease whereby colonisation and invasion are generally accompanied by allergic reactions. Invasive diseases usually begin with a primary infection in the lower respiratory tract but also occasionally in other places, such as the nasal sinuses and skin. Particularly at risk are patients with prolonged neutropenia, on treatment with steroids, patients who have undergone allogeneic haematopoietic stem cell transplantation and/or lung transplantation, patients under intensive care and patients with chronic granulomatous disease or advanced HIV infection. Disseminated infections in patients without apparent immune disorder are extremely rare. Traumatic infections are rare and are restricted to immune deficient patients, or cases in which wound-contamination is concerned. Non-pulmonary cavities can also be colonised. The species occurs commonly in the outer ear and is one of the prevalent agents of fungal sinusitis. Sinusitis may be allergic, lead to fungus balls or become invasive. In the latter case, complications may arise which may be of a cranial nature and therefore life-threatening. Invasion of adjacent tissues and dissemination is generally linked to innate immunosuppression, e.g. in transplant patients or after near-drowning. Cases of cerebral aspergillosis have been described. However, these infections are often caused by species other than *A. flavus* or *A. fumigatus* [72]. Other opportunistic infections

Table 3 Conidia diameter of the most common pathogenic species of *Aspergillus*

Species	Conidia diameter (μm)
Aspergillus ustus	3.0–4.5
Aspergillus terreus	2.0–2.5
Aspergillus flavus	3.5–5.0
Aspergillus niger	3.5–4.5
Neosartorya pseudofischeri	2.5–3.0
Aspergillus fumigatus	2.5–3.0
Aspergilus nidulans	3.0–3.5

caused by *A. fumigatus* include otomycosis, ocular infections, onychomycosis and endocarditis. These syndromes are all discussed in detail along this textbook. *A. fumigatus* is also capable of causing many animal infections in birds, dogs and other animals.

A. flavus is one of the main agents of human allergic bronchial aspergillosis and pulmonary infections in immunocompromised patients, where its pathology may be aggravated by the production of mycotoxins. It is the most common cause of superficial aspergillosis. In general, the disease spectrum is very similar to that caused by *A. fumigatus.* Particularly common clinical syndromes associated with *A. flavus* include chronic granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections and osteomyelitis following trauma and inoculation [13, 60]. *A. flavus* has only occasionally been associated with chronic infections such as chronic cavitary pulmonary aspergillosis [64].

A. niger is an osmotolerant species which is considered to be the prime aetiological agent of otomycosis, although its pathogenic role in this disease has not been clearly established. It is too frequently isolated as a secondary invader in cases of bacterial otitis externa. This species has also been involved in cases of pulmonary intracavitary colonisation, localised infections such as peritonitis, endocarditis, onychomycosis and disseminated infections generally after major surgery, which can be associated with environmental hygiene. *A. niger* seems to be a fungal species of low virulence that requires the presence of a severely immunosuppressed host to cause invasive disease [73].

A. terreus is a common cause of allergic or invasive bronchopulmonary aspergillosis, although it is also present in a wide variety of infections including aspergilloma (fungal ball), otomycosis, onychomycosis, aural cavity disease, subcutaneous abscesses and keratitis. Nosocomial infections have also been reported. Between 3 and 10% of cases of aspergillosis may be ascribed to this species [11, 74]. In the immunocompromised patient, it appears to cause higher morbidity. It can cause severe infections in pigeons and dogs.

A. calidoustus (formerly *A. ustus*) is apparently less common than the other species mentioned above, since only 15 cases of systemic infection have been reported [4, 75]. Most of these cases affected haematopoietic stem cell transplant recipients but there have been reports of cases of pneumonia, disseminated or primary cutaneous infection in immunocompromised patients, endocarditis, onychomycosis and endophthalmitis.

A. nidulans has been associated with numerous cases of chronic granulomatous disease where it was determined by a case review to be more virulent and more difficult to treat than *A. fumigatus* [1]. It is also reported to be the aetiological agent of diverse infections in humans and animals, either alone or in association with other opportunistic fungi. Amongst these are pulmonary infections, sinusitis, otitis, endophthalmitis, osteomyelitis, mycetoma in tropical areas, and superficial as well as disseminated infections. Animal cases concern ducklings and horses producing in the latter a typical guttural pouch disease.

10 Virulence (Animal Models)

Numerous animal models of aspergillosis have been developed, from the perspective of virulence and from the perspective of the evaluation of pharmaceutical agents, which included a wide range of animals such as rabbits, rats, guinea pigs, monkeys, sheep, ducks and chicks. However, in most of the studies only *A. fumigatus* was tested [76]. Fatal experimental infections leading to multiple organ involvement have been most frequently produced in mice with an intravenous injection of conidia and in the few studies that compared the virulence of different species, in general, *A. flavus* seemed to be more virulent than other *Aspergilli* of clinical interest [13]. However, most models used intraperitoneal inoculation with *Aspergillus* strains in mice, an approach that does not mimic the usual via of acquisition of *Aspergillus* conidia (e.g., by inhalation). Since laboratory animals normally show a high degree of resistance towards *Aspergillus* infections, host defense should be lowered by the administration of immunosuppressive agents or some others way in order to ensure establishment of persistent and/or invasive infections in these animals. However, in a comparative study testing different species of *Aspergillus* in a murine model of immunocompetent mice the order of virulence, estimated on the basis of survival of animals infected with inocula of 10^6 conidia, was: *A. flavus* > *A. niger* > *A. terreus* > *A. fumigatus* > *A. ustus*. But not only *A. flavus* was the most virulent since other related species of the *Flavi* section tested, as *A. parasiticus*, *A. tamari*, *A. foetidus* and *A. oryzae*, showed also a high virulence. All these species of the *Flavi* section were the only that consistently killed mice with doses as low as 10^4 conidia [66]. In another study the virulence of isolates of *A. terreus*, *A. flavus* and *A. nidulans* were compared in DBA/2 mice. Using an inoculum of 10^5 colony forming units (CFU), *A. flavus* killed 90% of animas at day 10, whilst 40% mortality was observed with a higher inoculum of *A. terreus* (10^6 CFU). *A. nidulans* showed a considerable lower virulence since the authors had to use pancytopenic mice to attain a similar mortality rate of 40% at 10 days [77]. Other studies in both normal and immunocompromised mice also confirmed the higher virulence of *A. flavus* over *A. fumigatus,* since the LD_{90} inocula (lethal dose required to kill 90% of animals) for the former were 100-fold lower than those required for the latter species [78]. In another study the tissue burden in different organs of mice infected by *A. terreus* were >100-fold higher than those of mice infected with *A. fumigatus* [79]. The results obtained for *A. terreus* in the study of Bowman et al. [77] were similar to those of Dannaoui et al. [80] who using inocula of 10^5, 10^6 and 10^7 CFU attained mortality rates of 10, 40 and 80%, respectively. In other cases by contrast *A. fumigatus* was revealed as more virulent than *A. terreus.* In a model of IPA in persistently neutropenic rabbits, the animals infected with *A. terreus* survived longer than those infected with *A. fumigatus.* The differences in virulence could be appreciated in mean pulmonary infarct scores and lung weights which were lower in animals infected with *A. terreus* [6]. These differences between the virulence of these two species could be explained by the fact that only one strain was tested in each study and that this pathologic trait

were strain-dependent. It is also possible that one fungal species was more virulent for one animal species than others and that *A. terreus* were more virulent in mice and *A. fumigatus* in rabbits.

A wide variety of murine models of aspergillosis by *A. fumigatus* including intravenous, intratracheal or intranasal challenge, have been described. Although having a wide range of models is a very positive factor for many specific questions, the methodological differences amongst them have made direct comparisons of individual studies difficult [81]. Furthermore, comparative studies in order to asses the interlaboratory variability of results obtained with the same experimental model are scarce. In an attempt of make available an experimental model of IPA by *A. fumigatus* which can be a reference for future studies on this disease recently a standardised murine model has been reported. It was based in an aerosol infection performed in two independent laboratories which found values of survival and fungal burden over time reproducible (Table 4) [81].

Table 4 Basic parameters and conditions of a standardized murine model of invasive pulmonary aspergillosis [80]

Basic parameters	Conditions
Strain	Af 293
Immunosuppression	Cyclophosphamide 250 mg/kg (on days –2 and +3) and cortisone acetate 200 mg/kg (on days –2 and +3)
Infection	Exposition to an aerosol (1 h) of 10^9 conidia/ml[a]
Survival at 14 days	33.4–42.7%
Tissue burden in lung	Mean $\log_{10}$ CFU/g of mouse: 2.92–3.81

Legend: Af 293, *Aspergillus fumigatus* reference strain Af 293; CFU, colony forming units.
[a]In an acrylic chamber and generated from 12 ml of phosphate-buffered saline supplemented with 1% Tween 80.

11 Phylogeny, Genetic Diversity and Population Structure

Recent molecular phylogenetic studies, which tested sets of isolates from different origins, have demonstrated that several *Aspergillus* species of clinical interest that have traditionally been considered as single species are indeed complexes of species. Some of these cryptic species have been characterised by using polyphasic studies, including morphology, multilocus gene sequences and extrolites profiles [3]. The discovering of numerous new species as a result of these studies open new perspectives of study in order to asses if these taxonomic groupings have clinical significance in terms of mode of infection, drug resistance or virulence. At the same time studies using population genetics statistical tests have allowed to discover evidence of past or ongoing recombination in some anamorphic *Aspergilli* with no association with any teleomorph and known to reproduce exclusively asexually in the laboratory. *A. flavus* constitutes a clear example of these fungi. The use of different molecular techniques has allowed the detection in *A. flavus* of highly polymorphic populations. Tran-Dhin et al. [82] demonstrated the existence

of two major Random Amplification of Polymorphic DNA (RAPD) patterns and Geiser et al. [83, 84] based on the level of fixation of polymorphisms amongst the different loci studied demonstrated the existence of two groups of Australian agricultural isolates (I and II) with a long history of reproductive isolation, which can represent two different biological species. However, the lack of concordance amongst gene genealogies amongst isolates of the group I was considered consistent with a history of recombination, whilst that the isolates of group II appeared to be more homogenous than those in group I, implying clonal dissemination [83, 84]. Important phenotypic variations have also been well documented in *A. flavus*. For example their isolates vary considerably in their abilities to produce aflatoxins, and in addition they can be grouped into two morphological groups, L strains and S strains, the former producing abundant conidia and sclerotia >400 μm in diameter, and the latter producing fewer conidia and sclerotia <400 μm in diameter [85]. However, no correlation was found between the phylogenetic groups obtained in the multilocus sequence analysis study and those phenotypic features. Most group I strains produced B aflatoxins, and none produced G aflatoxins, whereas in the group II a higher variation was observed since several subgroups produced both B and G aflatoxins and others not. By other hand and with respect to the sclerotial morphotypes all group II isolates were S whereas those of group I were both L and S [83].

A. fumigatus is a species of a relative recent origin as seems to be indicated by the low frequency of polymorphisms and low intraspecies variation detected within many genes scattered throughout its genome [86, 87]. Although this species has been traditionally considered as with exclusive clonal reproduction since any type of teleomorph has newer been observed, when the association of alleles at the five loci was explored, evidence was found to support recombination [88]. The *A. fumigatus* genome sequence project has also revealed genetic elements for sexual behaviour, and even genes for both mating types have been found opening the possibility that sexual recombination may occur in this fungus [87].

Similarly to that occurred in *A. flavus,* and in spite of the low *A. fumigatus* interstrain variation above mentioned, in a study based on DNA sequence data of five loci Pringle et al. [88] delineated two phylogenetic species in a set of isolates of *A. fumigatus* representing different continents. Both genetically isolated groups showed a global distribution with no evidence of a correlation between genotype and geographic location. But these new phylogenetic species are not an exception in the section *Fumigati* since recent molecular studies have demonstrated the existence of numerous well supported genetic clades, being some of them proposed as new species. It is likely that in a few years the spectrum of species of this section will be enlarged considerably. It is remarkable the fact that some of those isolates of *A. fumigatus* that sporulate poorly and that in may laboratories were discarded as contaminants currently and on the basis of molecular identification have been recognised as species different from *A. fumigatus*. Some of them are anamorphs such as *A. lentulus, A. fumigatiaffinis, A. fumisynnematus*, and *A. viridinutans* but others are teleomorphs that do not develop their ascomata in culture, as commonly occur with stressed clinical isolates, such as *N. udagawae* and *N. hiratsukae* [4, 5, 69–71].

A modern overview of the species belonging to the *Aspergillus* section *Fumigati,* based on analysis of macro-and micromorphology, extrolite profiles, and B-tubulin, calmodulin, ITS and actin gene sequences of the isolates, have been reported by Samson and Varga [3]. Some of these species have not been reported in clinical samples up to now but several others have and are probably more common that previously was thought.

In comparison to *A. fumigatus* and *A. flavus*, the population genetics of other less common species such as *A. terreus* is less known. Molecular typing techniques, testing isolates from two different countries from Europe and North America, have demonstrated that this species shows a high genetic diversity and that nosocomial acquisition of an *A. terreus* clonal strain was highly unlikely [89]. Considering only the morphological characteristics *A. terreus* had been traditionally considered as the only species of the section *Terrei.* However, molecular studies based on rRNA genes sequencing seems to indicate that this section should be expanded to include other species, and although in that study not cryptic speciation was found, more recent studies based on β-tubulin, calmodulin and enolase genes sequences indicated that several cryptic species probably exist within clinical isolates identified as *A. terreus* [4].

Scarce data are too on the phylogeny and genetic populations of *A. ustus.* Although in a RAPD analysis of the small isolates population obtained from six different patients haematopoietic stem cell transplant recipients in an outbreak of invasive infection in an USA hospital showed genetic similarity between them a large separation was noticed between these isolates and the control ATCC strain [75]. This seems to indicate that this species is also genetically variable as has been confirmed through sequence analysis of parts of the rRNA genes [4] or polyphasic studies based on chemical, molecular and morphological data [90]. The most recent studies have indicated that the section *Usti* currently encompasses 8 species, and that the clinical isolates traditionally included in *A. ustus* actually belong to the new species *A. calidoustus* [90].

12 Resistance

In different surveys that have compared the response of large sets of strains of *Aspergillus* spp. to the different traditional and newer antifungal drugs important differences have been noticed. In general, they responded well to the echinocandins, with the exception of *A. ustus* [75], and to amphotericin B with the exception of *A. ustus* [75, 91] and *A. terreus* [92]. In the case of azoles, significant differences amongst the different species were observed. *A. ustus* shows decreased susceptibility to azoles in general [75, 91]; *A. lentulus* to voriconazole and *Aspergillus fumigatus* and *A. niger* respond worse to itraconazole than to the other azoles [92].

But even amongst the *Fumigati* section important differences have been reported. Whilst *N. hiratsukae* and *A. fumisynnematus* were susceptible to all the antifungals tested, *A. lentulus* and *A. fumigatiaffinis* shows decreased susceptibility to amphotericin B, voriconazole and itraconazole [5, 71], *A. lentulus* to caspofungin

[69, 71], *N. udagawae* to amphotericin B, and *N. pseudofischeri* to amphotericin B and voriconazole [5, 69, 71].

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Immunology of *Aspergillus* and Aspergillosis: The Story So Far

Luigina Romani

Abstract Innate responses combine with adaptive immunity to generate the most effective form of anti-*Aspergillus* immune resistance, and T cells feedback onto a tenuous balance of diverse effects of inflammation on infection. Some degree of inflammation is required for protection – particularly in mucosal tissues – during the transitional response occurring temporally between the rapid innate and slower adaptive response. However, progressive inflammation worsens disease and ultimately prevents pathogen eradication. The newly described Th17 developmental pathway may play an inflammatory role previously attributed to uncontrolled Th1 responses and serves to accommodate the seemingly paradoxical association of chronic inflammatory responses with fungal persistence. The indoleamine 2,3-dioxygenase enzyme (IDO) and regulatory T cells help to tame overzealous and exaggerated inflammatory responses and have become an integral component of immune resistance to the fungus in infections and diseases. Recent data support a view in which interleukin (IL)-23/IL-17 antagonistic strategies, including the administration of synthetic kynurenines and IDO expressing dendritic cells, could represent a new means of harnessing progressive or potentially harmful inflammation in infection and allergy to the fungus in different settings, including haematopoietic transplantation.

Keywords Aspergillosis · IDO · Inflammation · Th17 · Tolerance

L. Romani (✉)
Microbiology Section, Department of Experimental Medicine and Biochemical
University of Perugia, Via del Giochetto, Perugia, Italy
e-mail: lromani@unipg.it

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Contents

1 Introduction

Aspergillus spp. are ubiquitous in nature. The spectrum of disease they cause is myriad, ranging from saprophytic colonisation of pre-existing cavities (fungal balls) to hypersensitivity pneumonitis, allergic bronchopulmonary aspergillosis (ABPA) occurring as a complication of asthma or cystic fibrosis [1], chronic cavitary pulmonary aspergillosis (CCPA), and disseminated invasive disease. The latter is associated with a high mortality rate and affects immunosuppressed patients such as those with haematological malignancies, recipients of solid organs and stem-cell transplants and patients admitted to the intensive care unit [2, 3]. While many viral and most bacterial infections can be managed successfully, the relative importance of fungal diseases, especially invasive aspergillosis (IA), has even increased. Despite the past decade has witnessed significant progress in the management of IA, the infection continues to be a deadly disease [4–6]. The main reasons for this include intrinsic or acquired antifungal resistance, organ dysfunction preventing the use of some agents and the deleterious effect of a deregulated inflammation [7–9].

This chapter will highlight how the past several years have seen remarkable advances in understanding the basic cellular and immunological mechanisms underlying resistance to the fungus but also organ dysfunction and failure to recovery relating to IA. Current understanding of the pathophysiology underlying *Aspergillus* infection and disease highlights the multiple cell populations and cell-signalling pathways involved in this complex condition, including the novel findings on the molecular connection between the failure to resolve inflammation, lack of

antifungal immune resistance and susceptibility to *Aspergillus* infections and diseases. The intricate cross-talk provided by temporal changes in mediators, metabolites, and cell phenotypes underlines the coordinated processes beyond the deregulated chaos in which fungal infection and disease is perceived. Applying systems approaches to these complex processes will permit better appreciation of the effectiveness or harm of treatments, and also will allow development not only of better directed, but also of more appropriately timed, strategies to improve outcomes from this still highly lethal infection.

2 Immunology of *Aspergillus*: Insights into the Failure of the Immune Response

2.1 Inflammation to Fungi: Friend or Foe?

Many of the diseases that afflict mankind are thought to result from dysfunctional innate and/or adaptive immune responses [10]. The inflammatory response, initiated by cells of the innate immune system, is followed by adaptive immunity, which responds to, and at the same time regulates, signals emanating from the innate system. Unresolved infection and inflammation are major epigenetic and environmental factors that contribute to chronic diseases and auto-immunity, and in specific settings, to an increased risk of cancer. Fungi can exploit or subvert a host's inflammatory response [11], and thus affect carriage [12] and pathogenicity. A hyperinflammatory response does, in fact, enhance virulence of *A. nidulans* infections [13]. Thus, although the inflammatory response to fungi may serve to limit infection, an overzealous or heightened inflammatory response may contribute to pathogenicity and paradoxically promote infection [8, 9, 14].

2.2 Immune Responses to Aspergillus*: From Protection to Disease Promotion*

Immunocompetent and non-atopic subjects are relatively resistant to *A. fumigatus* infections and disease occurs in the setting of host damage [15, 16]. Thus a high degree of coexistence occurs between the airborne fungi and their mammalian hosts, which deviates into overt disease only under specific conditions, most prominently deficits in immunity [17]. Innate and adaptive immune responses act to generate the most effective form of immunity for protection against *Aspergillus* species. The decision of how to respond is still primarily determined by interactions between the fungus and cells of the innate immune system, but the actions of T cells will feedback into this dynamic equilibrium to regulate antifungal effector functions and the balance between pro-inflammatory and anti-inflammatory signals (Fig. 1). As a matter of fact, the inherent resistance to infections caused by *A. fumigatus* suggests the occurrence of regulatory mechanisms that provide the host with adequate defence without necessarily eliminating the fungus or causing unacceptable levels of host damage [12, 18].

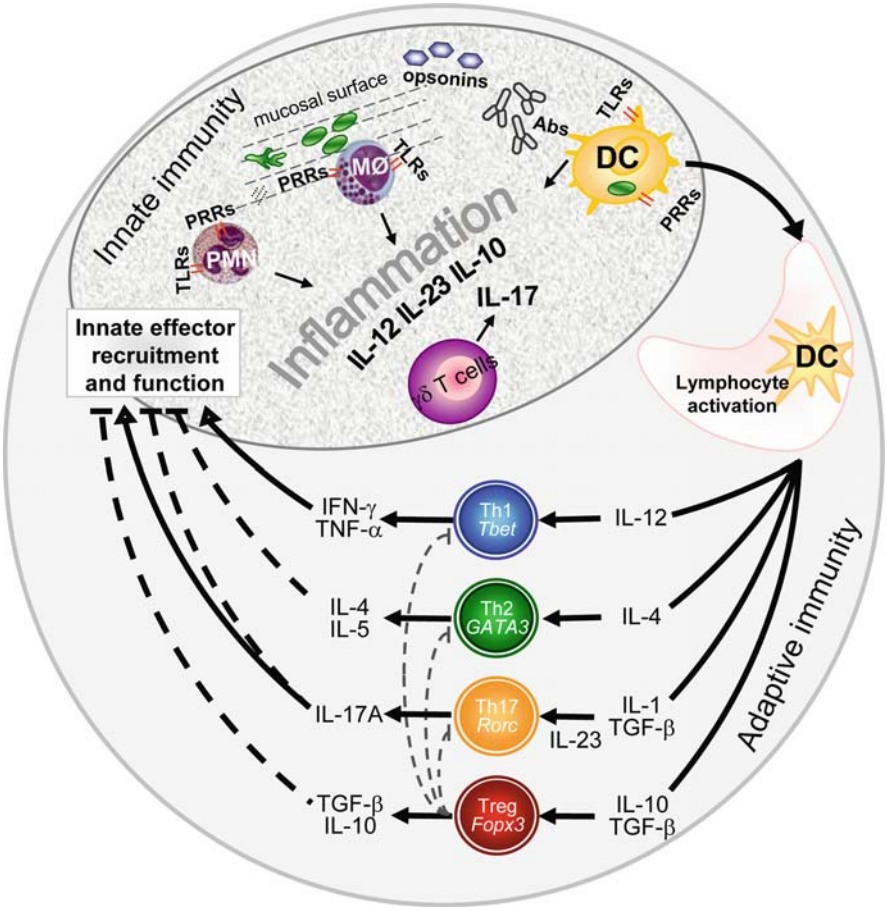

Fig. 1 Innate and adaptive cell-mediated immunity to *Aspergillus*. The figure is an update of the innate and adaptive responses mediated by different Th cell subsets, their transcription factors and possible effector functions in aspergillosis. Innate defence mechanisms act immediately and are followed by an early induced response that helps to keep infection under control. In vertebrates, however, an adaptive immune response will ensue, with generation of antigen-specific T helper (Th) effectors releasing a distinct panel of cytokines capable of delivering activating and deactivating feedback signals to effector phagocytes. Counter-regulatory T reg cells (Tregs) are needed to dampen the excessive inflammatory reactions. Thus, defective Treg cell accumulation to infected organs may apparently increased resistance to the fungus. Dendritic cells sample the fungus at the site of infection, transport it to the draining lymph nodes and activate disparate Th/Tregs in a morphotype-dependent fashion. However, an uncontrolled concomitant inflammation would later impair the development of protective Th1/Treg cell responses in the draining lymph nodes. Therefore, a tight control of both pathogen growth and host reactivity is needed in infection, to allow presentation of fungal antigens in the context of limited danger signals, a condition required for the successful activation of protective tolerance. DC, dendritic cells; MØ, macrophages; PMN, polymorphonyclear neutrophils; PRRs, pattern recognition receptors; TLR, Toll-like receptors. Solid and broken lines refer to positive and negative signals, respectively

There have been tremendous advances in the field of innate immunity over the last decade, including the elucidation of the toll-like receptors (TLR) that are activated by recognition of fungus-associated molecular patterns [19]. A compelling body of evidence indicates that hosts rely upon TLR and C-type lectins to sense the presence of and respond to *Aspergillus* ligands [20–23]. The different impact of TLR on the occurrence of the innate and adaptive Th immunity to the fungus is consistent with the ability of each individual TLR to activate specialised antifungal effector functions on innate immune cells. TLR influence specific antifungal programs of phagocytes, such as the respiratory burst, degranulation and production of chemokines and cytokines [20, 22]. As the quantity and specificity of delivery of toxic neutrophil products ultimately determine the relative efficiency of fungicidal activity versus inflammatory cytotoxicity to host cells, this implicates that TLR contribute to protection and immunopathology against the fungus. Recent evidence points to the importance of selected TLR polymorphisms in susceptibility to infection, in transplanted [24] and non-transplanted patients [25]. TLR may explain why polymorphonuclear neutrophils (PMN), although essential in initiation and execution of the acute inflammatory response and subsequent resolution [16, 26], may act as a double-edged sword, as the excessive release of oxidants and proteases may be responsible for injury to organs and fungal sepsis [20, 22]. As a matter of fact, despite extensive fungal growth, pulmonary pathology is reduced in the presence of PMN deficiency both in mice and humans [15, 27].

Overall, the current view suggests that TLR activation itself is also a double-edged sword. By over inducting pro-inflammatory cytokines, by facilitating tissue damage or by impairing protective immunity, TLR might also promote the pathogenesis of infections [28]. Another function of the innate immunity that is emerging is that it also has a role in sterile inflammation – that is, (auto-) inflammation caused by endogenous TLR ligands. Recent studies have provided new insights into the host pathogenic determinants responsible for inflammatory-driven diseases in *A. fumigatus* infections [11] and diseases [29].

Antigen-independent recognition of the fungus by the innate immune system leads to the immediate mobilisation of immune effector and regulatory mechanisms that provide the host with the rapid initiation of the immune response and creation of the inflammatory and co-stimulatory environment for antigen recognition; establishment of a first-line of defence, which holds the pathogen in check during the maturation of the adaptive immune response; and steering of the appropriate adaptive immune response to the fungus.

Generation of a dominant Th1 response driven by interleukin (IL)-12 is essentially required for the expression of protective immunity to *Aspergillus* species. Through the production of the signature cytokine interferon (IFN)-γ, the activation of T helper (Th)-1 cells is instrumental in the optimal activation of phagocytes at sites of infection (Fig. 1). The inflammatory allergic manifestations that follow the contact or inhalation of fungi all constitute compelling evidence of the pathogenic role of T-cell dysreactivity in fungal diseases. In patients with defective interleukin (IL)-12/IFN-γ pathway, such as those with hyperimmunoglobulinemia E syndrome, fungal infections and allergy are both observed [30]. Allergy is an overzealous Th2

response to environmental airborne allergens. Moreover, and consistent with the inflammatory state, patients with pulmonary aspergillosis are genetically low producers of both transforming growth factor (TGF)-β and IL-10 [31].

A major role of the immune system, innate and adaptive, is to maintain tissue homeostasis by stopping and/or preventing cell damage caused by invading pathogens and promoting tissue repair following a lesion. The control of the immune response is pivotal for preventing damage. Prolonged inflammation is a hallmark of a wide range of chronic diseases and auto-immunity [32]. In light of the above considerations, recent evidence point to a further complexity of cross-regulatory innate and adaptive Th pathways, beyond the dichotomous Th1/Th2 model, that operate from the state of continuous sensitisation to ubiquitous airborne fungi to infections and fungal diseases. In this regard, recent insights regarding the development of allergic diseases have suggested a role for "inflammatory T cells" or Th17 cells, producing IL-17, as a link between T cell inflammation and granulocytic influx as observed in allergic airway inflammation [8, 14, 29, 33]. The new entry, the Th17 pathway, playing an inflammatory role previously attributed to uncontrolled Th1 cell reactivity [8, 34, 35], and regulatory T cells (Tregs) [12, 14, 18, 36, 37], capable of fine-tuning protective antimicrobial immunity in order to minimize harmful immune pathology, have become an integral component of the immune response to the fungus (Fig. 1 and see below).

2.3 Aspergillosis as an Example of Immune-Related Pathology: Lesson from IRS and CGD

Although the inflammatory response to fungi may serve to limit infection, an overzealous or heightened inflammatory response may contribute to pathogenicity. This conceptual principle is best exemplified by the occurrence of severe fungal infections in patients with immunoreconstitution syndrome (IRS), an entity characterised by localised and systemic inflammatory reactions of varying degrees that have both beneficial and deleterious effects on infection [9, 38]. Intriguingly, IRS responses are also found in immunocompetent individuals and after rapid resolution of immunosuppression, indicating that inflammatory responses can result in quiescent or latent infections manifesting as opportunistic mycoses. Thus, although host immunity is crucial in eradicating infection, immunological recovery can also be detrimental and may contribute towards worsening disease in opportunistic and non-opportunistic infections [9, 39, 40]. Additionally, a high incidence of fungal infections and sensitisation to *Aspergillus* spp. has been described in the hyper-IgE syndrome [41, 42] in which increased levels of pro-inflammatory gene transcripts have recently been described [43]. For *A. fumigatus*, the association of persistent inflammation with intractable infection is common in non-neutropenic patients after allogeneic haematopoietic stem cell transplantation (HSCT) [44] as well as in allergic fungal diseases [45]. Therefore, paradoxically, increased inflammatory innate response may predispose to either fungal infections or deregulated immune responses to the fungus. As a matter of fact, the status of innate host immunity

also may contribute significantly to the histological patterns associated with fungal infections [15]. Thus, although host immunity is crucial in the eradication of infection, immunological recovery can also be detrimental and may contribute towards worsening disease expression.

Most prominently, in patients with chronic granulomatous disease (CGD), a hyperinflammatory phenotype and defective fungal – typically *A. fumigatus* – clearance have long been known to benefit each other [46]. The above observations highlight a truly bipolar nature of the inflammatory process in infection. Early inflammation prevents or limits infection, but an uncontrolled response may eventually oppose disease eradication. This condition is crucially exemplified by recent findings in CGD mice, in which an intrinsic, genetically determined failure to control inflammation to sterile fungal components determines the animals' inability to resolve an actual infection with *A. fumigatus* [14]. A main implication of these findings is that, at least in specific clinical settings, it is an exaggerated inflammatory response that likely compromises a patient's ability to eradicate infection, and not an "intrinsic" susceptibility to infection that determines a state of chronic or intractable disease. Because *Aspergillus*, through the subversion of host inflammatory pathways [11], may promote inflammatory responses, an inflammatory vicious circle seems to be at work the manipulation of which may offer strategies to control or prevent exacerbations of *Aspergillus* infections and diseases.

3 Pathogenic Inflammation: The Th17 Pathway

The mechanisms that link innate immunity, inflammation, and chronic infection are now beginning to be unravelled. Important components in this network are cytokines produced by activated innate immune cells, which stimulate adaptive responses that mediate inflammation [32]. Soluble mediators produced by fungi also recruit and activate inflammatory cells, which further increases inflammation. IL-12, by initiating and maintaining Th1 responses, was thought to be responsible for overreacting immune and auto-immune disorders. This was also the case in fungal infections where immunoregulation proved to be essential in fine-tuning inflammation and uncontrolled Th1/Th2 antifungal reactivity [8, 12, 14]. Over the past several years, the demise of a Th1/Th2 dichotomy paradigm has been accompanied by a renaissance in probing the basic tenets of CD4+ T cell biology. As a result, instead of only two distinct "fates" for developing T cells, research has identified alternative fates and more flexibility in T cell cytokine production than previously envisioned. Th17 cells are now thought to be a separate lineage of effector Th cells contributing to immune pathogenesis previously attributed to the Th1 lineage [47, 48]. Although the importance of the IL-17 cytokine family, and in particular of IL-17A and IL-17F, has been known for years, it is only recently that IL-17–producing T cells have been recognised as constituting a separate subset of T cells, termed Th17, in which STAT3 as well as retinoic acid receptor-related orphan receptor (ROR)γ – and more recently, RORα [49] – mediate lineage specification. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22. Th17 cell activation has been linked

with the pro-inflammatory cytokine IL-23, because IL-23-deficient mice contain very few Th17 cells and are protected from auto-immune diseases [50]. However, although IL-23 seems to be involved in Th17-mediated antimicrobial protection and in immune pathology, it is not required for the differentiation of Th17 from naive CD4 T cells in that they lack an IL-23 receptor [47]. In contrast, CD4+ T cells cultured with TGF-β, IL-1β and IL-6 express RORγ and become Th17 cells that are stabilised by dendritic cells (DC)-derived IL-23 [51]. In the relative absence of inflammatory innate stimuli, naive mouse CD4+ T cells upregulate expression of the transcription factor Foxp3 (forkhead box P3) in the presence of TGF-β and IL-2 (Fig. 1) and develop into "inducible" Tregs (iTregs), which suppress immune responses. Th17 cells, which produce IL-17A preferentially, although linked to the resistance to several bacterial and parasitic infections, correlate with disease severity and immunopathology in diverse infections [52]. With growing understanding of the contribution of the IL23/Th17 axis to various organ-related auto-immune and inflammatory diseases, there has been an interest in targeting many aspects of this pathway for therapeutic interventions [53].

IL-12 and IL-23 are members of a family of pro-inflammatory heterodimeric cytokines that share a common p40 subunit linked to the IL-12p35 chain or the IL-23p19 chain [28]. IL-17A has a non-redundant role in neutrophil recruitment [33], and IL-23 regulates PMN homeostasis [54]. However, the activity of IL-23 and IL-17A affects both the innate and adaptive response to fungi. Although IL-17A contributed to neutrophil mobilisation in disseminated candidosis [55] and participated in host defence to *Pneumocystis jirovecii* [56], it has been demonstrated that it is the Th17 pathway – and not the uncontrolled Th1 response – that is associated with defective pathogen clearance, failure to resolve inflammation and to initiate protective immune responses to *Aspergillus* (Fig. 2). Activation of pathogenic Th17 cells accounted for susceptibility to aspergillosis under conditions of deficient p35 [35], TIR8/SIGIRR [57], or functional NADPH oxidase expression [14] (e.g., in CGD mice). In all of these settings, Th17 pathway expression – rather than an unrestrained Th1 response – correlated directly with defective pathogen clearance, and failure to resolve inflammation as well as initiate protective responses to *Aspergillus* (Fig. 2).

3.1 IL-23/Th17 and the Vicious Circle

In experimental pulmonary aspergillosis, IL-23 and IL-17A are rapidly produced at sites of infections [14, 35], particularly under conditions of increased TLR signalling [57]. In vitro, inflammatory DC are a major source of IL-23 in response to *Aspergillus* [35]. However, PMN, and particularly those from IL-12 p35-deficient mice, appear to produce IL-23 when stimulated with *Aspergillus* in vitro. PMN also produce IL-17A in response to either organism in vitro. In vivo, however, the Vγ1+ subset of TCR γδ T cells is a major source of IL-17A in the lungs of conventional and CGD mice with invasive pulmonary aspergillosis [14]. Therefore, although their cellular sources may depend on sites of infection, the production

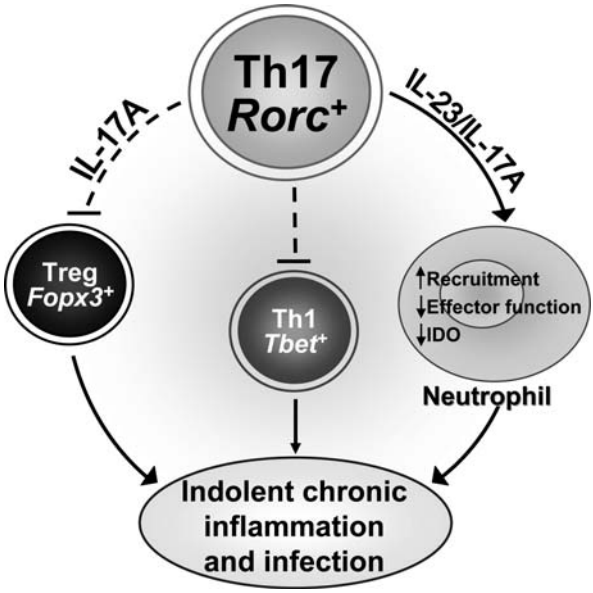

Fig. 2 The action of the Th17 pathway in *Aspergillus* diseases. The figure illustrates possible mechanisms of inflammatory activity, prevention of fungal clearance and subversion of regulatory T cell (Treg) and Th1 functions by Th17 cells in responses to *Aspergillus* (see text for details). Solid and broken lines refer to positive and negative signals, respectively

of IL-23 and IL-17A is augmented in condition of IL-12 p35 deficiency, and this worsens local disease [35]. Both IL-23 and IL-17 impaired the antifungal effector activities of neutrophils even in the presence of IFN-γ, a finding suggesting that the Th17 effector pathway prevails over the Th1 pathway. As a matter of fact, IL-12 and IL-23 cross-regulated each other and therefore, the relative levels of these cytokines may be the key to the outcome of infection. In addition, both cytokines activated the inflammatory program of neutrophils by counteracting the IFN-γ dependent activation of indoleamine 2,3-dioxygenase (IDO), known to limit the inflammatory status of neutrophils against fungi [18, 58] (see below), as well as by inducing the release of metalloproteinases and oxidants, which likely accounts for the high inflammatory pathology and tissue destruction associated with Th17 cell activation. The above findings, together with the ability of both IL-17 and IL-23 to reduce apoptosis of neutrophils, are thus consistent with the results obtained in IL-17 receptor-deficient mice with disseminated candidosis [55]. As neutralisation of IL-17 or IL-23 greatly reduced fungal burden and corrected inflammation in wild-type and more so p35-deficient mice [35], this suggests that while PMN ought to be present to control systemic infection and may become activated in an autocrine fashion, optimal protection requires a staged response, involving timely restriction of PMN inflammatory potential. Fungal persistence might otherwise occur and further stimulate IL-23 production by both DC and PMN, thus perpetuating inflammation and maintaining IL-17 production in a vicious circle [59]. Eventually, failure to

down-regulate microbe-induced expression of IL-17 could be one major link connecting infection with chronic inflammation.

The finding that IL-23 and IL-17 promote inflammation while subverting protective antifungal immunity may serve to accommodate the paradoxical association of chronic inflammatory responses with intractable forms of fungal infections where fungal persistence occurs in the face of an ongoing inflammation. In this scenario, the unrestricted fungal growth will result from the activation of not only pathogenic Th17 cells but also non-protective Th2 cells, whose activation is strictly dependent on fungal burden [14]. Ultimately, this may fit with the well known plasticity of human CD4+ T cell differentiation including the potential "interconvertability" of Th17 subsets [60]. Importantly, Th17 reactivity also limits the therapeutic efficacy of polyenes in experimental aspergillosis (author's unpublished observation), a finding suggesting that Th17 cells may adversely affect the clinical response to antifungal chemotherapy. Collectively, these findings point to strategies for immune therapy of infections that attempt to limit inflammation to stimulate an effective immune response. As a matter of fact, IL-23 or IL-17 neutralisation increased fungal clearance, ameliorated inflammatory pathology and restored protective Th1 antifungal resistance [8].

4 Dampening Inflammation and Allergy to *Aspergillus*: Crosstalk Between Treg Subsets

To limit the pathologic consequences of an excessive inflammatory cell-mediated reaction, the immune system resorts to a number of protective mechanisms. CD4+ T cells making immunoregulatory cytokines such as IL-10, TGF-β and IL-4 have long been known and discussed in terms of immune deviation or class regulation [61]. A number of clinical observations suggest an inverse relationship between IFN-γ and IL-10 production in patients with aspergillosis [62, 63]. High levels of IL-10, negatively affecting IFN-γ production, are detected in neutropenic or HSCT patients with aspergillosis. Recently, it has been suggested that, rather than causing the infection, IL-10 production may be a consequence of the infection [12]. This would predict that, in the case of chronic fungal infections, characterised by a state of chronic inflammation, IL-10 could be the homeostatic host-driven response aimed at keeping, however possible, inflammation under control. Tregs with tolerogenic activity have been described in fungal infections of both mice [18, 36, 64, 65] and humans [66]. Some cells with this function, such as CD4+ Foxp3+ natural Tregs (nTregs), originate in the thymus and pre-exist prior to infections whereas others may be induced as a consequence of infection (iTregs) or in conditions of impaired co-stimulatory signalling and in the presence of deactivating cytokines and drugs. As already mentioned, a reciprocal relationship has been described between the development of Foxp3+ Tregs and effector Th17 cells so that naive T cell activation in the presence of innate stimuli divert iTreg generation to Th17 generation.

With pathogens like fungi that have a complex pathogenesis (the reader is referred to the chapter by Drs. Ben-Ami and Kontoyiannis for more on the pathogenesis of IA), multiple types of regulatory cells could influence the outcome [12, 67]. A fine balance is established between Treg responses, effector components of immunity and the pathogen. The consequence of Treg activity is less damage to the host but also fungal persistence. However, the Treg responses may handicap the efficacy of protective immunity. Although the distinction between nTregs and iTregs in vivo is not always clear, particularly in highly inflammatory settings [68], recent data indicate that fungal growth, inflammatory immunity, and tolerance to *Aspergillus* in the respiratory tract were all controlled by the coordinate activation of nTregs – limiting early inflammation at the sites of infection – and pathogen-induced iTregs, which regulated the expression of adaptive Th immunity in secondary lymphoid organs. Early in infection, inflammation was controlled by the expansion, activation and local recruitment of nTregs suppressing PMN through the combined actions of IL-10 and cytotoxic T lymphocyte antigen (CTLA)-4 acting on IDO (see below). Late in infection, and similarly in allergy, the inflammatory immunity was modulated by iTregs, which acted through activation of IDO in DC and prevented Th17 cell development [18, 29]. Both IL-17A and IL-17F may contribute to the expression of airway inflammation and pulmonary hyper-reactivity; free soluble IL-17A is increased in asthma [33], and allergic cellular and humoral responses are suppressed in IL-17–deficient mice [69]. These findings indicate that the Th17 pathway may also be involved in fungal-associated allergic lung diseases. Given the contribution of the Th17 pathway to inflammation and deregulated immunity to the fungus, this finding further highlights the central role of Tregs in immunity and tolerance to the fungus and supports the concept of "protective tolerance" to fungi, implying that a host's immune defence may be adequate for protection without necessarily eliminating fungal pathogens or causing an unacceptable level of tissue damage [12].

5 Immunity or Tolerance to *Aspergillus*: The Contribution of the Tryptophan Metabolic Pathway

Continued integration of pro-inflammatory and anti-inflammatory stimuli is mandatory for a proper control of infection and T-cell homeostasis. The IDO mechanism has revealed an unexpected potential in the control of inflammation, infection and allergic airway inflammation [70]. IDO has a complex role in immunoregulation in infection, pregnancy, auto-immunity, transplantation, and neoplasia [71, 72]. IDO-expressing DC are regarded as regulatory DC specialised to cause antigen-specific deletional tolerance or induction of CD4+CD25+ Tregs [73]. These findings establish a mutual interaction between DC and Tregs for the upkeep of immunological tolerance. IDO expression is paradoxically up-regulated in patients with allergy or auto-immune inflammation, a finding suggesting the occurrence of a homeostatic mechanism to halt ongoing inflammation [59]. In experimental aspergillosis, IDO blockade greatly exacerbated infections and the associated inflammatory pathology

as a result of deregulated innate and adaptive immune responses caused by the impaired activation and functioning of suppressor CD4+CD25+ Tregs producing IL-10 [18].

Mechanistically, the level of inflammation and IFN-γ in the early stage of contact with the fungus set the subsequent adaptive stage by conditioning the IDO-dependent tolerogenic program of DC and the subsequent activation and expansion of tolerogenic Tregs preventing allergy to the fungus. More recently, while capable of inducing the Foxp3-encoding gene transcriptionally, tryptophan catabolites were also found to suppress RORγt, the Th17 lineage specification factor [34]. Thus, IDO and the IL-23/Th17 axis have opposite roles of in regulation of iTregs/Th17 balance at the host/pathogen interface. The detrimental side of the inflammatory action of an unopposed IL-23/IL-17 pathway, is – under physiological condition – restrained by IDO, through a mechanism leading to the sequential generation of regulatory and anti-inflammatory Vγ4+ γδ and CD25+ αβ T cells [14]. This further emphasizes the pivotal role of tryptophan catabolism and IDO in tolerogenesis and prevention of auto-immune inflammation and allergy to the fungus (Fig. 3).

Collectively, regulatory mechanisms operating in the control of inflammation and allergy to the fungus are different but interdependent as the level of the inflammatory response early in infection may impact on susceptibility to allergy, in conditions

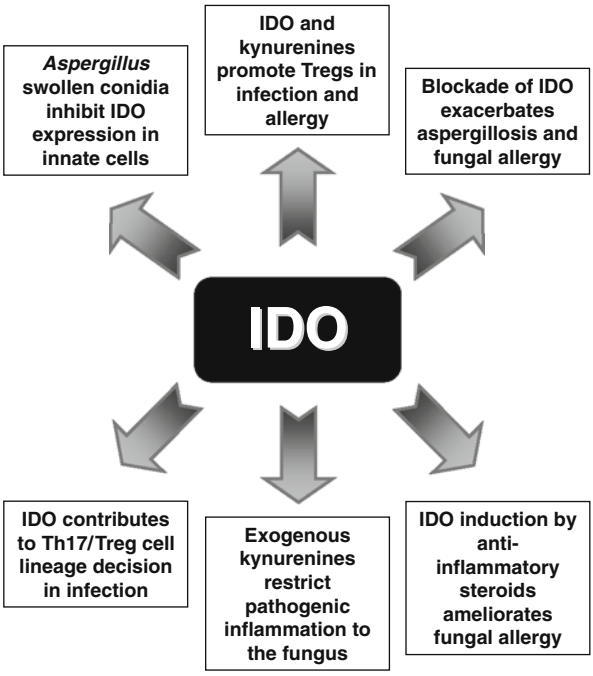

Fig. 3 The multiple activity of IDO in aspergillosis. The figure illustrates the multiple activities of IDO (indoleamine 2,3-dioxygenase enzyme) and tryptophan metabolites in *Aspergillus* infection and allergy (see text for details)

of continuous exposure to the fungus. Thus, a unifying mechanism linking nTregs to tolerogenic iTregs via IDO appears to be at work in response to the fungus and is consistent with the revisited "hygiene hypothesis" of allergy in infections – that is, an early reduction in microbial burden may predispose to allergy [74]. Because swollen *Aspergillus* conidia promote inflammatory responses by Treg inhibition [18], tolerance subversion may contribute to immunoevasion by the fungus.

6 Immunology of *Aspergillus*: Perspectives on Anti-inflammatory Strategies

6.1 Restraining Pathogenic Th17 Cell Inflammation by Exogenous Kynurenines

Inflammation regulated by different pathways is important for defence against a variety of mucosal pathogens. However, the recent evidence indicating the detrimental side effect of the inflammatory action of an unopposed IL-23/IL-17 pathway may represent ground-breaking observations in our understanding of the intricacies of inflammation and refractory fungal infections [8, 9, 14]. The main message is that, contrary to the established dogma, and at least in specific settings, it is an exaggerated inflammatory response that compromises a host's ability to eradicate infection. In addition, the data in the CGD experimental model reveal a new mechanism through which the absence of reactive oxygen species – otherwise associated with neutrophil-dependent oxidative stress – causes a distinct form of pathogenic inflammation and potential immune pathology via unrestrained γδ T-cell activity and IL-17 overproduction [14, 59]. In the experimental model of CGD, IL-17 neutralisation increased fungal clearance, ameliorated inflammatory pathology and restored protective Th1 antifungal resistance [14]. Perhaps more importantly, complete cure and reversal of the hyperinflammatory CGD phenotype were achieved by administration of supplemental l-kynurenine, an early amino acid catabolite of l-tryptophan in the IDO-dependent pathway [14].

Collectively, the new findings provide a molecular connection between the failure to resolve inflammation and lack of antifungal immune resistance and point to strategies for immune therapy of fungal infections that attempt to limit inflammation to stimulate an effective immune response. Further understanding of the cooperation of various multiple innate and adaptive immune responses in fungal infections will provide the basis for novel immunomodulatory therapies that are no longer just promising as adjuncts in the management of fungal infections but are strictly required to balance protective immunity and inflammatory pathology in infections. In this regard, IDO and tryptophan metabolites may prove to be potent regulators capable of taming overzealous or heightened inflammatory host responses to the benefit of pathogen eradication and host survival. Addition of recombinant IFN-γ to antifungal therapy may therefore result in not only augmentation but also modulation of immune responses through tryptophan metabolites (Table 1).

Table 1 Perspectives on anti-inflammatory strategy in invasive aspergillosis

Target	References
IL-17/IL-23 antagonists	[35]
Exogenous kynurenines	[14]
IDO targeting in allergy	[29]
IDO+DC as fungal vaccines in transplantation	[85]

Legend: IL, interleukin; DC, dendritic cells; IDO, indoleamine 2,3-dioxygenase enzyme.

6.2 Targeting IDO-Mediated Immune Homeostasis in Fungal Allergy by Steroids

ABPA is a Th2-sustained allergic condition of the lung that is responsive to steroid treatment [75]. This condition is discussed deeply in other sections of this book. Experimental models of the disease have been used to demonstrate a pivotal role for Treg cells [18], plasmacytoid DC [29] and tryptophan catabolism [70] in protecting mice from allergic airway inflammation. A recent study has shown the IDO-dependent effects of dexamethasone on the hypersensitivity response to *Aspergillus* antigens in the murine lung [29]. Consistent with the responsiveness of ABPA to steroid treatment [75], the Th2-allergic phenotype was greatly attenuated by dexamethasone, which enhanced production of IL-10 and enhanced Foxp3 transcripts, both markers of protective Treg activity in *Aspergillus* allergy. The data demonstrate that dexamethasone down-regulates exacerbating Th2 responses in ABPA by inhibiting the expansion and activation of Th2 cells and up-regulates the expression of Foxp3 via mechanisms that require tryptophan catabolism [29]. In this model, modulation of tryptophan catabolism via the glucocorticoid-induced tumour necrosis factor receptor (GITR) and its ligand, GITRL, inhibited Th2-cell responses and allergy and induced the expression of Foxp3+ Tregs through mechanisms dependent on IDO induction by components of the noncanonical NF-.B signalling pathway [70]. Thus induction of IDO could be an important mechanism underlying the anti-inflammatory action of steroids. Overall, these data suggest that IDO modulation by non-canonical NF-κB is essential for the maintenance of TLR-driven immune homeostasis in the airways. It is conceivable that fungal aeroantigen is prevented from initiating airway inflammation by the integrity and antimicrobial defence of the epithelium, in an environment in which TLR9-driven induction of IDO and consequent inhibition of Th2 cells will contrast the onset of allergic inflammation. Asymptomatic atopy associated with increased IDO activity and IL-10 production in seasonal allergen exposure has been described [76]. Clinical trials of TLR-9-based immunotherapy are presently ongoing, suggesting that "TLR-9 ligands may revolutionize the treatment of allergic diseases" [77]. As the effects of treatments with antifungal agents on symptoms and clinical findings in patients with allergic fungal diseases are controversial [75], targeting IDO-mediated immune homeostasis in *Aspergillus* diseases is a promising option (Table 1).

6.3 Exploiting IDO+DC as Fungal Vaccines in Transplantation

Developments in DC biology are providing opportunities for improved strategies for the prevention and management of fungal diseases in immunocompromised patients. The finding that *Aspergillus*-pulsed DC could act as a vaccine in haematopoietic transplantation [78] raised excitement about vaccination against aspergillosis [79]. At a time when vaccination of HSCT recipients is highly recommended [80], developing new strategies to expand and modulate the functions of distinct DC subsets associated with specific regulation of host immunity may provide novel immune-based therapies in HSCT [81]. Within the instructive model of DC-mediated regulation of the Th repertoire, it is conceivable that an improved understanding of the pathogen/DC interaction will allow the potential use of pathogen- or TLR-conditioned DC for the induction of patient-tailored Tregs with indirect anti-donor allospecificity. Over recent years experimental models have shown that it is possible to exploit the mechanism that normally maintain immune homeostasis and tolerance to self-antigens to induce tolerance to alloantigens [82, 83]. Like natural tolerance, transplantation tolerance is achieved through control of T cell reactivity by central and peripheral mechanisms of tolerance. We have recently found that this goal is achievable by the adoptive cellular therapy of *Aspergillus*-pulsed IDO+DC that could induce antifungal resistance within a regulatory environment [84]. Tolerogenic IDO+DC proved to be pivotal in the generation of some form of dominant regulation that ultimately controlled inflammation, pathogen immunity and tolerance in transplant recipients [85], eventually leading to prevention of graft versus host reaction and reduction of aspergillosis incidence rates (Table 1).

7 Concluding Remarks

Aspergillosis is an uncommon disease that predominantly affects patients with deregulated immunity. The heterogeneity of patient populations at risk for IA is another challenge in developing broadly effective immunotherapeutics. The newly described Th17 pathway may serve to support the pathophysiological hypothesis that severe aspergillosis belongs to the spectrum of fungus-related immune reconstitution inflammatory syndrome. The greatest challenge now is to pave the way from promising results in experimental models to the clinic. Clinical studies are needed to confirm whether combination antifungal therapy aimed at limiting inflammation in high-risk patients will result in better prevention and control of the infection, as already shown for chronic disseminated candidosis [86]. Similarly, as progress in gene therapy in CGD has been hampered by the appearance of insertional mutagenesis [87], the finding points to the need for alternative new therapeutic options for patients with CGD. In addition to newer classes of drugs and immune-modulating therapies [88], targeted anti-inflammatory strategies and vaccines are exciting future additions to the arsenal against IA in difficult-to-treat patients. This implicates that both fungal burden and inflammatory responses should be included

as pharmacodynamic endpoints in the preclinical evaluation of antifungal drugs and immunologic correlates should be included as secondary endpoints in future clinical trials [14].

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Part IA
Aspergillus and the Mycology Laboratory

The Importance of Conventional Methods: Microscopy and Culture

Elizabeth M. Johnson and Andrew M. Borman

Abstract Phenotypic identification of *Aspergillus* species centres on a careful examination of the colonial and microscopic morphology of an isolate and can commence as soon as a culture has been isolated from a clinical specimen. Colonial form, texture, colour and growth rate is noted. Microscopic mounts are made from the surface growth and a careful examination is made of the spores and spore-bearing structures. Until recently phenotypic morphological and physiological, including extrolite production, criteria were the only characteristics upon which classification and subsequent identification could be made. Even in the current genomic era, classification schemes based on phenotypic methods have proved remarkably resilient. However, variability in the phenotypic characteristics of certain common and important groups such as *Aspergillus* section *Fumigati* has led to problems in classification and errors in the literature. For this reason a polyphasic approach incorporating the phenotypic characters of gross and microscopic morphology, physiology, extrolite production and molecular data where all are given due consideration and equal weighting has been suggested as the best approach to species differentiation. It is therefore important that skill levels in the morphological recognition and identification of *Aspergillus* species are maintained.

Keywords *Aspergillus* · Colonial morphology · Identification · Microscopic morphology · Taxonomy

Contents

E.M. Johnson (✉)
Mycology Reference Laboratory, Health Protection Agency, Bristol, UK
e-mail: elizabeth.johnson@UHBristol.nhs.uk

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1 Introduction

Aspergillus is a large genus comprised of more than 250 species and this number is likely to increase as new species are identified and the concept of species is further refined [1]. It contains a number of important pathogens many of which are also regularly encountered as culture contaminants. As different *Aspergillus* species differ in their pathogenicity and response to different antifungal agents it is important to establish an accurate identification for a clinical isolate as rapidly as possible. The most comprehensive classification of *Aspergillus* species was published by Raper and Fennel in 1965 [2] in which they described 132 species subdivided into 18 groups based solely on detailed analysis of colonial and microscopic morphology and physiological characteristics. The groupings were re-examined by Gams et al. [3] and given formal taxonomic status as sections (18) with 6 subgenera added. Since that time there have been several modifications suggested based on molecular data and introduction of new species, see Geiser et al. [1] for an excellent summary of the current status of species recognition and identification. However, despite recent advances in the molecular identification of *Aspergillus* species, direct microscopy of a growing culture remains an essential tool for rapid identification to species level in most cases and complex level in others. Molecular methods will prove invaluable when an organism cannot be isolated in culture, when subtle differences within a complex are not readily visible under light microscopy or when unstable phenotypic characteristics have been lost as may happen in chronic infections. It is certainly recognised that some clinical isolates of *Aspergillus* species may be slow to sporulate or display aberrant microscopic characteristics, which can make phenotypic identification difficult or impossible. Molecular methods are also helping to elucidate relationships between currently recognized taxonomic sections. However, in most cases molecular methods applied to a growing culture are likely to be more time consuming than direct microscopy of that culture.

Phenotypic identification relies on careful examination of colonial morphology, including rate of growth, and the microscopic features of the spores and

spore-bearing structures in order to help differentiate the complex into which the isolate under examination belongs. Analysis of extrolites can also provide useful information although routine analysis of these is more often employed for isolates used in the food industry or causing food spoilage than for those causing infection. Use of a stereoscopic binocular microscope can reveal additional information about the spore distribution over the vesicle. Heads can be seen to be globose, globose splitting into columns or truly columnar. The increasing sophistication of photographic equipment and software packages capable of image analysis by pattern recognition may serve as a truly objective record of colonial form i.e. texture and colour and these have been used in the identification of *Penicillium* species [4].

Light microscopy is usually sufficient to identify an isolate to complex level but more sophisticated scanning electron microscopy may be necessary to distinguish subtle differences required for differentiating species within each complex. Recent genotypic studies also suggest that some distinctions are only possible at the molecular level when several genetically distinct species exist within a single morphospecies [5]. On occasion such isolates have been found to differ in their susceptibility to different antifungal agents so such distinction may be of clinical as well as scientific interest [6, 7]. This is discussed in more detail in the chapter on molecular methods for identification of *Aspergillus* species.

2 Culture Methods

Aspergillus species grow readily on most culture media and much can be ascertained from direct examination of the colonial morphology. Most current texts contain descriptions of colonies cultured on defined laboratory media such as Sabouraud agar (glucose peptone agar), Czapek agar, Czapek yeast autolysate agar, oat meal agar, potato dextrose agar or malt extract agar with or without additional glucose. As the composition of the medium may affect certain colonial characteristics such as colour and growth rate it is important to be cognisant of the growth medium. It is also important to consider the age of the culture under examination as some structures only develop with time. The temperature of incubation can also help to distinguish between species, although many are recognised as being thermotolerant, a temperature of 42°C can help to distinguish the more pathogenic *Aspergillus* species such as *Aspergillus fumigatus* group. Diagnostic specimens should routinely be cultured at 30°C and 37°C but for more accurate identification prolonged incubation at 25°C may be necessary for some species.

Asporic or poorly sporulating cultures may be encouraged to sporulate by subculture onto rich media such as malt agar or potato sucrose agar and incubation in a site where they will be exposed to natural rhythms of light and dark (diurnal cycles). Such manipulations may prove time-consuming as even under these conditions it can often take several weeks for cultures to initiate sporulation, if at all. This is clearly one of the circumstances when molecular identification methods can prove time efficient.

3 Generic Description of *Aspergillus* Species

Aspergillus species usually produce rapidly growing, more or less powdery or granular white, green, black, brown or yellow colonies. On microscopic examination unbranching conidiophores are produced from foot cells. Colourless or pigmented, rough or smooth conidiophores produce a swollen vesicle of variable shape at their tip, which bear conidium-forming cells known as phialides either directly on the vesicle (uniseriate) or on a layer of cells known as metulae (biseriate). Long chains of dry spores (conidia) are produced in basipetal succession (youngest spore at the base of the chain) from each phialide, either over the entire surface of the vesicle resulting in radiate sporing heads, or just from the top portion of the vesicle resulting in columnar sporing heads. Spores are globose to sub-globose in shape and range in surface ornamentation from smooth to echinulate (spiny).

Some *Aspergillus* species have a recognised sexual state (teleomorph) and in these isolates sexual structures known as cleistothecia (ascomata) may be seen producing asci containing ascopsores which are variously ornamented. Where a sexual and asexual state is encountered the name of the sexual form usually takes precedence in identifying the organism. Thick-walled Hülle cells are produced by several *Aspergillus* complexes mainly as a protective structure for cleistothecia. Sclerotial bodies, formed by aggregations of hyphae into hard structures, are also a feature of several complexes as are aggregations of conidiophores known as coremia.

3.1 Features of Colonial Morphology Useful for Identification

- Colour of surface and reverse
- Rate of growth (colony diameter)
- Texture (velvety, powdery, granular, floccose)
- Zonation
- Form of sporing heads (columnar, radiate)
- Diffusing pigment
- Sclerotial bodies
- Cleistothecia
- Coremia
- Odour (suitable only for corroborative evidence)

4 Analysis of Extrolite Production

Although not routinely performed by many laboratories those with an interest in the phenotypic identification of *Aspergillus* species may at times find that analysis of extrolite production is a useful adjunct to morphological methods. Most often it is analysis of the production of secondary metabolites but also the mycotoxins: aflatoxin, gliotoxin and ochratoxin A, by pure cultures that proves to be a reliable

and consistent characteristic for each species and is helpful in species differentiation [8]. Important extrolites that aid species identification have been comprehensively summarised and tabulated by Samson et al. [9]. Newer analytical methods for determining the composition of different fungal species such as Matrix-Assisted Laser Desorption/Ionisation – Time Of Flight (MALDI-TOF) and Surface Enhanced Laser Desorption/Ionisation – Time Of Flight (SELDI-TOF) mass spectrometry have not been widely validated on fungal species but may prove a useful future addition to morphological methods [10].

5 Examination of Microscopic Morphology

The microscopic morphology of *Aspergillus* spp. is best examined by preparing a mount in a specialist mounting fluid such as lactophenol cotton blue or lactofuchsin. Lactofuchsin is more viscous than the former so may facilitate separation of hyphae for clearer microscopy. This should then be viewed initially under the × 10 objective, but × 40, × 60 or × 100 with oil immersion can help to reveal minute differences in the smoothness of a conidiophore or the surface ornamentation of spores.

5.1 Needle Mounts

A needle mount is prepared by picking up a small amount of the surface of the colony on the tip of a sharp needle. This is placed in a drop of mounting fluid on a glass slide, and then whilst holding it still with the first needle gently teasing out the mycelium with a second needle. Finally a coverslip is placed over the drop of fluid and tapped sharply several times with the blunt end of the needle to produce shock waves within the mounting fluid and thus further help to distribute the mycelium. This method is useful for heavily sporing cultures where the needles can be used to dig down below the surface layer of spores to pick up the conidiophores or to select macroscopically visible fruiting bodies for further examination. A modification of this technique can be used when the presence of multiple spores is obscuring observation of their mode of production. This entails gently washing the tip of the first needle with its adherent mycelium in a drop of mounting fluid before proceeding with preparation of the mount in a second drop of fluid.

5.2 Sellotape Mounts

A second method of preparing microscopic mounts, and one which has the advantage of preserving intact surface structures, is to make sellotape mounts. Double-sided sticky tape in a tape dispenser is easier to manipulate than single-sided tape. A small flag of tape is stuck to the tip of a needle and gently laid on the surface of the colony under examination a second needle is used to gently press the tape onto the culture. The first needle with its adherent tape is then lifted away from

the colony at which point visible material comprising fungal mycelium and spores can be seen adhering to the underside of the tape. The tape is then mounted on a microscope slide in a drop of mounting fluid with the fungal material uppermost, a second drop of mounting fluid is placed on the surface and covered with a coverslip which is gently pressed down to expel small air bubbles. The purpose of the initial drop of mounting fluid is to improve the optics so that there are no air bubbles under the tape. Inverting the tape before mounting means that fungal structures can be viewed directly through the glass coverslip without looking through a layer of tape, which may obscure details such as delicate roughening. If a culture is heavily sporing then tape mounts are best prepared from the advancing edge of the colony where the fungal mycelium is youngest and fewer spores are produced, in the older parts of the colony the tape may merely succeed in capturing a layer of chains of spores.

5.3 Slide Culture

Slide culture is more time-consuming as it requires further sub-culture rather than direct examination of the available culture. A block of agar about 1 cm^2 cut from a plate of any agar designed to enhance spore production is placed on the surface of a sterile glass slide then, by means of a loop or needle, inoculated around the edges with the fungus under study. A sterile coverslip is placed over the agar and the slide incubated in a moist chamber for a week or more until there is visible growth at the sides of the agar block and spread as a single layer onto the underside of the coverslip and onto the glass slide. The coverslip is harvested by placing it on a drop of mounting fluid on a glass slide, the block of agar is discarded and a drop of mounting fluid placed on the original slide and covered with a coverslip. This results in the formation of two slide-culture preparations in which the structures have been subjected to minimal disruption and therefore remain intact and in a single plane of view.

5.4 Preservation of Microscopic Mounts

Needle mounts or slide culture mounts can be preserved for later examination, photography or for addition to a library of fungal species by blotting off excess mounting fluid and sealing around the edges of the coverslip with a proprietary mounting fluid. It is not possible to preserve sellotape mounts for later examination as after just a few hours the mounting fluid starts to degrade the tape and can obscure delicate microscopic features.

5.5 Features of Microscopic Morphology Useful for Identification

- Size and shape of vesicle
- Colour, surface roughening and length of conidiophore

- The foot cell
- Presence or absence of metulae
- Formation of sporing heads
- Size and shape of spores
- Surface ornamentation of spores
- Cleistothecia
- Sclerotial bodies
- Size, shape and surface ornamentation of ascospores
- Hülle cells

5.5.1 Conidiophore and Vesicle

The conidiophore, or stalk that bears the conidiogenous (conidia or spore-forming) cells, swells at the tip to form a vesicle the shape of which can vary from globose to the elongated club-shaped seen in *A. clavatus* and is a useful identification feature (Fig. 1). The vesicle thereby provides a structure with a large surface area for the production of spore-bearing cells (phialides). Conidiophores and vesicles may be hyaline or pigmented and rough or smooth – such features are useful for identification purposes. Conidiophores and vesicles are usually upright but in some species may bend slightly towards the substrate (nodding heads) or may be definitely curved towards the substrate (deflected heads). In some species such as *A. versicolor* (Fig. 2) the heads with typical "spoon-shaped" vesicles may be accompanied by reduced heads, which can be mistaken for those of *Penicillium* spp. as they lack the swollen vesicle at the tip of the conidiophore.

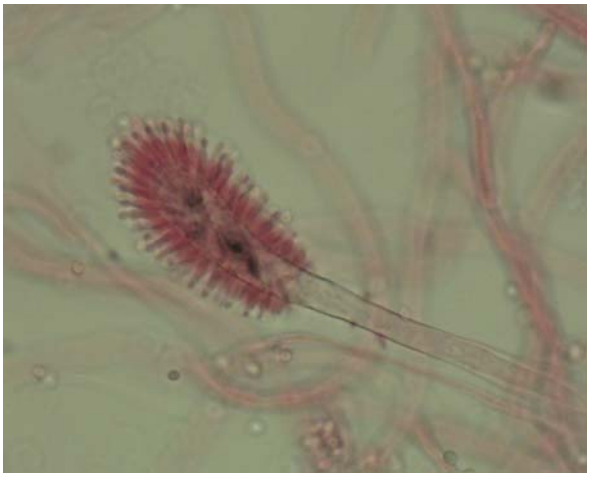

Fig. 1 *Aspergillus clavatus* clavate or "club-shaped" vesicle with a single layer of phialides

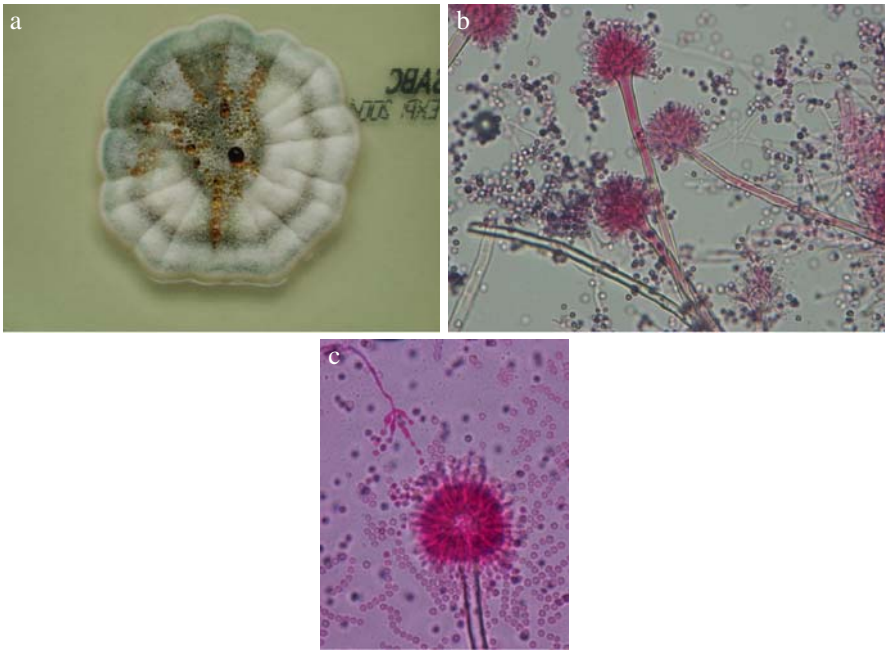

Fig. 2 *Aspergillus versicolor* (**a**) colonial form; (**b**) "spoon-shaped" vesicle; (**c**) reduced or *Penicillium*-type heads

5.5.2 Foot Cells

In some *Aspergillus* species there is an obvious and recognisable foot cell at the base of the conidiophore. These are thick-walled cells from which the conidiophore is produced usually at right angles to the mycelium.

5.5.3 Metulae

Metulae (singular – metula) is the name given to the layer of cells surrounding the vesicle from which phialides are formed. Not all *Aspergillus* species produce metulae and their presence or absence can be a very useful feature to aid identification. However, even within a single strain of some species e.g. *Aspergillus glaucus,* some heads do produce metulae whilst in others the phialides are produced directly from the vesicle. In order to determine whether there is a single (monoseriate) or double layer of cells (biseriate) between the vesicle and the start of the spore chains, it may be necessary to vary the focus. Another clue that a second layer of cells, the metulae, may be present is the distance between the vesicle and the start of the chains of spores (Fig. 3a).

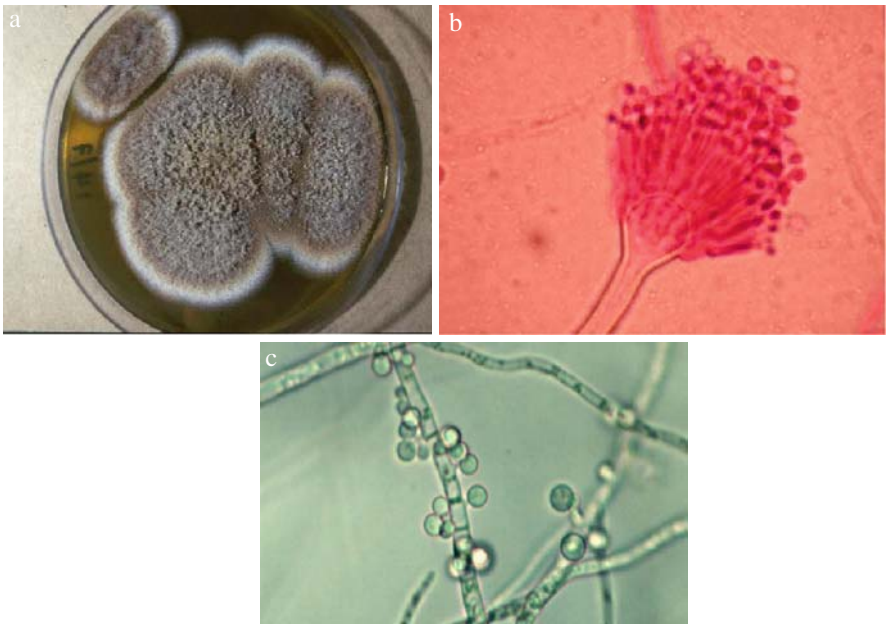

Fig. 3 *Aspergillus terreus* (**a**) colonial form; (**b**) conidial heads showing the presence of metulae and long, delicate phialides fertile only over the upper surface of the vesicle; (**c**) sub-agar chlamydospores

5.5.4 Phialides

Phialides are the bottle-shaped structures, formed directly on the vesicle at the tip of the conidiophore or separated from it by a layer of cells known as metulae, through which long chains of enteroblastic spores are produced in basipetal (youngest at the base) succession. Spores are formed from the inner cell wall only and are extruded through a pore at the tip of the phialide.

5.5.5 Spore or Conidium

The asexual spores or conidia of *Aspergillus* species are formed from within phialides, they are produced in long, dry chains suited for air dispersal. They may be variously pigmented which determines the colour of the heads and therefore the colony colour, and display degrees of surface ornamentation ranging from smooth to obviously echinulate (spiny), features that should be visible under the light microscope. The conidia are formed in chains with more or less prominent disjunctor regions however this feature is not helpful in species separation. The colour, form and ornamentation of the spores are useful features to note in phenotypic identification.

5.5.6 Cleistothecia

Cleistothecia (or ascocarps) are the sexual structures formed by the teleomorphs of *Aspergillus* species. They are closed fruiting bodies containing asci, which produce the sexual ascospores by meiosis. At maturity the walls of the cleistothecia are composed of a thin layer of flattened cells which split open to release the asci and ascospores. In *Emericella nidulans,* the teleomorph of *A. nidulans,* cleistothecia are dark purple, about 150 μm in diameter and often surrounded by Hülle cells, whilst in the *A. glaucus* group they are small, yellow and naked borne on a single hypha. Cleistothecia are often visible to the naked eye. Figure 4 depicts a cleistothecium of *E. nidulans* containing numerous asci, surrounded by Hülle cells and alongside a characteristic, darkly pigmented conidiophore with metulae and phialides in order to give an idea of the relative sizes of the two structures. With the exception of three known species, the first described being the aptly named *Aspergillus heterothallicus* [11], the cleistothecia of *Aspergillus* species appear to be homothallic requiring only one mating type.

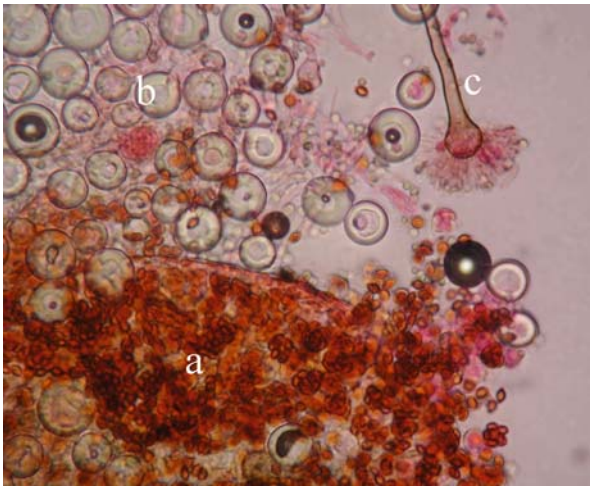

Fig. 4 *Emericella nidulans* (**a**) cleistotecium containing numerous asci; (**b**) Hülle cells surrounding the cleistothecium; (**c**) darkly pigmented conidiophore (note the relative sizes of cleistothecium and conidiophore)

5.5.7 Ascospores

These are the sexual spores formed by meiosis within asci enclosed in cleistothecia. With few exceptions the asci contain eight ascospores. In different species of *Aspergilli* they are variously ornamented; roughened, echinulate (spiny) or ridged often with single or double equatorial crests formed at the junction of what looks like a double convex lens, however these features are often only visible under scanning electron microscopy (see Table 1).

Table 1 Phenotypic features of some key species within *Aspergillus* Section *Fumigati*

Taxon	Colony[a]	Conidial shape/ornamentation[a]	Ascospores[a] (decoration/equatorial crests)	Max. growth temp			References
				45°C	48°C	50°C	
A. brevipes	Restricted, velvety, yellow–green–olive	Globose, echinulate	NR[b]	–	–	–	[21]
A. duricaulis	Restricted, lily/olive green	Globose, echinulate	NR	–	–	–	[2]
A. fumigatus	Velvety to powdery; blue–green–grey	Globose, lobate-reticulate	NR	+	+	+	[22]
A. fumigatiaffinis	Floccose, white/dull green, poorly sporing	Globose, smooth	NR	–	–	–	[23]
A. fumisynnematous	Floccose; grey/green	Ellipsoidal, microtuberculate	NR	+	–	–	[24]
A. lentulus	Whitish, velvety; poorly sporing	Globose, microtuberculate	NR	+	–	–	[6]
A. novofumigatus	Floccose, deep green/grey green	Ellipsoidal, smooth	NR	–	–	–	[23]
A. thermomutatus (N. pseudofischeri)	Whitish, velvety; poorly sporing	Sub-globose, smooth	Ribbed, 2 flexuous crests	+	(+)[c]	–	[25]
A. turcosus	Velvety, grey–turquoise–green	Sub-globose, smooth	NR	+	+	+	[26]
A. viridinutans	Velvety; sage green; Nodding vesicles	Globose, smooth/delicately roughened	NR	–	–	–	[27]
A. unilateralis	Velvety, slate-olive	Globose, coarsely echinulate	NR	–	–	–	[27]
N. assulata	Floccose, white, heavily sporing	Sub-globose, smooth	Large oval surface flaps, 2 crests	NA[d]			[26]
N.aureola	Floccose; yellow–orange–buff	Globose, lobate-reticulate	Echinulate, 2 prominent crests	NA			[28]
N. denticulata	Floccose, white; poorly sporing	Globose, smooth	Denticulate, prominent furrow	NA			[26]

Table 1 (continued)

Taxon	Colony[a]	Conidial shape/ornamentation[a]	Ascospores[a] (decoration/equatorial crests)	Max. growth temp			References
				45°C	48°C	50°C	
N. fennelliae	Floccose, green-blue	Globose, finely roughened	Delicately roughened, 2 crests	NA			[29]
N. fischeri	Floccose, deep olive/grey green	Sub-globose, finely roughened	Ridged, 2 ruffled crests	+	+	?[e]	[30]
N. galapagensis	Funiculose, white, sporing	Globose, usually smooth	Micro-tuberculate, 2 crests	NA			[26]
N. hiratsukae	Restricted, white, velvety, poorly sporing	Globose, delicately roughened	Reticulate, 2 compressed crests	?	–	–	[31]
N. quadricinta	Powdery, mineral/olive grey	Globose, delicately roughened	Spinulose/reticulate; 4 crests	–	–	–	[32]
N. udagawae	Floccose, dull green	Globose, lobate-reticulate	Tuberculate, irregular crests	(+)	–	–	[33]

[a]Where possible the descriptions of colonial appearance, and conidial and ascospore surface structure/decoration were taken from the original description of the species. Species associated with human and animal infections are in bold face.
[b]NR – not relevant. Ascomata and ascopsores have not been evidenced for these anamorphic states.
[c](+) poor growth at this temperature, or conflicting data from different authors.
[d]NA– data for the maximum growth temperature are not available for this species.
[e]?– unknown.

5.5.8 Hülle Cells

Hülle cells are a type of chlamydospore and are often found in large numbers surrounding cleistothecia (ascocarps) produced by the ascosporic species of *Aspergillus* and may provide a protective layer against desiccation and attack by soil dwelling animals although their true function is uncertain. Hülle cells are large thick-walled structures that may be terminal or intercalary and are ellipsoidal, globose and sometimes even horse-shoe shaped (Fig. 4). As the Hülle cells of different groups assume quite characteristic shapes they can be useful in the phenotypic identification process. They are capable of germination and thus may act as reproductive cells [2].

5.5.9 Coremia

Coremia formation is an uncommon feature formed by the aggregation of erect conidiophores. These can be visible to the naked eye as crown-shaped structures produced on the surface of the colony.

5.5.10 Sclerotia

Sclerotia are formed by the aggregation of parenchymal cells into characteristically-shaped compact hard masses. They are found in some members of certain groups but not others. There are also pseudo-sclerotial structures found in some groups and together with sclerotial bodies if present may help to provide clues to identification but are not definitive as their presence or absence is influenced by precise cultural conditions.

6 Descriptions of the Most Clinically Relevant *Aspergillus* Sections

Although there are many *Aspergillus* species from several different complexes that have been implicated in human disease [12] what follows is a discussion of the key sections that contain the most common clinically relevant species. For detailed descriptions of colonial and microscopic morphology of other *Aspergillus* species see references [2, 12, 13].

6.1 Aspergillus *Section* Fumigati

A review of the literature to January 2008 reveals that *Aspergillus* section *Fumigati* now comprises in excess of 29 species of *Neosartorya* and 14 species of *Aspergillus*. Unfortunately, the descriptions and available phenotypic data for many of these species rely on single isolations, and no systematic comparison of all putative members of the section has been performed. For the better characterised species, some key phenotypic and morphological features are summarised in Table 1. It is clear that

certain of the criteria suggested to distinguish between the various species (conidial and ascospore surface decoration/ornamentation) are open to the vagaries of personal interpretation and also require careful electron microscopic examination. Additional characters, including detailed extrolite and mycotoxin profiles, homo-/heterothallism and the sequences of combinations of the β-tubulin, calmodulin, actin, and hydrophobin genes and internal transcribed spacer regions have been evaluated for certain of the species. However, once again, these are not approaches that are readily amenable to the majority of microbiology/mycology laboratories. This chapter will thus describe the general morphological features of *Aspergillus fumigatus sensu strict* (s.s.), with mention of the key differences between *A. fumigatus* s.s. and the better characterised of the other members of *Aspergillus* section *Fumigati*.

Aspergillus fumigatus Fresenius is characterised by spreading velvety to floccose colonies that are white at first and rapidly become blue–green to dark grey–green upon sporulation (Fig. 5). Conidial heads are typically columnar, with smooth conidiophores that are frequently greenish and are borne from submerged hyphae or arise as short branches from emergent aerial hyphae. Conidiophores flare gradually to form an apical, clavate or flask-shaped vesicle, typically 20–30 μm in diameter. The upper half to two thirds of this vesicle directly bears uniseriate phialides, arranged closely parallel to each other and to the axis of the conidiophore. Conidia, which are green to blue-green in mass, are globose to sub-globose and echinulate, and typically measure 2.5–3 μm (Fig. 6). Cleistothecia and ascomata are never seen.

A. fumigatus however exhibits extremely variable cultural characteristics, and both white (*A. fumigatus* var. *albus*) and brown (*A. fumigatus* mut. *helvola*) colonial variants, which nonetheless are heavily sporulating, have been described (Fig. 5; and see for example reference [2]). White, poorly sporing colonies are also a frequent feature of *A. fumigatus* isolates cultured from deep, usually sterile body sites,

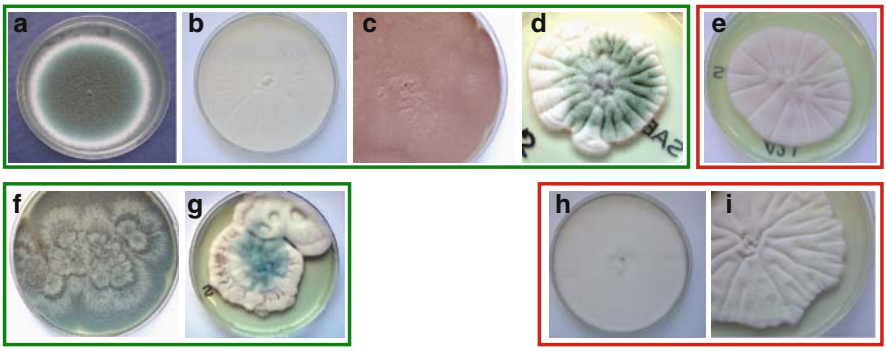

Fig. 5 Colonial appearance of *Aspergillus fumigatus*, its variants, and selected species within *Aspergillus* section *Fumigati*. Colonies were grown on Sabouraud agar at 30°C for 7 days. (**a**) *A. fumigatus sensu stricto*; (**b**) *A. fumigatus* var. *albus*; (**c**) *A. fumigatus* mut. *helvola*; (**d**) poorly-sporulating *A. fumigatus* (frequently seen in patients with chronic cavitary pulmonary aspergillosis); (**e**) non-sporing *A. fumigatus*; (**f**) *Neosartorya quadricinta*; (**g**) *A. fumigatiaffinis*; (**h**) *A. thermomutatus*; (**i**) *N. hiratsukae*. Colonies a–d, f and g produced typical *A. fumigatus* conidiophores and conidia; colonies e, h, i showed no evidence of sporulation

including the lungs of cystic fibrosis patients (Fig. 5). Our own molecular studies have confirmed that the vast majority of such isolates are *A. fumigatus* s.s. (unpublished data); unfortunately however, such colonial appearance, coupled with poor or slow sporulation are also features of several other members of *Aspergillus* section *Fumigati*, including *A. lentulus, A. fumigatiaffinis, A. thermomutatus, N. hiratsukae* and *N. denticulata* (Table 1).

It is also generally accepted that extensive variations in micromorphology are frequent in *A. fumigatus* s.s., with common variables including conidiophore colouration (which ranges from colourless to dark smoky grey) and vesicle size and shape, which can vary dramatically even within a single colony (Fig. 6). Asymmetrical, or nodding, vesicles are recognised characters shared by *A. brevipes, A. duricaulis, A. viridinutans* and *A. unilateralis*, but are not usually observed with *A. fumigatus* s.s.

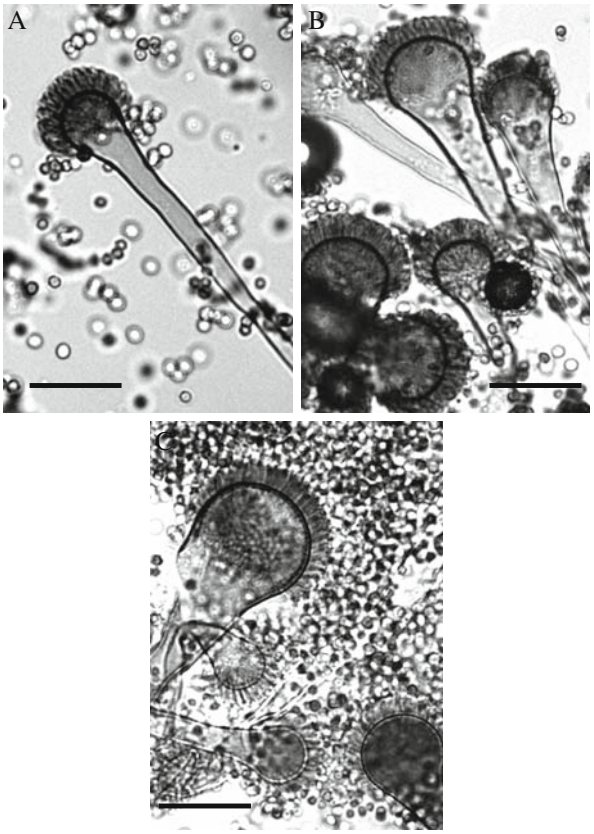

Fig. 6 Conidiophores and conidia of *Aspergillus fumigatus* sensu stricto. (**a**) a single typical conidiophore terminating in a *flask-shaped* vesicle bearing uniseriate phialides. Panels (**b**) and (**c**): highly variable vesicle sizes and conidiophore colorations observed with different isolates of *A. fumigatus*. Bar = 25 μm

A. fumigatus s.s. is thermophilic, and grows well in vitro at or even above 50°C, which is a useful, though not perfect, aid to identification. With the exception of *A. turcosus* (which can be distinguished from *A. fumigatus* by its smooth conidia), the reported maximum growth temperatures of the other members of *Aspergillus* section *Fumigati* lie in the 40–48°C range (Table 1). However, it is worth noting that at least one isolate of *A. fumigatus* s.s. studied at the Bristol Mycology Reference Laboratory repeatedly fails to grow at 50°C (unpublished data).

Finally, it is worth considering whether identification of members of *Aspergillus* section *Fumigati* to species level is of clinical benefit. At present, the clinical relevance of many of the species in *Aspergillus* section *Fumigati* is unclear. Excluding *A. fumigatus*, only *A. lentulus, N. pseudofischeri, N. fischeri, A. viridinutans, N. hiratsukae*, *A. fumisynnematus, A. fumigatiaffinis*, *N. quadricinta* and *N. fennelliae* have been isolated from clinical samples or proven mammalian infections, with the remaining species isolated sporadically from environmental sources. Moreover, one retrospective study revealed the mis-identification of *A. lentulus* as *A. fumigatus* in only 4 of 128 isolates from 82 stem cell transplant patients over a 10 year period [14], suggesting that these hitherto cryptic species within *Aspergillus* section *Fumigati* are relatively uncommon in the clinical setting. Our own unpublished data would support this contention: genetic analysis of 40 clinical isolates of "*Aspergillus fumigatus*" specifically selected on the basis of poor sporulation and/or unusual colonial appearance or micromorphology demonstrated that only 4 isolates were species other than *A. fumigatus* s.s (one example each of *A. thermomutatus, A. fumigatiaffinis, N. hiratsukae* and *N. quadricinta*). However, several recent studies have indicated that *A. lentulus, A. fumigatiaffinis, N. pseudofischeri* and *A. viridinutans* exhibit elevated antifungal susceptibility profiles when compared to *Aspergillus fumigatus sensu stricto* [6, 7, 14, 15], suggesting that identification of *Aspergillus* section *Fumigati* to species level might eventually be important for optimal therapeutic decisions.

6.2 Aspergillus *Section* Flavi

Aspergillus flavus Link ex Fries is the commonest pathogen of a very large group of related fungi, which show similar physiological behaviour [16]. Colonies are usually granular and an intense yellow–green colour with a colourless to dark red–brown reverse (Fig. 7a). Colonies may display large, dark red-brown to black sclerotial bodies which are visible to the naked eye. Under the microscope they are typified by heavy walled, uncoloured, coarsely roughened conidiophores covered in metulae and phialides or phialides alone producing a radiate spore configuration (Fig. 7b), commonly strains produce a mixture of vesicles with and without metulae and occasionally a mixture of metuale and phialides or phialides only produced on a single vesicle. The conidia themselves are globose and also conspicuously roughened. Together with *A. parasiticus, A. flavus* is the most common aflatoxin producer, which has important implications in the contamination of foods and animal feeds and may play a role in increased pathogenicity [16, 17].

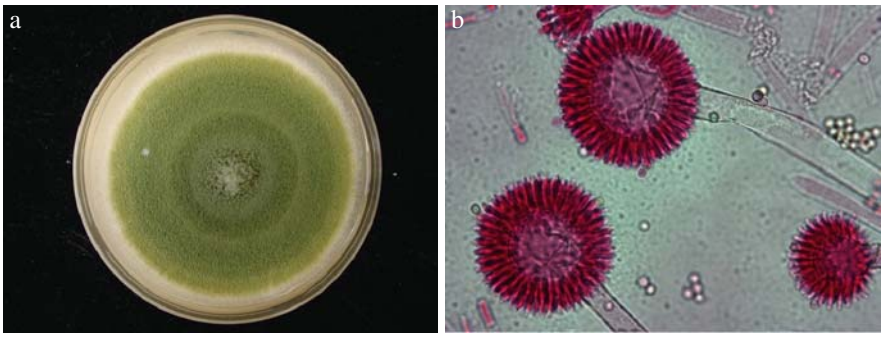

Fig. 7 *Aspergillus flavus* **(a)** colonial form**; (b)** conidial head showing the presence of metulae and phialides

6.3 Aspergillus *Section* Terrei

Aspergillus terreus Thom is readily distinguished from other *Aspergillus* species by its buff to cinamon colour, the presence of delicate long metulae and the production of small, smooth conidia borne in long columns (Figs. 3a and 3b). A very useful feature to confirm the identity of members of the *A. terreus* complex is to look for the presence of globose, heavy-walled hyaline cells singly or in clusters along the sides of the submerged mycelium (Fig. 3c). As they are also formed in vivo it is possible that they may play a role in haematogenous dissemination and thus in the increased pathogenicity of *A. terreus* species noted in vivo. From a clinical perspective this is one of the species that it is important to identify as rapidly as possible due to its frequently observed in vitro and in vivo resistance to amphotericin B [18]. Until recently *A. terreus* was the only recognized species in this section, although there were also two rarely encountered varieties. However, recent publications suggest that one of these varieties should be raised to species status and the presence of several cryptic species within this section indicate that the current classification should be expanded [5].

6.4 Aspergillus *Section* Nigri

The group previously referred to as *A. niger* species group [2] and classified into section *Nigri* by Gams et al. [3] are characterised by the rapid formation of black conidia on white to pale yellow mycelium (Fig. 8a) sometimes showing concentric zonation. Currently 14 species are accepted but as with most of the complexes this is under flux and several new species may soon be described [19]. *Aspergillus niger* van Tieghem is distinguished by the presence of metulae (biseriate) whilst in some other members of the group, such as *Aspergillus japonicus,* the phialides are formed directly on the vesicle (uniseriate). Spores are formed over the entire surface of globose vesicles producing radiate heads (Fig. 8b). Conidia are dark black-brown,

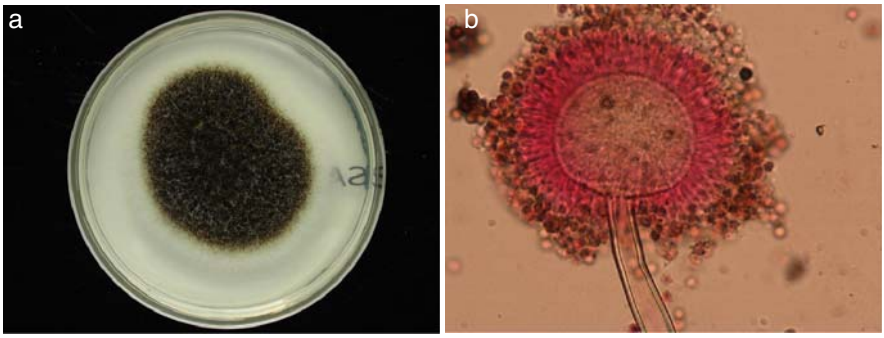

Fig. 8 *Aspergillus niger* (**a**) colonial form; (**b**) conidial head showing the presence of metulae and phialides over the entire surface of the vesicle and darkly pigmented, roughened spores

more or less globose with a diameter of 4–6 μm and often very rough or echinulate. Occasionally large white sclerotia are formed that are visible to the naked eye. The taxonomy of this section is amongst the most complex within the genus and as some of the species remain difficult to differentiate solely with phenotypic methods it is sensible that most non-taxonomists refer to *A. niger* complex. For a recent review and identification key see Abarca et al. [20].

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Molecular Methods for Identification of *Aspergillus* Species

Eszter Deak and S. Arunmozhi Balajee

Abstract Using molecular methods for *Aspergillus* species identification can be a cost-effective, rapid, discriminatory, and objective approach for delineating *Aspergillus* species in a clinical microbiology laboratory. Identification of *Aspergillus* to species level is important for therapeutic decision-making, since different species have variable susceptibilities to available antifungal drugs. Standardization of loci and methods, availability of appropriate guidelines for species definitions using sequence information, and robust sequence databases will make these technologies more conducive to use in a clinical microbiology laboratory. This book section focuses on utilization of molecular methods, specifically comparative sequence identification methods, for the identification of *Aspergillus* at the species level.

Keywords *Aspergillus* · DNA sequencing · Molecular methods · Species identification

Contents

S.A. Balajee (✉)
Mycotic Diseases Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA
e-mail: fir3@cdc.gov

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1 Introduction

The incidence of invasive fungal diseases (IFD) continues to rise in conjunction with the increasing population of susceptible individuals. In particular, patients with neutropenia induced by chemotherapy or solid organ and haematopoietic stem cell transplantation, are high-risk populations where IFD can be serious, difficult-to-treat, and rapidly fatal. Consequently, empiric antifungal treatment of these patients has become a common practice [1]. However, treatment failure may necessitate alternative therapeutic approaches which may be costly and, as potentially effective therapy is delayed, can also involve significant utilization of hospital resources (prolonged hospitalization and drugs) [2, 3]. Although historically *A. fumigatus* has been the predominant aetiological agent of invasive aspergillosis (IA), other species within this genus are being increasingly reported as causes of IA [4, 5]. Importantly, these species have variable susceptibility profiles to several antifungals. For instance, *A. ustus* has high minimal inhibitory concentrations (MIC) to amphotericin B, voriconazole, itraconazole, ravuconazole, and posaconazole [6–8], whilst *A. terreus* has a propensity for higher MICs to amphotericin B [9]. Taken together, distinction of *Aspergillus* species is important as it may guide therapy and in the end reduce the cost of treatment. Accordingly, laboratory methods used for *Aspergillus* identification should be capable of distinguishing the several species within this genus rapidly and accurately.

2 Why Molecular Methods?

2.1 Progression Towards Molecular Methods for Aspergillus *Species Identification*

The genus *Aspergillus* is comprised of approximately 250 members and is divided into 7 subgenera; each of the subgenera is subdivided into several sections; each section is composed of several closely related species. For instance, section *Fumigati*, subgenus *Fumigati*, genus *Aspergillus* comprises a number of species that include the clinically relevant *A. fumigatus*, *A. lentulus*, and *A. udagawae*. For a detailed understanding of the recent classification of the genus *Aspergillus,* the reader is directed to recent reviews [10–12]. Habitually, mycologists have relied on morphological characteristics to delineate species within this genus (explained in detail in the previous chapter by Drs Johnson and Borman). Although most microbiology laboratories continue to rely on such morphology-based identification methods, recent studies have highlighted the limitations of discriminating fungal species by morphology alone [13–23]. Problems in identification associated with slow sporulation or failure of isolated fungi to sporulate, atypical presentations of *Aspergilli* recovered from clinical specimens, and overlapping morphologies of similar looking but genetically distinct aspergilli are amongst the primary drawbacks of such morphology-based identification methods [13–23]. In addition, these

methodologies can be time-consuming, labour-intensive, require a certain level of experience/expertise, and are often subjective.

Molecular methods such as comparative sequence-based methods offer a rapid species identification format with the ability to detect single nucleotide differences and differentiate fungal isolates down to the species/strain level. Over the last decade, molecular technology has supplemented conventional methods, played a significant role in delineating *Aspergilli* at both the species and strain levels [13, 24], and has been instrumental in resolving relationships amongst species within this genus [25]. The focus of this chapter will be *Aspergillus* identification at the section and species level using comparative sequence-based methods, as strain identification using other molecular typing methods will be covered in a later chapter by Dr Klaassen. Before presenting the currently available molecular methodologies for *Aspergillus* species identification, a few representative case studies are presented below. These case studies, drawn from available literature, clearly demonstrate the utility and importance of molecular methods in delineation of *Aspergillus* species.

2.2 Case Studies

2.2.1 Case study 1: Molecular Methods Reveal Cryptic Species with Decreased In Vitro Susceptibility to Antifungal Drugs

Aspergillus ustus, a member of the *Aspergillus* section *Usti*, is a rare human pathogen and can cause invasive infections in immunocompromised hosts. A recent study screening several clinical and environmental isolates of *A. ustus* using molecular, morphological and physiological methods demonstrated the presence of a cryptic species, *A. calidoustus* [21]. All available *A. calidoustus* isolates have low in vitro susceptibilities to several triazoles including the new triazole posaconazole. Posaconazole has demonstrated good antifungal activity against several *Aspergillus* species and has been useful in preventing IA in high risk patients with acute myeloid leukaemia, myelodysplastic syndrome, or graft-versus-host disease [21]. Given the high in vitro MICs of *A. calidoustus* to posaconazole, it can be postulated that this species may cause breakthrough infections in patients receiving posaconazole prophylaxis. In this study, all of the isolates of *A. calidoustus* were originally misidentified as *A. ustus* by various microbiology laboratories using morphological methods. This is not surprising given the fact that *A. calidoustus* and *A. ustus* have very few reliable morphological differences to delimit these two species. Detailed physiological examination of these isolates did indicate differences in growth temperature requirements and limited biochemical differences between these species. However, in addition to *A. ustus*, the section *Usti* is comprised of several other species. Although growth at high temperatures and positive Ehrlich reaction differentiate *A. calidoustus* from *A. ustus*, these tests are insufficient for discrimination of *A. calidoustus* from other species within the section *Usti*. For example, *A. pseudodeflectus* and *A. granulosus* both can grow at high temperatures like *A. calidoustus,* and at least four out of eight species within the section *Usti* produce a

positive Ehrlich reaction [26]. Only molecular methods can delineate this clinically important species from all other species within the section *Usti*. Since *A. calidoustus* demonstrates higher in vitro MICs to several antifungal drugs, this may be an important species to delineate in the clinical microbiology laboratory.

2.2.2 Case study 2: Morphological Characteristics Inconclusive for Species Identification

Phenotypic variation is common to *Aspergillus* species and can be observed even when examining an individual isolate. A recent study describing a fungal isolate from a corneal ulcer highlighted the atypical morphological characteristics of *Aspergillus* species and supports the use of sequence-based methods for species confirmation. In this case, the recovered isolate appeared representative of *Aspergillus tamarii* by morphological examination; however, several morphological inconsistencies conflicted with the known species description [19]. Specifically, in addition to the normal thick-walled and coarsely roughened conidia or conidia spiked with protuberances, hyaline, smooth walled structures were also observed in the isolate examined. Additionally, isolates of *A. tamarii* normally grow at both 37°C and 42°C, but this particular clinical isolate was unable to grow at 42°C. Hence, identification based exclusively on phenotype was not definitive. However, DNA sequencing of the ribosomal regions, in addition to two protein coding regions, showed 100% homology to the sequences of an isolate of *A. tamarii* in the GenBank database using the BLASTn sequence similarity search. Thus, this isolate was confirmed to be *A. tamarii* using molecular methods of identification.

That was only the fourth reported case implicating *A. tamarii* in keratitis infection, and the first known recovery of *A. tamarii* from an ocular infection. *Aspergillus* species, primarily *A. flavus*, *A. terreus*, *A. fumigatus* and *A. niger*, are common etiologic agents of fungal keratitis (more detail on *Aspergillus* keratitis are presented in the chapter by Dr Thomas in this book). The recovery and confirmation of *A. tamarii* from a case of keratitis using molecular methods simultaneously demonstrates the changing epidemiology of fungal keratitis infections and the need for molecular methods to clearly determine the etiologic agent when morphological assessment proves to be inconsistent.

2.2.3 Case study 3: Lack of Sporulation Hinders Species Identification

Another study bought into focus the value of using sequencing-based strategies along with morphological methods to identify a non-sporulating clinical isolate as *Petromyces alliaceus* (anamorph *A. alliaceus*). This rare *Aspergillus* species was recovered as the etiological agent of invasive pulmonary aspergillosis, with low in vitro susceptibilities to the antifungal drugs amphotericin B and caspofungin, correlating with clinical failure of therapy with both these drugs. Initially, this fungus could not be identified by morphological methods because growth occurred on laboratory media as non-sporulating sterile hyphae, with occasional ascomata (sexual bodies). Subsequent comparative sequence analysis of the ribosomal regions

revealed the identity of the isolate to be *P. alliaceus* [27]. *Post hoc* morphological assessment of the cultures confirmed the identity of this organism as *P. alliaceus*, since on special laboratory media it produced smooth conidiophores with vesicles that gave rise to radiating, biseriate orange–yellow conidial heads producing smooth-walled, globose conidia. The isolate grew well at 30, 35, and 37°C, sporulated abundantly in the presence of light, and produced abundant ascomata, which is the hallmark of *P. alliaceus*. This study as well as other studies with *Aspergilli* [27, 28], reinforce the fact that the induction of sporulation for morphological analysis is not always easy, and may require testing under multiple conditions without necessarily resulting in success. Importantly, in cases of fungi that initially do not sporulate, extensive effort can be involved to induce the "diagnostic" sporulating bodies for identification of the unknown organism. In such cases, molecular methods can quickly and efficiently indicate (depending on the locus) the genus/species to which the fungus belongs. Once this is known, the microbiologist (if need be) can narrow down the steps to further confirmatory identification using morphological methods of identification. As evidenced by several recent publications, non-sporulating fungi are a problem not only restricted to the genus *Aspergillus*, but also in other fungi recovered from clinical specimens [29–31]. In these cases, the recalcitrant non-sporulating fungi were identified using molecular strategies; such rapid identification of these fungi may have an immediate impact on patient management.

2.2.4 Case study 4: Overlapping Morphologies Confound Species Identification

In 2005, seven clinical isolates previously identified as *Aspergillus fumigatus* "variants" by morphology were examined closely using molecular methods. Unlike most *A. fumigatus* isolates, all these "variant" isolates sporulated poorly and exhibited high MICs to several antifungal drugs, including amphotericin B, itraconazole, voriconazole, and caspofungin. Detailed phylogenetic analyses using sequences from multiple protein coding regions revealed that four of the isolates were genetically distinct from *A. fumigatus* and were assigned as a new species, *A. lentulus,* within the section *Fumigatus* [15]. Subsequent studies have revealed that *A. lentulus* is geographically widespread and can be isolated both from clinical and environmental sources. Both *A. lentulus* and *A. fumigatus* have very few morphological differences that can distinguish these species from each other using only morphological analyses. However, these two species are genetically distant from each other and have also been shown to have distinct secondary metabolite profiles [32].

Aspergillus fumigatus and other closely related species within the section *Fumigati* have overlapping morphologies, making species identification within this section notoriously difficult. Accordingly, in the last few years several studies have reported cases where closely related species such as *A. udagawae* and *N. pseudofischeri* have been misidentified as *A. fumigatus* [14, 16]. Given that these species are morphologically similar, it is difficult to distinguish these species using only morphological features as indicators. Results of another study showed that *A. lentulus* and *A. fumigatiaffinis* have high in vitro MICs to amphotericin B,

itraconazole, voriconazole and ravuconazole. In addition, *N. pseudofischeri* and *A. viridinutans* had high MICs to itraconazole, voriconazole and ravuconazole, whilst *N. hiratsukae* and *A. fumisynnematus* were susceptible to all antifungals tested [33]. In contrast, most *A. fumigatus* isolates have low in vitro MICs to these antifungals. Thus, given the variable (and sometimes high) MICs of these species to several antifungals, species identification within this section could be clinically relevant.

3 Molecular Approaches

Numerous recent articles and reviews have exhaustively discussed the various molecular techniques available for *Aspergillus* species identification [24, 34] and hence, in the proceeding sections of the chapter, an overview of sequence-based identification methodologies only, specifically their role in *Aspergillus* species identification, will be presented. Whole or partial genome sequences are now available for most *Aspergillus* species (http://www.broad.mit.edu/), thus enabling the design of useful DNA sequence-based methods for identification. With today's sequencing technology, identification of an unknown fungal species can be obtained within 24 hours of growth in culture and at a cost of under 10 US dollars [24, 35]. Ease of use, reproducibility, sensitivity, and specificity of comparative DNA sequencing has resulted in its emergence as the gold standard for fungal molecular identification [36].

Several steps are involved in a DNA sequence-based scheme. In brief, DNA is extracted from fungal cultures and specific genes or portions of genes (locus/loci) are amplified using polymerase chain reaction (PCR). The PCR amplicon is subjected to DNA cycle sequencing reactions that are a variation of PCR, and the sequence of the desired gene region is determined using capillary electrophoresis. The resulting sequence is then submitted to an appropriate database to obtain matching sequences to which it can be aligned for species identification, with numerous available software programs such as ClustalW and BLASTn. The value of using sequence-based identification formats relies on (i) the level of diversity exhibited by the locus and the suitability of the region to PCR amplification and sequencing; (ii) the reliability of interpretation of the results; and (iii) availability of an appropriate sequence database for sequence comparison.

Depending on the levels of identification required (subgenus, section, or species level identification), the choice of the locus could be different [24, 34, 37]. Multiple genes, ranging from the ribosomal DNA internal transcribed spacer (ITS) regions and the large ribosomal subunit D1-D2 to protein encoding genes such as the β-tubulin and calmodulin, have been evaluated to delimit species within the *Aspergilli*. Subgenus and section within the genus *Aspergillus* can be identified through PCR amplification and sequencing of the conserved 28S and 18S nuclear ribosomal subunits, and the variable D1 and D2 regions of the 28S ribosomal subunit [38–40], as well as the variable intergenic (IGS) [41] and ITS1 and ITS2 regions [42, 43]. Mitochondrial cytochrome B and the RNA polymerase B genes have also been utilized for this purpose [44–47]. Multilocus sequence typing (MLST) of protein-encoding

genes, including β-tubulin, rodlet A, actin, calmodulin, as well as several other markers has proved useful for demarcation of species within a section [48–52].

The *Aspergillus* community, at the "International Workshop on *Aspergillus* Systematics in the Genomic Era" meeting at Utrecht (The Netherlands) in early 2007, proposed the use of the ITS regions for subgenus or section identification, whilst comparative sequence analysis of β-tubulin was concluded to have the best utility for species discrimination within the sections [12]. For instance, identification of *A. terreus* and *A. flavus* can be performed by using the ITS regions, whilst identifying species within sections *A. terreus* and *A. flavus* can be achieved only by sequencing the β-tubulin region. The Clinical Laboratory Standards Institute (CLSI, USA) recently published interpretive criteria for identification of bacteria and fungi by DNA target sequencing (MM18-A; www.CLSI.org). The ITS1 and ITS2 regions were also recommended as targets for subgenus and section level identification, and two protein coding loci, β-tubulin and calmodulin, were recommended as alternative DNA targets for species identification of *Aspergillus*.

Once the sequence(s) is/are obtained, comparisons can be made against previously identified sequences within a database. The publicly available GenBank sequence database (http://www.ncbi.nlm.nih.gov/BLAST), maintained by the USA National Library of Medicine in collaboration with EMBL (the European Molecular Biology Laboratory), is most commonly used for *Aspergillus* identification [36]. GenBank contains a web-based interface where users can both submit sequences to be added to the database and/or interrogate the database(s) with an unknown sequence using the BLASTn (basic local alignment search tool) algorithm to find regions of homology between two sequences. Unfortunately, because sequences are submitted by users and are not validated externally, these databases can be open to errors. However, several databases confirm submissions both phenotypically and taxonomically so that the sequences provided are more likely to be accurate (http://www.cbs.knaw.nl/aspergillus/ DefaultPage.aspx). BLAST searches yield a pair-wise alignment of the queried sequences matched with sequences in the database, represented as ranked scores. The species identification of the unknown isolate can be determined if there is a high sequence similarity (99–100%) to one or more reliable sequences of a known species in the database. Problems in interpreting sequencing results can arise when the percentage gene homology is less than 99%. Here, more detailed phylogenetic analysis evaluating sequences from multiple independent loci and numerous closely related species can be used to better assess and assign genotypes, and subsequently, determine species identification. An area of immediate focus should be the building of validated databases with "clean" sequences from "type" isolates as well as sequences from authenticated isolates.

In spite of the promise of sequence-based methodologies, additional work is required before these technologies can be available for routine use in clinical microbiology laboratories. Presently, given the availability of novel and cost-effective PCR/sequencing technologies, generation of sequences can be done rapidly and efficiently. The rate-limiting step is actually in understanding and interpreting the resultant data to obtain a meaningful species identification. The definition of

a genetically distinct species using comparative sequence-based methodologies continues to be ambiguous, with no true criteria for the number of nucleotide differences required to call an isolate a new species, and conversely, the level of homology necessary to assign an isolate to an existing species.

4 Summary

Treatment of *Aspergillus* infection can be challenging, as discussed in other sections of this book. Rapid species identification is extremely helpful to direct an appropriate treatment regimen resulting in a positive clinical outcome. *Aspergillus* species identification by conventional phenotypic methods continues to be the primary mode of diagnosis of an unknown isolate in the clinical microbiology laboratory. However, as early as 1965, Raper and Fennell noted that ". . . in studying aspergilli, it is important to realize that strains may vary within a species, that species may vary within a group and that groups overlap within the genus" [53], alluding to the limitations of the conventional approaches of microscopy and culture for species identification. The case studies presented in this section clearly highlight the fact that conventional methods of identification based on morphology have limitations, and are oftentimes inadequate. Reliance on this method of identification alone is becoming less efficient with the increasing incidence of antifungal resistant *Aspergillus* species and the concomitant increase in the spectrum of *Aspergillus* species that can cause invasive infections.

Molecular methods have proven to be highly discriminatory compared to morphological methods. DNA-based molecular techniques are specific and have a rapid turnaround time, whilst maintaining overall cost-effectiveness. Currently, there is an extensive array of molecular technologies at our disposal, which are constantly being improved and modified. As this process continues, we anticipate that adjustments will be made in *Aspergillus* taxonomy with cryptic, as well as new or previously recognized species, placed correctly within the genus.

The potential for incorporating molecular techniques in clinical mycology for quicker and more accurate species identification is promising. These methods are currently in use in research and reference laboratories, as evidenced in the mycology literature. However, these technologies are difficult to incorporate in routine clinical laboratories, largely because of a lack of standardization of DNA-based methods and lack of clear consensus to define species and validate sequence databases. As these inconsistencies are addressed, standardized, automated, high-throughput, multiplex molecular-based technologies for *Aspergillus* identification will find a mainstream application in clinical microbiology laboratories.

5 Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC, USA).

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The Pathology of *Aspergillus* Infection

Richard Kradin

Abstract *Aspergillus* spp. are responsible for a broad spectrum of human disorders ranging from benign colonisation of mucosal surfaces to life-threatening angioinvasive infection. Diagnosing the array of disorders caused by *Aspergillus* spp. can be challenging. In the case of the hypersensitivity responses to *Aspergillus* species, i.e., allergic bronchopulmonary aspergillosis, bronchocentric granulomatosis, and hypersensitivity pneumonitis, the surgical pathologist must be cognisant of their relationship to infection, as fungal organisms may be rare or even absent from the biopsy specimens. Within the gamut of opportunistic infections, *Aspergillus* species must be distinguished from other fungal mimics; additionally, one must assess whether infection is limited, progressive, or immanently life-threatening. With respect to diagnosis, certain *Aspergillus* spp. exhibit characteristic morphologic features in tissue that suggest their identity. This chapter examines the pathological changes in tissues due to infection by *Aspergillus* spp.

Keywords Aspergillosis · Diagnosis · Histopathology · Tissue

Contents

R. Kradin (✉)
Infectious Disease Pathology, Departments of Pathology and Pulmonary/Critical Care Medicine, Massachusetts General Hospital, Boston MA, USA
e-mail: rkradin@partners.org

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1 Introduction

Aspergillus species are the cause of abnormalities ranging in severity from benign commensal growth in tissues to life-threatening vascular invasion leading to disseminated infection and sepsis. The spectrum of pathologic conditions attributable to *Aspergillus* spp. requires that surgical pathologists be acquainted with their diagnostic features, as well as with their clinical implications [1]. The development of increasingly specific fungal antibiotics necessitates distinguishing *Aspergillus* spp. from other filamentous fungi that can mimic their morphological features. According to the revised EORTC/MSG consensus [2], the diagnosis of invasive aspergillosis (IA) can only be proved in the presence of a positive histopathology. As confirmatory microbial cultures are not available in all cases, establishing expertise in discerning the specific morphological features of *Aspergillus* infection is critical in determining optimal therapy.

Aspergillus spp. are ubiquitous within the environment. There are more than 300 species within the genus but 8 species cause the vast majority of human disease [3]: *A. fumigatus* is the most common pathogen, followed by *A. niger, A. nidulans, A. terreus, A. clavatus, A. flavus, A. niveus, and A. ustus*, account for the remainder of infections [4].

Aspergillus spp. are readily isolated from soil and decaying vegetation, but they have also been identified from several sources, including foodstuffs, paints, medications, refrigerator walls, and dialysis bags. The fungal spores of *Aspergillus spp*. are released into the ambient air, and viable organisms can survive extreme climactic conditions, e.g.., Antarctica, the Saharan desert, and high-altitude. Additionally, *Aspergillus* spp. can endure extremes of acid-base balance.

2 Microscopic Morphology

Aspergillus spp. are reliably demonstrated in tissue section with silver histochemical stains, e.g., Gridley or Gomori methenamine silver (GMS) [5]. These decorate the fungal wall grey–black. The hyphae of *Aspergillus spp*. range in diameter from 2.5–4.5 μm and exhibit frequent septation. The hyphae of *Aspergillus* spp. tend to branch dichotomously, progressively, and at acute angles of ~45°, mimicking an arborizing tree branch (Fig. 1). When cut in cross-section, the hyphae can be mistaken for yeast or spores; however, the absence of budding suggests their true nature (Fig. 2). In areas of mycelial growth, hyphae frequently become tangled, bulbous, and distorted, so that it may be difficult to confirm the diagnosis with accuracy based exclusively on morphology (Fig. 3). This is why the diagnosis of

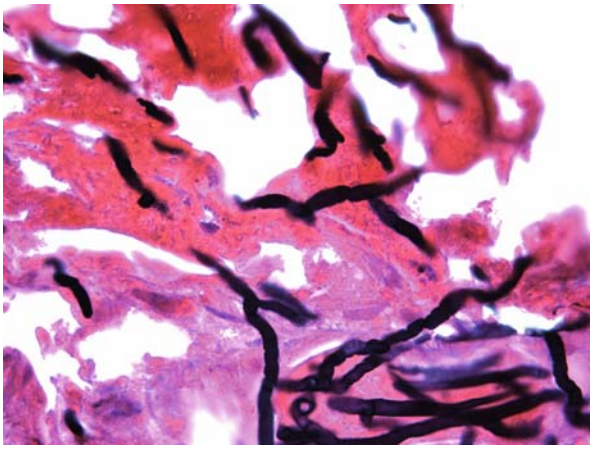

Fig. 1 Progressive acute angle dichotomous branching of *Aspergillus* hyphae. Gomori Methenamine Silver (GMS). ×600

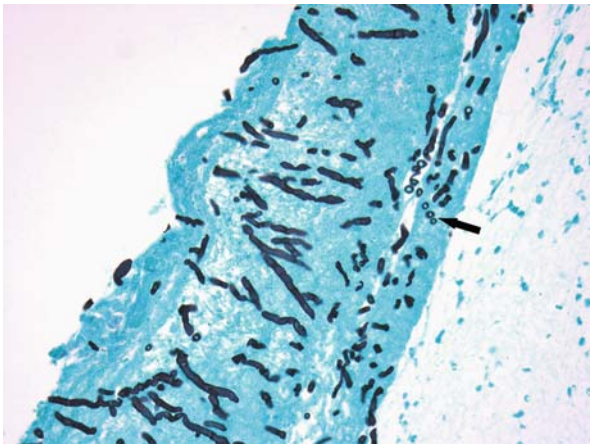

Fig. 2 *Arrow* indicates hyphae cut in cross section mimicking yeast forms. Gomori Methenamine Silver (GMS). ×250

"proven IA" can only be established in the presence of *both* positive histopathology and culture for *Aspergillus* species – if culture is negative or is not performed and hyphae are observed in tissue, the infection should be classified as "proven mould invasive fungal disease" [2]. The morphological features of *Aspergillus* spp. are summarized in Table 1 and contrasted with those of common fungal mimics.

The *aspergil*, a ritual instrument used in the Roman Catholic mass, resembles the fruiting body, and gives rise to the name of the fungus (Fig. 4). Fruiting bodies develop from mycelia in areas of high oxygen tension, such as the trachea, lung or sinus cavities, but rarely develop in tissues. The fruiting body is composed of

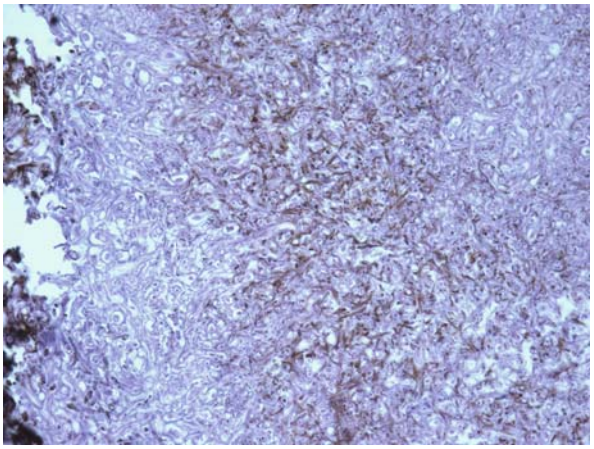

Fig. 3 Bulbous hyphal form seen in mycelia. Gomori Methenamine Silver (GMS). ×600

Table 1 Morphological features of *Aspergillus* and its mimics

	Width of hyphae	Septations	Pattern of branching
Aspergillus spp.	3–6 μm	Frequent	45 degrees, progressive
Pseudallescheria spp.	2–5 μm	Frequent	Acute angle, random
Fusarium spp.	3–8 μm	Frequent	Right angle, branch point constriction
Zygomycetes	6–25 μm	Pauciseptate	Right angle, random

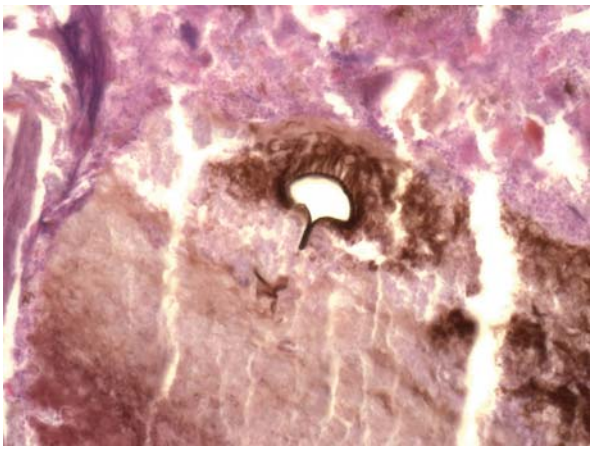

Fig. 4 Aspergil (conidial head) of *A. fumigatus* with conidia. Gomori Methenamine Silver (GMS). ×600

a vesicle with either one or two layers of phialides that produce conidia, and the morphology of the fruiting body allows *Aspergillus* spp. to be accurately speciated in situ.

3 Inflammatory Response to *Aspergillus*

Aspergillus species produce a variety of putative virulence factors including endotoxins, heparin-like factors, and oxalic acid. This is reviewed in more detail in the chapter on the pathogenesis of IA by Drs. Ben-Ami and Kontoyiannis. The hyphae of *Aspergillus* spp. evoke histological responses by the host that reflect the level of

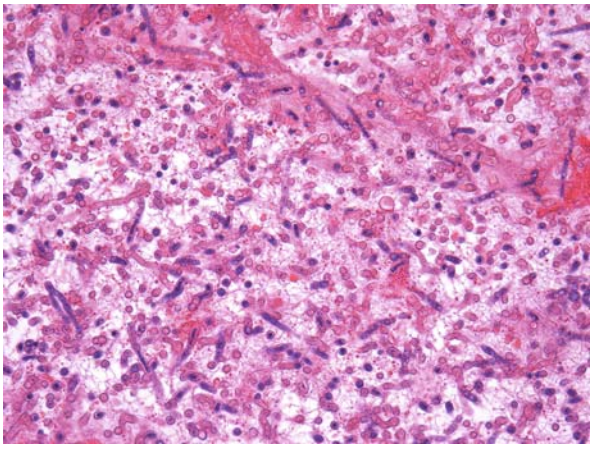

Fig. 5 Hyphae of *Aspergillus fumigatus* are seen in H&E stained section. Hyphae are invested in coat of eosinophilic plasma proteins. Haematoxyline and Eosin (H&E). ×150

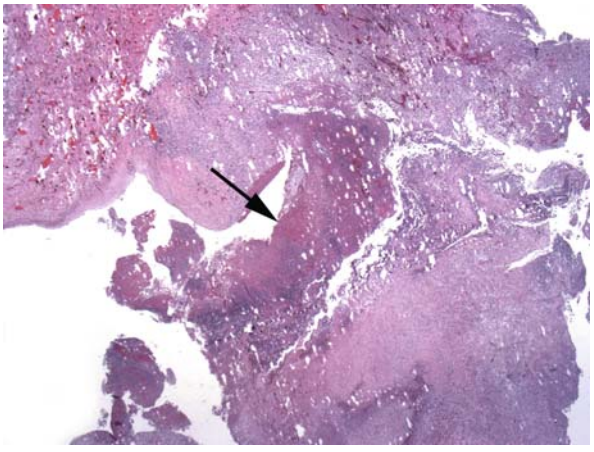

Fig. 6 Dense eosinophilic matrix – the *Splendore-Hoeppli* phenomenon – surrounds mycelia in fungal ball. Haematoxyline and Eosin (H&E). ×100

immunocompetence. In chronic infections, hyphae often show evidence of coating by plasma proteins, including immunoglobulin and complement. This can range in extent from individualized sheaths around hyphae (Fig. 5) to extensive amorphous hypereosinophilic coatings around mycelia, a phenomenon termed the *Splendore-Hoeppli* phenomenon (Fig. 6).

Invasive *Aspergillus* infections are characterized by tissue necrosis accompanied by neutrophilic exudates. Eosinophils are variably present. Macrophages are invariably present and granulomatous inflammation with giant cell formation is common (Fig. 7). Non-necrotising sarcoidal granulomas may develop in the walls of non-invasive fungal balls.

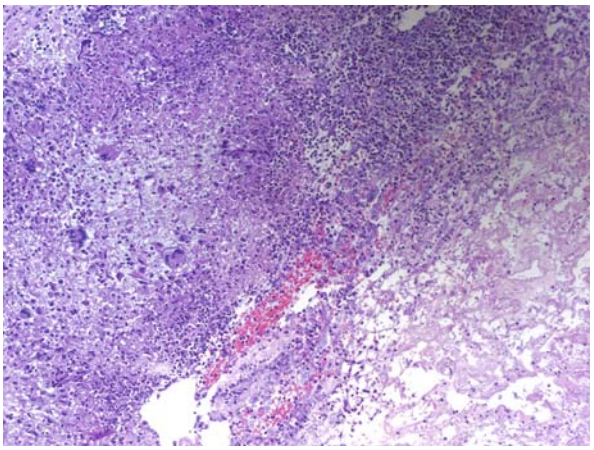

Fig. 7 Granulohistiocytic inflammatory response with scattered multinucleate giant cells in case of invasive aspergillosis. Haematoxyline and Eosin (H&E). ×100

4 Immunological Disorders Due to *Aspergillus* Infection

Allergic bronchopulmonary aspergillosis (ABPA) includes chronic reversible airways disease (asthma) and bronchiectasis with non-invasive colonisation of the airways by *Aspergillus* spp [6]. It is not certain whether the fungi play an opportunistic role in exacerbating atopic responses or are primary in the pathogenesis of this disorder [7]. Patients develop intractable bronchospasm, with blood eosinophilia and elevated serum IgE levels. The disorder is characterized by central cystic bronchiectasis with mucoid impaction of the proximal airways of the upper lung [8]. The impacted mucus is viscid and may form a cast of the airways, a disorder termed plastic bronchitis. Microscopically the mucus plugs show layers of degenerating eosinophils interspersed within the mucin (Fig. 8). In addition, the lung may show patchy peribronchiolar eosinophilic pneumonia.

Most cases of ABPA are diagnosed clinically and do not come to biopsy. However, the disease may be diagnosed following the microscopic examination of an

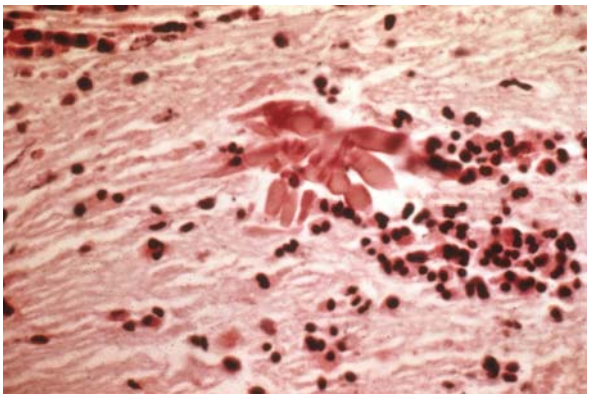

Fig. 8 Allergic mucus with sheets of eosinophils in patient with allergic bronchopulmonary aspergillosis. Haematoxyline and Eosin (H&E). ×100

expectorated mucus plug showing allergic mucus and fragmented fungal hyphae, or in lungs surgically resected for other diagnoses.

Fragmented fungal hyphae can be difficult to identify in ABPA, and silver stains should be applied routinely to the evaluation of allergic mucus plugs. Whereas the clinical and histologic features of this disorder are most frequently caused by *Aspergillus* spp., other fungi, e.g., *Candida* spp., can yield comparable changes. A subset of patients with cystic fibrosis may develop concomitant ABPA, and establishing the diagnosis in this setting can be difficult [9]. The clinical presentations, diagnosis and treatment of ABPA are discussed in detail elsewhere in this book.

5 Bronchocentric Granulomatosis

Bronchocentric granulomatosis reflects an abnormal cell-mediated response to *Aspergillus spp*., in which small calibre airways develop circumferential granulomatous inflammation, loss of the normal lining respiratory epithelium, and impaction of the airway lumen by a granular basophilic mucin and cellular debris [8] (Fig. 9). The disorder may be first noted radiographically as isolated or multiple airway centred nodules [10]. At times, it may be difficult to distinguish radiographically from neoplasia. Bronchocentric granulomatosis may be part of the spectrum of ABPA or occur as an isolated disorder. As in ABPA, *Aspergillus spp*. are often fragmented hyphae and difficult to identify. Surrounding areas of eosinophilic pneumonitis are common. When the disease is suspected clinically, it can be treated non-invasively with steroids. However, definitive resection may be indicated, in order to exclude neoplasia.

6 Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis reflects a combined abnormality of humoral and cell-mediated immunological responses to organic antigens. Most cases of hypersensitivity pneumonitis are caused by thermophilic actinomycetes but hypersensitivity

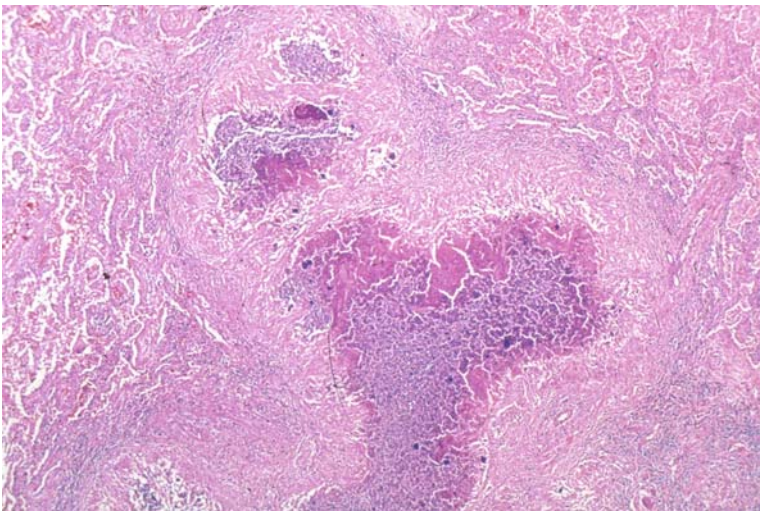

Fig. 9 Wall of bronchiole is partially replaced by granulomatous inflammation in bronchocentric granulomatosis. Haematoxyline and Eosin (H&E). ×150

to *Aspergillus* spp. is well documented. Sources include mouldy hay, compost, esparto grass, home humidifiers, and malt (*A. clavatus*). The pulmonary pathology of hypersensitivity pneumonitis depends on whether the disease is acute, subacute, or chronic. Upper lobe predominance is the rule in hypersensitivity pneumonitis and may be a helpful feature in establishing the diagnosis. Although the diagnosis is based primarily on establishing a historical link between antigen exposures and clinical findings, lung biopsies can be helpful as certain histopathologic features strongly suggest hypersensitivity pneumonitis, particularly in it subacute phase. The lung shows bronchiolocentric lymphohistiocytic interstitial infiltrates with poorly formed microgranulomas and giant cells that contain birefringent crystals [11] (Fig. 10). Whereas CD8+ lymphocytes characteristically predominate in bronchoalveolar lavage fluid specimens, immunostains may reveal dominance of either CD4+ or CD8+ lymphocytes in situ. The presence of interstitial and alveolar eosinophils is characteristically seen in hypersensitivity pneumonitis due to *Aspergillus* antigens but is rare in the response to other antigens

Whereas these pathological features strongly correlate with the clinical diagnoses of hypersensitivity pneumonitis, at times it may be difficult to establish antigenic causation. In addition, other histopathologies, including, non-specific cellular interstitial pneumonitis (NSIP), obliterative bronchiolitis, lymphoid interstitial pneumonitis (LIP), and non-necrotising granulomatous inflammation resembling sarcoidosis, can be seen with hypersensitivity pneumonitis.

The diagnosis is confirmed by careful history taking, a syndrome of repetitive episodes of pneumonia-like illnesses following exposures, and serological testing for serum precipitins to the offending antigens. In chronic hypersensitivity

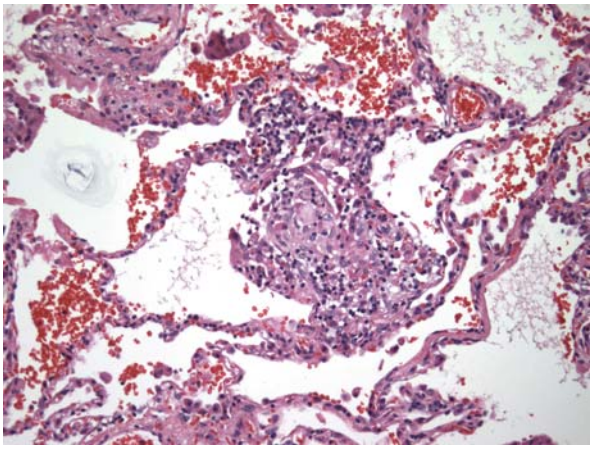

Fig. 10 Micrograniuloma in alveolus in patient with hypersensitivity pneumonitis secondary to *Aspergillus fumigatus*. Haematoxyline and Eosin (H&E). ×200

pneumonitis, the lung shows a pattern of interstitial fibrosis that mimics usual interstitial pneumonitis (UIP) except for the presence of rare microgranulomas. The prognosis of chronic hypersensitivity pneumonitis is guarded, and patients may show progressive lung scarring leading to respiratory failure that is not responsive to steroids or removal of the offending antigen.

7 *Aspergillus* Bronchitis and Chronic Necrotising Aspergillosis

The presence of bronchial and bronchiolar infection by *Aspergillus spp*. is a poorly recognized entity. It is generally seen in the setting of modest immunosuppression accompanying disorders like diabetes mellitus or the use of aerosolised steroids in asthmatics. In many cases, it is a precursor lesion for invasive disease. Fungal hyphae may be observed filling the lumen of airways, without evidence of chronic commensal growth, e.g. the *Splendore-Hoeppli* phenomenon, or frank tissue invasion. Elastic stains are helpful in determining whether organisms have begun to transgress normal tissue barriers (Fig. 11).

8 Fungal Balls

The colonisation of old fibrocavitary disease, e.g., areas of bronchiectasis due to healed tuberculosis or sarcoidosis [12], or emphysematous bullae, by *Aspergillus* spp., is the cause of pulmonary aspergilloma formation. The presence on chest radiography of an intracavitary density that is positionally mobile strongly suggests the diagnosis. The term mycetoma accurately applies to soft tissue infections

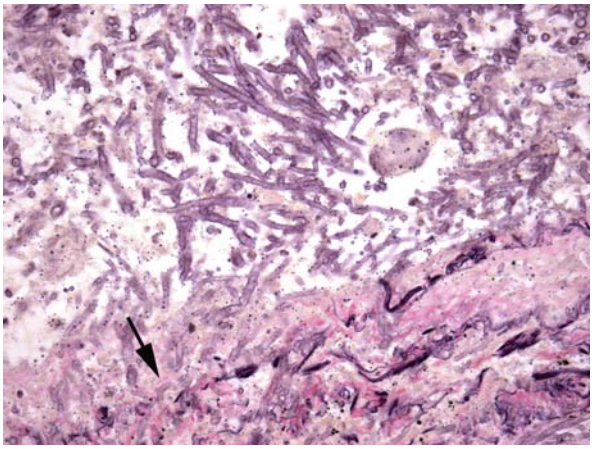

Fig. 11 *Arrow* indicates microinvasion of airway wall in airway showing overgrowth by *Aspergillus fumigatus*. Elastic stain. ×400

and should not be applied to intracavitary fungal mycelial growth. Fungal balls and chronic cavitary pulmonary aspergillosis are discussed in more detail elsewhere in this book. Patients may be asymptomatic or present with episodes of haemoptysis that can be massive, necessitating emergent bronchial arterial embolisation of feeding vessels or definitive resection of the fungal ball. Long-term antifungal therapy is usually required.

The morphology of the hyphae in fungal balls is often distorted and, at times, it can be impossible to identify the septate acute angle branching hyphae of *Aspergillus* spp. Fungal balls show areas of alternating staining intensity that is evi-

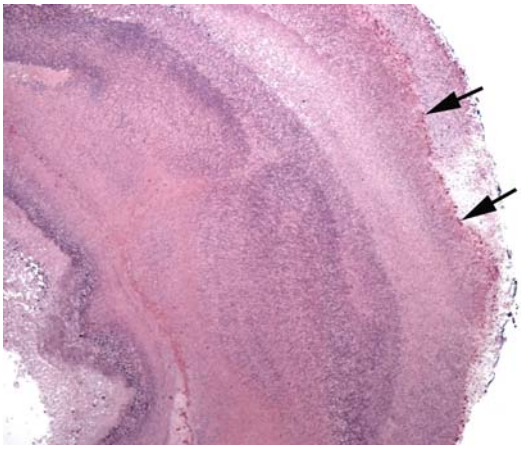

Fig. 12 Zonation of mycelial staining in fungus ball. *Arrows* indicate *Splendore-Hoeppli* phenomenon. Haematoxyline and Eosin (H&E). ×100

dent on haematoxylin and eosin (H&E) stained sections (Fig. 12), giving the impression of alternating zones of fungal growth. The *Splendore-Hoeppli* phenomenon is invariably seen. The walls of the cavity are often superficially ulcerated and lined with granulation tissue, granulomatous inflammation, or metaplastic squamous epithelium, depending on the activity of the lesion. The occasional presence of germinative fruiting bodies of *Aspergillus* spp. with characteristic phialides and conidial forms allows definitive speciation.

When the fungal ball is caused by *A. niger*, one may occasionally observe rapid expansion of cavitary disease due to vascular thrombosis induced by calcium oxalate crystal deposition, a disorder termed chronic pulmonary oxalosis (Fig. 13). Oxalic acid is produced by a variety of *Aspergillus* spp. but is most commonly a feature of *A. niger* infection [13]. Diffusion of oxalate into surrounding blood vessels can be prothrombotic and lead to extensive ischemic necrosis. These patients may also show oxalate crystal deposition in the renal tubules [14]. Resection of the fungal ball is definitive treatment.

The walls of all "benign" fungal balls in patients with chronic cavitary pulmonary aspergillosis must be carefully examined in order to exclude chronic necrotising aspergillosis, in which there is evidence of fungal invasion of adjacent lung parenchyma but no angioinvasion. This condition is also named "subacute invasive aspergillosis" and affect moderately immunosuppressed patients. The application of silver and elastic stains is helpful in excluding fungal invasion. In addition, it is important to distinguish fungal balls that may form in areas of active necrotising cavitary disease due to angioinvasive infection from "benign" intracavitary disease.

Fungal balls commonly develop in chronically inflamed and anatomically distorted nasal sinuses. As in the lung, the wall of the sinuses must be carefully examined for evidence of invasion. The differential diagnosis between *Aspergillus* spp. and the *Zygomyces* (Mucor) can be difficult and at times immunohistological or culture confirmation must decide the question. Fungal balls can develop at a variety of sites of chronic infection but rarely progress to fruiting body formation except in tissues with high oxygen tension.

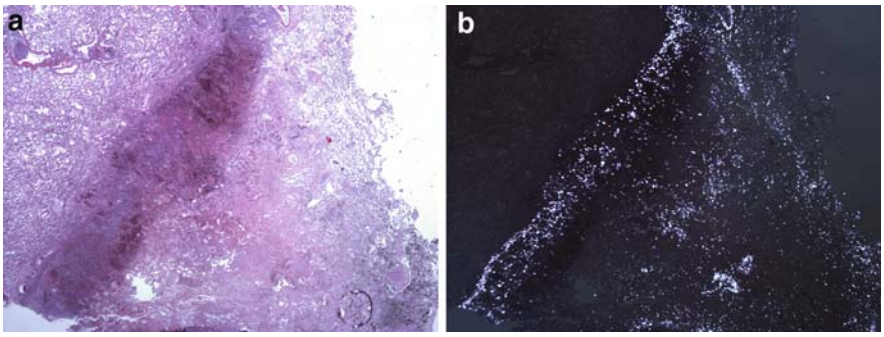

Fig. 13 Pulmonary oxalosis due to fungus ball of *Aspergillus niger*. The band of necrosis seen in "**a**" is due to the diffusion of oxalate crystals as evidenced by their birefringence under polarized light seen in "**b**.". Haematoxyline and Eosin (H&E). ×100

9 Angioinvasive Aspergillosis

This life-threatening infection is seen in patients who have been chronically immunosuppressed and/or neutropenic [15]. It is a feared complication of haematological stem cell and solid organ transplantation, as well as anti-leukemic chemotherapies resulting in prolonged neutropenia. Interestingly, angioinvasive aspergillosis is an uncommon complication of HIV-related illness, despite profound immune deficiency [16]. The lung is generally the primary site of infection and shows necrotising pneumonia with areas of haemorrhage, acute and granulomatous inflammation (Fig. 14). Grossly, targetoid lesions with central thrombosed vessels secondary to angioinvasion surrounded by a rim of consolidated lung (Fig. 15), confluent bronchopneumonia, or dense lobar consolidation, may be seen. Identification of fungal angioinvasion is enhanced with the aid of silver and elastic stains. Rarely, foci of infarcted lung yield an infected pulmonary sequestrum (Fig. 16).

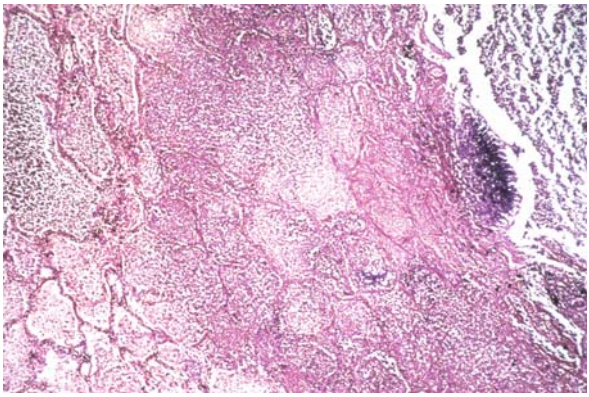

Fig. 14 Acute pneumonia in patient with invasive aspergillosis. Haematoxyline and Eosin (H&E). ×100

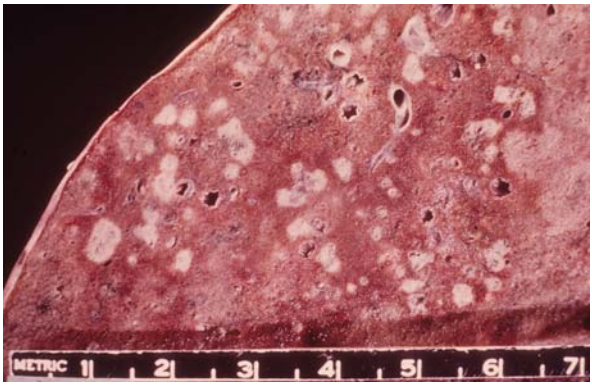

Fig. 15 Lung shows round area of targetoid necrosis in angioinvasive aspergillosis

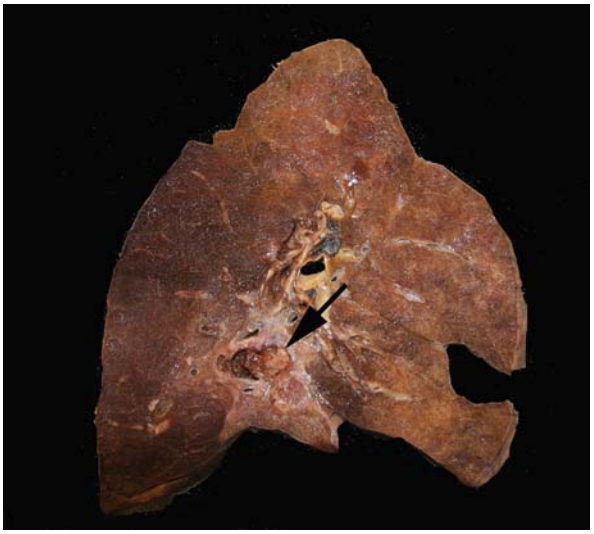

Fig. 16 *Arrow* indicates a sequestrum of necrotic lung in case of angioinvasive aspergillosis

The fungal hyphae have a propensity to invade blood vessels and to "metastasize" to other organs. The presence of sun-burst vasculocentric hyphal growth is diagnostic of "metastatic" foci of infection (Fig. 17). Virtually any organ may be secondarily involved following vascular invasion within the lung. The thyroid is frequently involved at autopsy but it is generally clinically silent pre-mortem. *Aspergillus* spp.

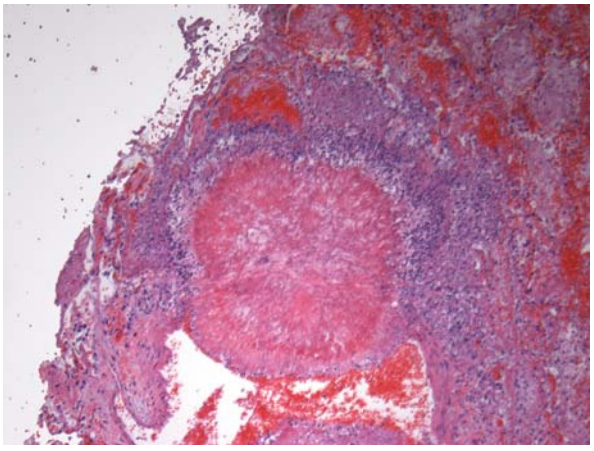

Fig. 17 Sunburst appearance of fungal growth is pathognomonic for blood vessel borne infection. Haematoxyline and Eosin (H&E). ×150

can seed abnormal cardiac valves to produce endocarditis and dissemination to brain may lead to fatal hemorrhagic infarctions.

10 Diagnostic Findings

The gold-standard for the diagnosis of *Aspergillus* infection is isolation and culture of the organisms in the microbiology laboratory. However, certain *Aspergillus* species show characteristic morphologies. *A. terreus* is an opportunistic fungus that frequently infects patients with chronic granulomatous disease [17], as well as other immunocompromised hosts [18]. Its hyphae are characteristically fragmented and pyriform and show orthogonal branches (Fig. 18). The presence of pigmented conidia and oxalate crystals suggests infection by A. *niger* (Fig. 19). Fungal balls due to *A. nidulans* have a propensity to produce pale staining thick-walled non-viable *Hülle* cells that show Maltese cross birefringence when examined under polarized light (Fig. 20). *A. nidulans* is the most common pathogen producing otomycosis and examination.

Although the majority of acute-angle branching septate hyphae encountered in medical practice are *Aspergillus* spp., the exceptions can be difficult to distinguish by conventional light microscopy and histochemical staining. Organisms that mimic *Aspergillus* include the *Zygomyces, Pseudallescheria boydii* (*Scedosporium*), and *Fusarium* spp. The *Zygomyces* are broad, pauciseptate, and show predominantly orthogonal right angle branching (Fig. 21) although acute angle branching is not uncommon and does not exclude the diagnosis. The hyphae of the *Zygomyces* are particularly well stained by standard H&E. All hyphate fungi, including the

Fig. 18 Club-shaped right angle branching of *Aspergillus terreus*. Gomori Methenamine Silver (GMS). ×400

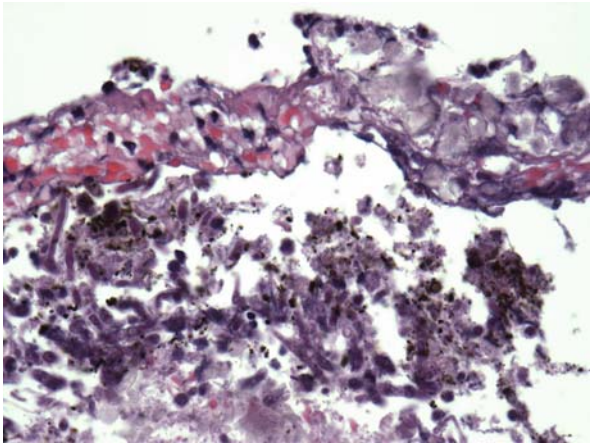

Fig. 19 Multiple pigmented conidia of *Aspergillus niger.* Haematoxyline and Eosin (H&E). ×250

Zygomyces can produce fungal balls in the lung. Invasive infection by *Zygomyces* invariably requires both antibiotics and surgical resection.

The hyphae *of Pseudoallescheria* are generally smaller than those of *Aspergillus spp*. The fungus branches predominantly at acute angles, but tends to branch haphazardly rather than progressively. Terminal chlamydospores mimicking the appearance of a "tennis racket" may be frequent (Fig. 22). The conidia produced by *Pseudoallescheria* in fungal balls are ovoid and pigmented but cannot be reliably distinguished from those of *A. niger*. The septate hyphae of *Fusarium* spp. branch irregularly, generally at right angles, and often show constrictions at branch points (Fig. 23). Whereas distinguishing these pathogens from one another in tissue may be exceedingly difficult it is important, as the efficacy of available fungal antibiotics is dependent on diagnosis. Immunohistochemical reagents are available that

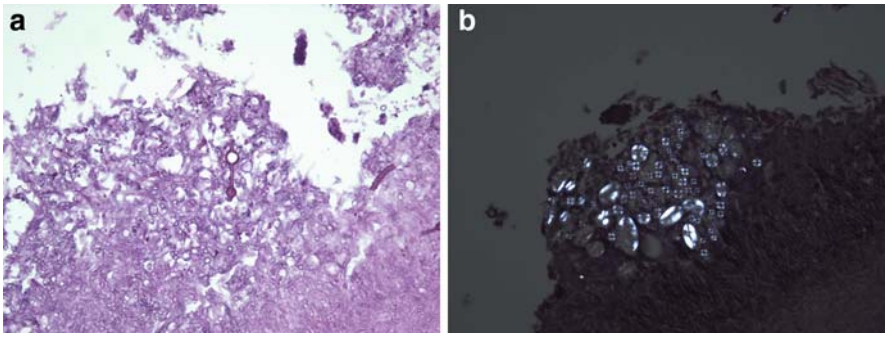

Fig. 20 (**a**) Large *Hülle* cells of *Aspergillus nidulans* showing (**b**) "maltese cross" birefringence. Haematoxyline and Eosin (H&E). ×250

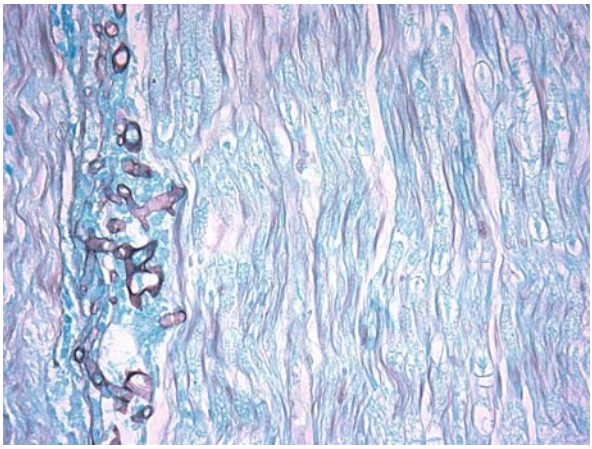

Fig. 21 Broad pauciseptate hyphae of *Zygomyces*. Gomori Methenamine Silver. ×400

may assist in the identification of *Aspergillus* spp. (Fig. 24). Currently available polymerase chain reaction (PCR) techniques out of tissue sections have not generally proven reliable mostly due to lack of standardisation. Whereas both *Aspergillus* spp. and *Pseudallescheri*a are sensitive to voriconazole, *Pseudallescheria* is resistant to amphotericin. The sensitivities of *Fusarium* spp. to most antifungal antibiotics are ill-defined but appear to be limited.

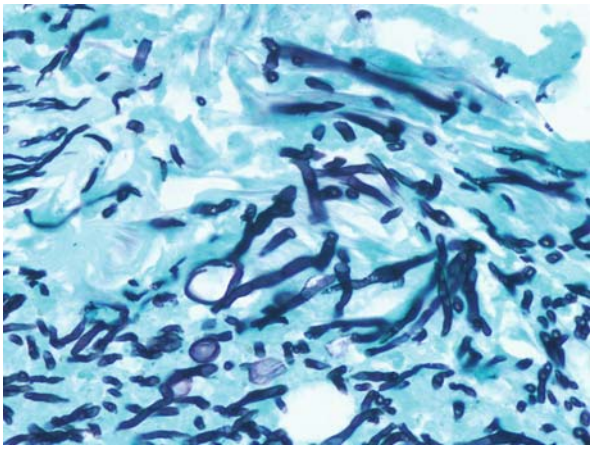

Fig. 22 Terminal chlamydospore formation in *Pseudallescheria boydii*. Gomori Methenamine Silver (GMS). ×400

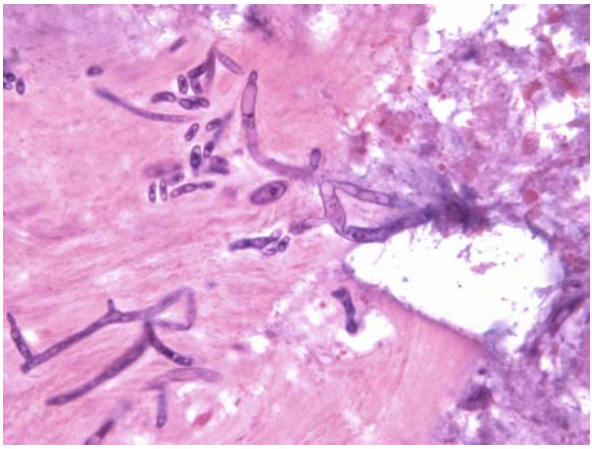

Fig. 23 Right-angle branching hyphae of *Fusarium* spp. with branch point narrowing. Haematoxyline and Eosin (H&E). ×400

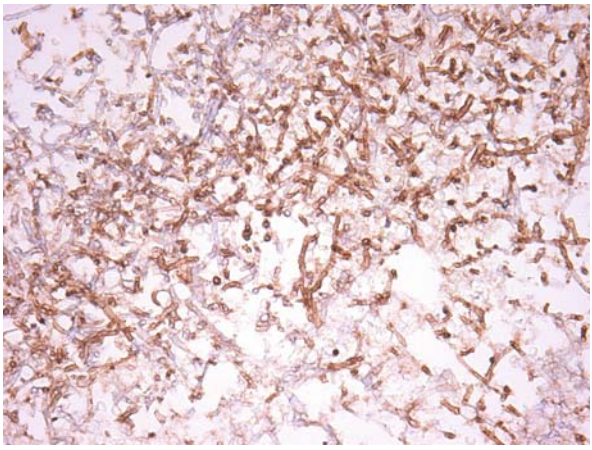

Fig. 24 Immunostain decorates hyphae of *Aspergillus fumigatus*. Peroxidase. ×200

11 Conclusions

Differentiating the effects of *Aspergillus* spp. in tissue biopsies can be challenging. However, as prognostic and therapeutic implications of diagnosis are often substantial, it is imperative that the surgical pathologist establish expertise in this area. Consultations with treating infectious diseases physicians, internists, surgeons, and radiologists can be of invaluable assistance in confirming histological impressions;

however, the treating physicians will often rely on the surgical pathologist to establish the final diagnosis in complicated cases. In order to properly diagnose *Aspergillus*-related syndromes, precise clinical information should shared between all elements of the team.

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Galactomannan Testing

Johan Maertens, Koen Theunissen, and Katrien Lagrou

Contents

1 Introduction

Inhaled conidia (spores) of *Aspergillus spp.* are usually effectively cleared by phagocytic cells, such as alveolar macrophages, neutrophils and monocytes, without clinical consequence [1]. However, a depressed immune system presents risks for invasive aspergillosis (IA), mainly affecting the lungs, and, to a lesser extent, the sinuses [2]. At particular risk for invasive disease are allogeneic haematopoietic stem cell transplant (HSCT) recipients [3–5], and patients who have received intensive myelotoxic therapy for haematological malignancies, resulting in deep and prolonged neutropenia [6].

The diagnosis of IA is based on clinical suspicion, imaging studies, and collection of affected tissue for pathologic and microbiologic examination [7]. Because the prognosis of the disease improves with early commencement of specific therapy, diagnosis should be made as quickly as possible. However, suggestive clinical signs and symptoms such as persistent (neutropenic) fever, progressive dry cough, dyspnoea, pleuritic chest pain, and haemoptysis lack sensitivity and specificity [8].

J. Maertens (✉)
Department of Haematology, University Hospitals Leuven, Leuven, Belgium
e-mail: johan.maertens@uz.kuleuven.be

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In addition, physical examination is often unrevealing and laboratory studies are non-specific [7]. Chest radiography is too insensitive to reliably diagnose IA at an early stage. Chest computerised tomography (CT) scan has therefore become an important diagnostic tool. In neutropenic patients, the CT-graphic presentation of pulmonary IA has been well characterised. Classically, these infections present as macro-nodular lesions or infiltrates [9]. Approximately 80% of these nodular lesions will be surrounded by areas of lower attenuation, or "halo signs" which represent areas of hemorrhagic inflammation. The lesion eventually may cavitate during the course of therapy and following neutrophil recovery, creating what is commonly referred to as "air crescent signs" [10]. Unfortunately, these characteristics are neither highly sensitive, nor specific for IA; other infections that elicit such inflammatory responses, including other moulds, bacteria, and viruses can present with halo signs. In addition, IA can also present as isolated nodules, ground glass opacities mimicking viral infection, or dense lobar infiltrates. To further impair diagnosis based on radiographic presentation alone, recent studies have noted that the radiographic presentation in the post-engrafted allogeneic HSCT recipient are yet more diverse, with a large proportion of patients developing focal or lobar consolidations [11].

The antifungals that are currently used for invasive fungal infections have differential activity against the organisms that cause sinopulmonary disease; specifically, voriconazole, the drug of choice for IA [12], does not display activity against the emerging group of Zygomycetes [13]. This increases the importance of establishing a diagnosis to the genus level. Blood cultures are notoriously negative despite the use of sensitive detection techniques and the propensity for widespread disease via haematogenous route [7]. Recovery of *Aspergillus* species (by culture or microscopy) from a respiratory tract specimen (sputum, tracheal aspirate, bronchoalveolar lavage sample [BAL]) has a high predictive value in the setting of haematological malignancy (including HSCT) with suspected IA, in contrast to immunocompetent hosts, in whom it is usually considered an indicator of colonisation. Unfortunately, a positive culture (or microscopy) from respiratory samples is seldom obtained [14].

Currently, only one test can be considered reference standard for establishing a definitive or "proven" diagnosis of IA: demonstration of the organism, with culture confirmation, from biopsied tissue. However, the sensitivity and specificity of fungal specific stains is unpredictable. In addition, utilizing biopsy to diagnose IA is also hampered by the physiologic condition of these patients, as most cannot tolerate invasive procedures, due to thrombocytopenia, coagulation disorders and/or their critical condition. The yield of invasive diagnostic tests in the diagnosis of IA is discussed elsewhere in this book.

Given these diagnostic dilemmas and lack of a clinically feasible gold standard to establish diagnosis, a consensus committee was recently established to define categories of certainty of IA diagnosis, based on the constellation of host, clinico-radiologic, and laboratory characteristics [15]. In these definitions, it was recognised that the most common method of diagnosis was to culture the organism directly from BAL fluid; in the setting in which the patient demonstrates consistent radio-

graphic abnormalities, culture of *Aspergillus* species from BAL is thought to provide a "probable" diagnosis. Unfortunately, sensitivity of culturing the organism in BAL fluid is low (20–60%), and variable depending on the methods in which the lavage is performed and the methods in which the sample is processed in the laboratory.

However, use of adjunctive tests to detect *Aspergillus* in either serum samples or in BAL-samples, e.g. by the galactomannan enzyme immunoassay (EIA), has been shown to increase sensitivity compared to culture.

2 Detection of Galactofuranose-Antigens

The detection of circulating anti-*Aspergillus* antibodies has been considered of little use for the diagnosis of acute IA in neutropenic patients and HSCT recipients, mainly because these populations are not capable of mounting an adequate and timely antibody response [16, 17]. Whether antibody detection may be useful for other risk groups has yet not been carefully assessed [18].

The low sensitivity and low predictive value of antibody detection has prompted the development of immunoassays for the detection of circulating fungal antigens. *Aspergillus* antigenaemia was first detected and characterised in the late 1970s [19]. After immunising healthy rabbits with serum of an experimentally infected rabbit with disseminated aspergillosis, the resultant immune serum formed an immunoprecipitate with an antigen present in an extract of *Aspergillus* hyphae. Physicochemical analysis showed that this immunoreactive component was galactomannan (GM), a heat-stable heteropolysaccharide present in the cell wall of most *Aspergillus* and *Penicillium* species. The molecule was comprised of a non-immunogenic mannan core that was heavily substituted with side chains of varying lengths containing immunodominant galactofuranosyl (Galf) units [20–22]. In vitro, the antigen could be released as pure polysaccharide, but the number of epitopes varied between genera and strains, as well as with the conditions used for its extraction and purification. More recently, Mennink-Kersten et al. described several other fungal glycoproteins that also contain immunogenetic galactofuranose units and proposed the term "galactofuranose-antigens" for all molecules that could be detected by galactofuranose-specific immunoassays, including GM [23].

Refinement of immunodiagnosis led to the development of more sensitive radioimmunoassays (RIA) and EIAs. However, RIA has never gained widespread acceptance due to its limitations, including the inability to detect antigen concentrations in the low ng/ml range, the short shell life of radioisotopes, and the lengthy procedure which makes the technique less suitable for routine clinical use.

The results of specific EIAs, often with a lower limit of detection ($\sim$10 ng/ml), were in general comparable with RIAs [24–26]. Figures of sensitivity vary but are generally rather low. Also, studies on serial samples from single patients have demonstrated the transient nature of antigenaemia.

It was hypothesised that an increased efficiency would only be obtained by regular screening using immunoassays with highly specific and sensitive antibodies. The production and purification of monoclonal antibodies and the creation of

standardised and reproducible assays were considered necessary steps towards better immunodiagnosis.

3 The Introduction of Monoclonal Antibodies

In Europe, a latex agglutination test using beads sensitised with the monoclonal anti-GM antibody EB-A2 (Pastorex^{TM} *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-coquette, France) became commercially available in the early 1990s. The antibody was produced by immunizing rats with a mycelial extract of *A. fumigatus* and recognised the β-(1→5)-linked galactofuranose epitopes of GM. The assay detected purified antigen at concentrations as low as 15 ng/ml from several *Aspergillus* species, including *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *A. versicolor*. In addition, cross-reaction with exoantigens from several other moulds was noted, including potential laboratory contaminants such as *Penicillium chrysogenum*, *Cladosporium herbarum*, *Acremonium* spp. and *Alternaria alternata*, as well as rare fungal pathogens such as *Fusarium oxysporum*, *Wangiella dermatitidis*, *Rhodotorula rubra*, and *Penicillium marneffei* [27, 28]. When evaluated in a guinea-pig model of IA, sensitivity was 97.6% and specificity 96.2% [29]. These encouraging initial results were also confirmed in human sera; the kit was positive in 13 of 15 proven cases and 17 of 18 patients with a high probability of IA [30]. However, subsequent evaluations in larger series of neutropenic patients yielded disappointing results: clinical sensitivity was usually low (∼30%) and circulating antigen was detected only in the late stages of disease, negating the primary objective of developing fungal diagnostic methods [31–38].

In the early 1990s, serodiagnosis for IA faced many challenges [31–38]:

- Although highly specific in neutropenic patients, detection of GM in serum and urine was only moderately sensitive and not reliable at all in non-invasive presentations of *Aspergillus* infections.
- The temporal relationship of a positive assay and early symptoms of disease or initiation of treatment was not accurately assessed. Antigenaemia preceded clinical diagnosis or antifungal therapy in some patients, but failed in many others to diagnose disease before other conventional means or start of empiric therapy, making the test clinically redundant.
- Single sera tested often negative so that serial sampling was required. However, an optimal sampling frequency was not determined.
- Antigen levels in body fluids correlated with the extent of fungal involvement and serological response correlated well with clinical response, but the use of GM as a prognostic marker or its use in therapeutic monitoring had never been properly addressed.
- Reproducibility was suboptimal.

  Most importantly, the observed variability in the performance often resulted from methodological shortcomings that undermined virtually all of the clinical studies (for a detailed review see [39]).

4 Detection of Galf-Containing Molecules by a Sandwich EIA

In recent years, increased experience has been gained with the Platelia™ *Aspergillus* (Bio-Rad Laboratories, Marnes-La-Coquette, France and Bio-Rad laboratories, Hercules, CA) [40]. Available in Europe since the mid 1990s, this sandwich immunoassay was cleared by the Food and Drug Administration (FDA) for diagnostic use in the United States of America (USA) in 2003 [41]. In addition, a positive assay has been incorporated as a microbiological criterion in the first (2002 [15]) and updated version (2008 [42]) of the EORTC-MSG consensus definitions of invasive fungal disease for clinical research. In these consensus definitions, a positive EIA in specimens of BAL fluid, cerebrospinal fluid, or $\geq$2 serum samples carries the same diagnostic weight as a positive culture or microscopy for *Aspergillus* species. Importantly, these results do not define disease when considered in isolation, but only in the presence of other clinical signs and symptoms of disease, and in the setting in which appropriate host criteria are met.

4.1 General Characteristics of the Sandwich EIA

The Platelia™ *Aspergillus* EIA is a one-stage immunoenzymatic sandwich microplate assay which detects circulating Galf-containing molecules in body fluids. The assay also uses the rat monoclonal antibody EB-A2. The monoclonal antibody is used to coat the wells of the microplate and bind the antigen, and as the detector antibody in the conjugate reagent. By using the antibody both as captor and detector antibody, the detection limit of GM could be lowered to 0.5–1.0 ng/ml of body fluid, resulting in an earlier detection compared to the previous latex agglutination assay [36–38].

Test samples are heat-treated in the presence of 4% EDTA in order to dissociate immune complexes and to precipitate proteins that could interfere with the test. The treated samples and conjugate are added to the wells coated with monoclonal antibody, and incubated. In the presence of *Aspergillus* antigen, a monoclonal antibody-galactomannan-monoclonal antibody-peroxidase complex is formed. The strips are washed to remove any unbound material. Next, the substrate solution is added, which will react with the complexes bound to the well to form a blue colour reaction. The enzyme reaction is stopped by the addition of acid, which changes the blue colour to yellow. The optical absorbance of specimens and controls is determined with a spectrophotometer set at 450 and 620/630 nm wavelength. Results of the test are calculated as optical density (OD) index, which is the OD of the clinical sample divided by the mean OD of two threshold controls. The average turn-around time varies between 6 and 8 h. However, the immunoassay lends itself to automation, allowing better execution of the test at reduced technician time and lesser use of consumables. Intra-assay and inter-assay reproducibility at low index levels using different kit lots was good between laboratories and on different days [43, 44]. Significant variability does seem to occur between runs/lots, which may be associated with different threshold control values in different kits. These usually

do not affect GM test interpretation, although caution is required for single samples with low-positive indices [43]. Finally, animal studies have shown that the results of the EIA correlate with tissue fungal burden [45, 46].

4.2 Intended Use: Screening Assay or Diagnostic Test?

In line with results of studies that have assessed the utility of the Platelia™ EIA, the test should be used as a screening tool for the early detection of IA in neutropenic patients with haematologic malignancies and/or HSCT recipients. A single positive assay or consecutive positive assays with increasing OD index can serve as a microbiological criterion to define probable IA in patients with appropriate radiographic abnormalities. As such, the test was cleared as an aid to diagnose IA in cancer patients, using serum as the primary sample. However, recent data has demonstrated excellent sensitivity of the Platelia™ EIA when used on fluids retrieved during the course of the illness (BAL fluid, cerebrospinal fluid, and pleural fluid), both in haematology and non-haematology patients (e.g. solid organ transplant recipients, intensive care unit patients, patients with auto-immune disorders, AIDS patients) [47–51]. In these settings, the test is used as a confirmatory (diagnostic) assay in patients with unexplained radiological features, rather than for the early detection of IA. To date, use of these assays cannot yet be recommended to assess the outcome of antifungal therapies, although preliminary analyses look very promising [52–55].

4.3 Characteristics of the GM EIA

Results of published studies that prospectively evaluated performance of the Bio-Rad GM EIA applied to serum samples from haematology patients are summarised in Table 1. As shown in this table, the *sensitivity* for proven and probable IA varies between 27.5 and 100%. However, a comparison of the results of these different studies is difficult since many factors relating to the host, the study design and the methodology influence the performance characteristics of the test [56].

4.3.1 Type and Severity of Immunodeficiency

Studies that have reported high sensitivity with the serum EIA primarily included haematology patients such as those with acute leukaemia, myelodysplastic syndrome, and those undergoing allogeneic HSCT or treatment for graft versus host disease (GVHD). In contrast, studies that have enrolled large numbers of low-risk neutropenic episodes (such as autologous transplants and solid tumour patients [57]) and non-neutropenic immunosuppressed patients (e.g. organ transplant recipients and critically ill patients [50, 58, 59]) have invariably reported low sensitivities (15–30%). This observation is in line with recently recognised differences in pathogenesis and disease progression of aspergillosis between animals with and without

Table 1 Prospective studies in haematology patients evaluating the performance of the Platelia™ *Aspergillus* EIA in serum samples. Proven and probable cases were defined according to the 2002 EORTC-MSG consensus criteria [15]

References	Number of proven and probable cases *vs*. controls	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)
Package insert [97]		81	89	55	97
Rohrlich et al. [98]	Cases: 10 probable Controls: 27	100	92.5	83.3	100
Sulahian et al. [34]	Cases: 25 proven; 15 probable Controls: 163	82.5	92.6	73.3	95.5
Bretagne et al. [99]	Cases: 3 proven; 3 probable Controls: 21	100	76.2	54.5	100
Machetti et al. [36]	Cases: 1 proven; 3 probable Controls: 17	60	82.3	50	87.5
Maertens et al. [64]	Cases: 27 autopsy proven Controls: 44	92.6	95.4	92.6	95.4
Sulahian et al. [100]	Cases: 5 proven; 4 probable	100	89.9	20	100
	Cases: 22 proven; 22 probable	88.6	97.5	79	98
Maertens et al. [65]	Cases: 30 proven; 9 probable Controls: 264	89.7	98.1	87.5	98.4
Ulusakarya et al. [101]	Cases: 10 proven; 6 probable Controls: 117	68.7	99.5	68.7	99.5
Maertens et al. [73]	Cases: 18 proven Controls: 73	94.4	98.8	94.4	98.8
Herbrecht et al. [57]	– FUO ($n = 261$)	100	95	7	100
	Cases: 1 possible	27.5	98.6	95.2	58.3
	– Suspected pulmonary infection ($n = 297$)	40	100	100	88
		100	91.3	10	100
	Cases: 25 proven; 67 probable; 53 possible	29.4	94.8	57.7	84.9
	– Suspected extrapulmonary ($n = 28$)				
	Cases: 5 proven				
	– Surveillance in transplant recipients ($n = 211$)				
	Cases: 1 proven; 1 possible				
	– Overall ($n = 797$)				
Becker et al. [95]	Cases: 2 proven; 11 probable; 4 suspected Controls: 117	47	93	73	82
Pinel et al. [102]	Cases: 3 proven; 31 probable Controls: 751	50	99.6	85	97.7
Rovira et al. [103]	Cases: 1 proven; 5 probable; 2 possible Controls: 66	75	100	100	97
Maertens et al. [66]	Cases: 16 proven; 13 probable Controls: 74	96.5	98.6	98.6	98.4
Kawazu et al. [104]	Cases: 9 proven; 2 probable Controls: 125	100	93	55	100

Table 1 (continued)

References	Number of proven and probable cases *vs*. controls	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)
Marr et al. [45]	Cases: 13 proven; 11 probable Controls: 35	87.5 (no AF) 81.8 (AF)	77.1 (no AF) 77.1 (AF)	–	–
Buchheidt et al. [105]	Cases: 8 proven; 3 probable Controls: 104	33.3	98.9	–	–
Pazos et al. [106]	Cases: 5 proven; 3 probable Controls: 29	87.5	89.6	70	96.3
Marr et al. [92]	Cases: 20 proven; 26 probable Controls: 269	43.4	93.3	52.6	90.6
Yoo et al. [107]	Cases: 2 proven; 12 probable Controls: 114	85.7	78	32.4	97.8
Busca et al. [108]	Cases: 2 proven; 7 possible	100	93	64	100
Suankratay et al. [109]	Cases: 5 proven; 12 probable Controls: 33	88.2	97	93.8	94.1
Steinbach et al. [110]	Cases: 1 probable Controls: 63	–	87.3	–	–
Maertens et al. [67]	Cases: 19 proven; 19 probable Controls: 201	92.1	97.5	87.5	98.4
Lai et al. [111]	Cases: 5 proven; 9 probable Controls: 149	78.6	93.9	55	97.9
Yoo et al. [112]	Cases: 7 probable Controls: 56	71.4	98.2	83.3	96.4
Penack et al. [69]	Cases: 17 proven and probable	100	93.8	60.7	100

Legend: AF, antifungal prophylaxis; EIA, enzyme immunoassay; FUO, fever of unknown origin; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.

neutropenia [60, 61]. In neutropenic animals, pulmonary disease is characterised by intensive hyphal growth, high fungal burden and angioinvasion. This usually results in high GM concentrations, both in the lungs and the serum [61]. By contrast, in steroid-immunosuppressed animals, pulmonary inflammation is the main cause of disease progression. The fungal burden is low with little angioinvasion, resulting in GM levels close to the detection threshold [61].

4.3.2 Timing and Intensity of Sampling

Most prospective studies have assessed serial screening throughout the period of increased risk. However, some studies primarily addressed the utility of "focused" GM screening (e.g. up to 3 consecutive days) in patients with neutropenic fever of unknown origin or with clinically suspected pulmonary infection [57], resulting in significant differences in the mean number and timing of measurements. This will affect the performance of the assay.

4.3.3 Disease Manifestation of IA

Invasive aspergillosis represents a continuous spectrum of disease manifestations with distinct histological and radiological appearances that are based primarily on the immune status of the host. Clearly, leakage of GM into the circulation will differ in different disease manifestations. For example, the presence of haemorrhagic infarctions due to angioinvasion may facilitate leakage of antigens, whereas locally invasive (e.g. *Aspergillus* tracheobronchitis) [50] or encapsulated manifestations (such as abscesses) [62] may hamper the release of GM. Chronic non-invasive forms of aspergillosis such as chronic cavitary pulmonary aspergillosis (CCPA) and allergic bronchopulmonary aspergillosis (ABPA) are typically associated with negative serum GM test results [63].

4.3.4 Case Definition of IA

Given the rigid gold-standard definition of proven IA, stringent criteria should be used in the analysis and should preferably include the histopathologic control of positive and negative test results [64]. However, autopsy-based evaluations are biased towards selecting manifestations of *Aspergillus* disease with greater organ burden and antigenaemia and omit many unverified, though relevant study episodes. Stratifying study episodes or patients according to their probability of disease whilst using standardised diagnostic criteria overcomes at least partly these methodological obstacles. The recently proposed EORTC-MSG set of research-oriented consensus criteria for defining degrees of diagnostic certainty has greatly improved the uniform classification of patients with IA [15, 42]. Nevertheless, despite the use of these definitions in most recent studies, variable sensitivities have been reported, largely due to the addition of "possible" cases to "proven and probable" cases [65]. "Possible" infection is usually defined by clinico-radiological findings without microbiological confirmation. Numerous studies have concluded that the inclusion of "possible" infections in the case definition underestimates the sensitivity; they should therefore be omitted from the sensitivity analysis [56].

The *specificity* of the assay largely depends on the definition of a positive result. A specificity of 98.8% was demonstrated if two consecutive results were required (index $\geq$1.0) *versus* 85.4% if only one was required for classification as true-positive [65]. However, false-positive results have been reported in up to 20% of cases, even in studies requiring consecutive positive samples. The cut-off for positivity is the second major variable that affects specificity. When launched in the 1990s, the manufacturer recommended a serum cut-off ratio of 1.5. In their analysis of reproducibility of the test, Verweij et al. already suggested to lower the threshold for negative samples from 1.0 to 0.8 and for positive samples from 1.5 to 1.0 [44]. Meanwhile, many European investigators have gradually lowered the index cut-off in serum to 1.0 or less, especially in an attempt to make an earlier diagnosis [57, 65, 66]. More recently, the performance of the Platelia™ *Aspergillus* EIA test was also assessed in the USA and was found to be both sensitive (81%) and specific (89%) at a low cut-off. Therefore, in May 2003, the FDA accepted an index cut-off

for positivity of ≥0.5 [45]. This latter cut-off has recently also been accepted in Europe based on receiver-operating characteristic (ROC) analyses from two major centres [67].

Although there is general agreement on the best cut-off for serum GM testing, the ideal threshold for testing BAL or other body fluids remains a matter of debate. In an experimental model, Hope et al. [68] showed that GM is detected earlier and at higher levels in the alveolar compartment, in comparison to the endothelial compartment. In the clinical practice, many studies have shown that testing GM in BAL fluid samples result in higher optical densities than testing sera [49, 69]. That occurs for both haematological and non-haematological patients. Using 0.5 as the cut-off for BAL testing will probably result in many false-positive results, which have been demonstrated in several publications [70], particularly when lung transplant recipients are tested [49, 71].

The problem of unique false positive samples at a predefined ("static") cut-off can be handled by retesting the original sample or by requiring a subsequent positive sample ("dynamic" cut-off) for that patient. However, given the gradual rise in antigenaemia in proven and probable cases of IA – as opposed to the stable or fluctuant results in false positivity – a "dynamic" threshold seems clinically more meaningful. The demonstration of two sequential sera with an OD ≥0.5 increases the specificity and the positive predictive value of the assay to 98.6% and the clinical efficiency to 98% [66].

The incidence of *false-positive results* varies from ~5% in adults to as much as 83% in premature infants [23]. Different causes are summarised in here:

- In neonates, false-positivity can be partly explained by gastrointestinal translocation of GM [72]. Besides, GM is widely distributed throughout nature, has been detected in food and drinks, and is commonly found in human faeces. Smaller GM molecules might translocate in patients with impaired integrity of their intestinal mucosa, such as neonates. An identical mechanism probably applies to cancer patients with therapy-induced mucositis and might explain the higher occurrence of false-positive results in allogeneic HSCT recipients during the first weeks after conditioning, although other have failed to demonstrate this [73]. Recently, it was demonstrated that the origin of persistent antigenaemia in neonates without IA might be due to the presence of a lipoteichoid acid of *Bifidobacterium* spp., a major coloniser of the neonatal gut. This molecule shares epitopes with GM that can be recognised by the EB-A2 monoclonal antibody [74, 75].
- The intravenous administration of the antibiotic piperacillin-tazobactam [76–82] and the intravenous and oral formulation of amoxicillin-clavulanate [83, 84] have been shown to result in false-positive EIA reactivity. The precise origin for this reactivity remains unknown. However, piperacillin and amoxicillin are semi-synthetic drugs derived from natural compounds produced by fungi of the *Penicillium* species. Because this latter species contains GM in the cell wall, it has been hypothesised that detectable levels of antigen may be carried through the production process into batches for therapeutic use.

- Cyclophosphamide and its metabolites have been suspected of causing false-positive results. However, others were unable to detect false-positivity in a series of transplant patients conditioned with a cyclophosphamide-containing regimen [73].
- False EIA reactivity has occasionally been observed in patients infected with fungal organisms that share cross-reacting epitopes with *Aspergillus* species, such as *Penicillium* chrysogenum, *Paecilomyces lilacinus*, *Nigrospora oryzae*, *Trichothecium roseum*, *Cryptococcus neoformans, Cryptococcus gattii, Geotrichum capitatum*, *Paracoccidioidomycosis brasiliensis* and *Histoplasma capsulatum* [85–87]. Fortunately, many of these pathogens are rare in immunocompromised patients. False-positive assays have been found in neutropenic patients with bacteremia caused by *Escherichia coli*, Staphylococci, Enterococci, *Corynebacterium jeikeium*, and *Pseudomonas* species [28]; however, exoantigens from these organisms were not reactive in the assay and others failed to confirm this observation [65, 73].
- In liver transplant recipients, patients with underlying auto-immune liver disease were more likely to have false-positive tests [59]. This observation is in line with the occurrence of false reactivity in other conditions associated with autoimmune phenomena, such as chronic GVHD [88]. In that same study, patients on dialysis were also more likely to have false-positive assays [59].
- The use of gluconate-containing plasma-expanders (Plasma-Lyte®) may result in false-positive readings [89].
- Finally, miscellaneous other causes of false-positivity have been reported, such as the presence of glucopyranose in cotton swabs, lab contamination, and technical problems.

Because GM is released primarily by growing hyphae, and not by conidia that are colonising the airways, it is thought that detection of GM may provide better evidence of actual *Aspergillus* infection (notable by growth of hyphae) as opposed to either culture or polymerase chain reaction. Studies performed in cystic fibrosis patients with culture-proven airway colonisation have demonstrated repeatedly negative test results. However, some studies have suggested that false-positive results may be seen with GM BAL testing when lung transplant recipients colonised with *Aspergillus* species are tested [71][also Dr. Pasqualotto, Preliminary data presented at ECCMID, 2009].

Major variables affecting *false-negativity* include the presence of anti-*Aspergillus* antibodies and the prophylactic and empiric use of mould-active antifungal agents. Contrary to the widely accepted belief that immunosuppressed patients are not able to mount a significant antibody response, Herbrecht et al. detected anti-*Aspergillus* antibodies in one third of the patients with possible, probable, and proven IA at the onset of infection and demonstrated a higher sensitivity in patients without neutralising antibodies (64% for proven and probable infection) than in patients with anti-*Aspergillus* antibodies (37%) [57]. However, a large group of this study population was not severely immunosuppressed and was probably immunised before the development of invasive disease.

The impact of the prophylactic or empirical use of mould-active agents appears to be much more important. Animal studies have showed that a single administration of a mould-active agent (such as itraconazole, voriconazole, posaconazole, caspofungin, and amphotericin B) reduces the maximum concentration of antigen in the blood, resulting in a delayed detection of GM [90]. However, in several prospective studies patients received antifungal prophylaxis with itraconazole. In addition, empirical antifungal therapy is considered standard of care for the treatment of antibiotic-resistant or relapsing neutropenic fever and has been prescribed in many studies [91]. These confounding factors may have hampered the interpretation of those studies. As recently evidenced by Marr et al. sensitivity of the assay is highest in patients who do not receive preventative mould-active agents [92].

Finally, false-negativity may be related to technical issues [93].

Table 2 summarizes the studies that have evaluated the performance of the GM EIA applied to BAL samples, from haematology patients as well as non-haematology patients [48, 51, 70, 71, 94–96]. In general, the assay shows good sensitivity and excellent negative predictive value; GM can be detected in patients with IA with higher sensitivity than is the case with culture, as well as early in the course of infection. However, the evidence is largely based on retrospective studies that often include heterogeneous patient populations and limited numbers of cases of proven disease. Clearly, well-designed prospective studies with systematic sampling and use of consensus case definitions are needed to compare the performance of antigen detection in samples other than serum specimens with that in serum specimens [47].

Table 2 Performance of the Platelia™ *Aspergillus* EIA in BAL samples. Proven and probable cases were defined according to the 2002 EORTC-MSG consensus criteria [15]

References	Proven and probable cases *vs*. controls	EIA cut-off	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)
Sanguinetti et al. [94]	Cases: 20	1.5	100			
Becker et al. [95]	Cases: 17	1.0	100	100	100	100
	Controls: 143	1.0	85	100	100	88
	Cases: 22					
	Controls: 176					
Musher et al. [96]	Cases: 50	0.5	76	94	84	96
	Controls: 50					
Clancy et al. [71]	Cases: 5	1.0	100	90.8	41.7	100
Husain et al. [51]	Cases: 6	1.0	60	95	–	–
Nguyen et al. [70]	Cases: 6	1.0	100	88.1	42.9	100
	Controls: 67					
Meersseman et al. [48]	Cases: 26	0.5	88.1	87	–	–
	Controls: 43					

Legend: BAL, bronchoalveolar lavage fluid; EIA: enzyme immunoassay; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; spec, specificity.

5 Conclusions

Current data support the use of the serum (and BAL) GM EIA as a biomarker to be used in conjunction with other diagnostic tests (radiographic procedures, clinical findings) for diagnosis of probable IA and patient inclusion in clinical trials for IA. The revised EORTC/MSG definitions for IA are consistent with this conclusion, with incorporation of serum and BAL GM EIA as a microbiologic criterion to define disease.

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β-D-Glucan Testing

Minoru Yoshida

Abstract β-D-glucan is a fungal cell wall component circulating in the blood of patients with invasive fungal infections besides zygomycosis. This chapter reviews Japanese, European, and North-American experience with β-D-glucan testing in patients with invasive aspergillosis. Japanese testing results using a G-test, Fungitec G-test MK, and β-glucan Test WAKO indicated a positive rate of 82.5% in 85 of 103 cases of invasive pulmonary aspergillosis. European and North-American results using the Fungitell assay were equivalent, with a positive rate of 73.5% in 83 of 113 cases. Testing for β-D-glucan requires caution with regard to false positives, regardless of the assay method used. In addition, β-D-glucan testing offers promise for preemptive/presumptive therapy of invasive aspergillosis, as well as in monitoring response to therapy.

Keywords Beta-glucan · Diagnosis · Fungitell · Invasive aspergillosis · Preemptive therapy

Contents

M. Yoshida (✉)
Fourth Department of Internal Medicine, Teikyo University School of Medicine, Kawasaki city, Japan
e-mail: myoshida@med.teikyo-u.ac.jp

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1 Introduction

Invasive aspergillosis (IA) is a serious infection affecting mostly immunocompromised patients such as those with haematological malignancies and haematopoietic stem cell transplantation (HSCT) recipients. Recent increases in the incidence of IA have been noted. Much of this incidence is in the form of invasive pulmonary aspergillosis (IPA) – early diagnosis of such cases is extremely difficult because of the dearth of specific clinical manifestations [1]. Pathologically or microbiologically definitive diagnosis usually requires the performance of invasive medical procedures, which is not usually feasible in thrombocytopenic patients with haematological malignancies. New antifungal drugs with exceptional antifungal activity against *Aspergillus* spp. have been marketed in recent years, and yet there is an urgent need for novel non-invasive methods allowing for an early diagnosis of IA. β-D-glucan (BDG) is contained in the cell wall of most fungi besides the *Zygomycetes*, so it is an useful target for the serological diagnosis of IPA. This paper summarises the Japanese experience with BDG testing results for the diagnosis of IA, in conjunction with the European and North-American data accumulated so far.

2 History of BDG Testing in Japan

The G-test, a method of serological diagnosis that tests for BDG, was developed in Japan in 1992 by Obayashi T et al. [2]. Factor G has been found to be a constituent that reacts with BDG from fungi, so the principle of the G test is to use the coagulation cascade mediated by this factor in a Limulus amebocyte lysate (LAL) test, which is conventionally used to detect endotoxin [3]. In Japan, an assay specific to endotoxin was first developed by excluding this factor G from the lysate of the Asian horseshoe crab (*Tachypleus tridentatus*) [4], and this assay was used in the diagnosis of gram-negative bacterial infections [5]. The G-test, a glucan-specific assay, was subsequently developed and used in the diagnosis of fungal infections [2, 6]. Multicentre clinical trials have been conducted primarily in the fields of haematology and respiratory medicine, and exceptional results have been announced, i.e. positive G-test results for 37 of 41 cases of deep mycosis with sensitivity of 90% and specificity of 100% [7]. Reports also indicated that BDG is detectable in relatively rare mycoses such as those due to *Trichosporon, Fusarium, Acremonium,* and *Saccharomyces* in addition to *Candida* and *Aspergillus* infections [8]. *Cryptococcus* has a lower BDG content than other fungi, so negative results may be seen in patients with cryptococcal pneumonia, while positive results may occur in cases of fungaemia (probably due to the large amount of fungi in the blood) [2, 9]. In zygomycosis, in contrast, there is no reaction since these fungi contain no BDG.

The G-test was initially performed by pre-treating plasma with perchloric acid (PCA) and then measuring BDG levels by chromogenic assay [2, 7], but the currently popular form is the Fungitec G-test MK using alkaline pre-treatment followed by kinetic chromogenic assay [10]. In addition, the β-glucan Test WAKO,

Table 1 Comparison of β-D-glucan assay kits

Kit	G-test[a]	Fungitec G test-MK	β-glucan test WAKO	Fungitell
Manufacturer	Seikagaku Corporation	Seikagaku Corporation	Wako Pure Chemical Industries	Associates of Cape Cod Inc.
Lysate	*Tachypleus tridentatus*	*Tachypleus tridentatus*	*Limulus polyphemus*	*Limulus polyphemus*
Pre-treatment	Perchloric acid	Alkaline	Dilution-heating	Alkaline
Method	Endpoint chromogenic	Kinetic chromogenic	Kinetic turbidimetric	Kinetic chromogenic
Standard glucan	Pachyman (*Poria cocos*)	Pachyman (*Poria cocos*)	Curdlan (*Alcaligenes faecalis*)	Pachyman (*Poria cocos*)
Detection range	2–60 pg/ml	3.9–500 pg/ml	6–600 pg/ml	31.25–500 pg/ml
Cut-off value	20 pg	20 pg	11 pg	60–80 pg
Approval year	1995	1996	1996	2004

[a] This test was removed from the marked and replaced by the kinetic formulation (Fungitec G test-MK).

an assay that pre-treats plasma by the dilution-heating method and then measures BDG levels by kinetic turbidimetric assay, was also developed. This method was reported to have results comparable to those of the G-test, indicating a positive in 29 of 37 cases of deep mycosis (78.4%) [11]. Characteristics of both tests are shown in Table 1. Both utilize the same principle but have different methods of measurement and standard glucan, so their measurements do not usually coincide.

3 BDG Testing Results for IPA

Since the G-test was approved as a diagnostic test in Japan in 1995, BDG has been widely used as a non-invasive method of diagnosing deep mycosis in this country [9, 12–18]. In the field of haematology, for instance, a survey found that BDG testing is performed as part of routine diagnosis for invasive fungal diseases (IFD) at some 90% of the Japanese facilities [19]. Besides IA, BDG is detectable in many other relevant fungal infections including candidosis, so there are reports of such testing in a variety of fields from surgery and emergency medicine. However, most reports of IPA are from the fields of respiratory medicine and haematology, including HSCT; consolidating the literature indicates that testing was positive in 85 of 103 cases (82.5%) of IPA. These results are summarized in Table 2, stratified by the method of testing [20–24]. Several reports evaluated the performance of multiple methods for BDG testing [12, 15, 16, 18, 25]. Results of these comparisons show that the G-test had a positive rate of 82.4% in 14 of 17 cases, the Fungitec G-test MK had a rate of 84.6% in 66 of 78 cases, and the WAKO test had a rate of 72.7% in 24 of 33 cases. A comparison of BDG, galactomannan (GM) and *Aspergillus* DNA

detection by polymerase chain reaction (PCR) technique has also been performed. In the study by Kami et al., the positive rates were 67% for BDG, 58% for GM, and 79% for PCR [13]. Kawazu et al. reported that GM had the highest positive rate at 100%, using a cut-off value of 0.6, while BDG and PCR had similar rates (55%) [14]. These 2 studies were conducted at different times in the same facility. In addition, the BDG test used by the former was the Fungitec G-test MK, while the latter used the WAKO test. The WAKO test tends to show poorer sensitivity for BDG detection, a finding that was confirmed by the results from other studies (Table 2). This was also observed in experimental animal models of *Candida* and *Aspergillus* infections [25]. Nowadays, the WAKO test is no longer recommended for the early diagnosis of IPA.

Recently Senn et al. evaluated the performance of BDG detection by screening 95 patients with 190 episodes of neutropenia complicating acute leukaemia. BDG was detected using a colorimetric assay (β-glucan test Maruha) [26]. In the presence of two positive tests (using a cut-off value of ≥7 pg/ml), the positive and negative predictive values for the diagnosis of IFD were 0.79 and 0.91, respectively. BDG detection occurred much earlier than radiological manifestations of IFD, and also before the diagnosis was performed using the EORTC/MSG criteria. However, 6 of 15 cases of proven/probable IPA were not diagnosed by this test despite the use of a reduced cut-off value of ≥7 pg/ml (manufacturer's cut-off: 11 pg/ml [27]).

Table 2 Summary of β–D-glucan determination in patients with invasive pulmonary aspergillosis

		Number of positive samples/Number of patients			
References	Year	G-test	Fungitec G test-MK	WAKO	Fungitell
Obayashi et al. [7]	1995	7/8	–	–	–
Yoshida et al. [17]	1997	1/1	–	–	–
Hossain et al. [25]	1997	1/1	–	1/1	–
Mori et al. [11]	1997	–	–	4/4	–
Mori et al. [15]	2000	3/5	5/5	5/5	–
Kami et al. [13]	2001	–	22/33	–	–
Moro et al. [16]	2003	2/2	2/2	2/2	–
Kawazu et al. [14]	2004	–	–	6/11	–
Horiguchi [12]	2004	–	7/8	5/8	–
Odabasi et al. [20]	2004	–	–	–	4/4
Ostrosky-Zeichner et al. [22]	2005	–	–	–	8/10
Pazos et al. [23]	2005	–	–	–	7/8
Koo et al. [21]	2006	–	–	–	16/21
Persat et al. [24]	2008	–	–	–	48/70
Obayashi et al. [9]	2008	–	26/26	–	–
Yoshida [18]	2008	–	4/4	1/2	–
Total		14/17 (82.4%)	66/78 (84.6%)	24/33 (72.7%)	83/113 (73.5%)

4 Development of a BDG Assay in the USA

In 2004, the United States of America (USA) firm Associates of Cape Cod developed Fungitell, a kit for BDG detection in serum that was cleared by their regulatory agency, FDA (Food and Drug Administration) [20]. This assay is based on the coagulation cascade of the American horseshoe crab (*Limulus polyphemus*), but the methods of plasma pre-treatment and testing are similar to those for the Fungitec G-test MK (Table 1), and the latter has almost the same sensitivity. This test has been accepted as an adjunct for the diagnosis of IFD and included in the revised EORTC/MSG criteria for the diagnosis of IA, in parallel to GM testing [28]. In addition, it was included amongst the non-culture-based methods in the latest guidelines for the treatment of IA by the Infectious Disease Society of America (IDSA, USA) [29]. The assay provides satisfactory results with a positive rate of 73.5% (Table 2). While Japanese results are similar, much of the available data results from studies evaluating definitive cases of IPA, so there are insufficient data with regard to the assays' utility in early diagnosis.

5 False-Positive Results for BDG

Several precautions are necessary when using BDG as an indicator of IFD. False-positive results have been reported in Japan and it is quite likely that this will become a problem in Europe and the USA as well. Sufficient recognition of this problem is needed in order to appropriately evaluate BDG testing. False-positive results may occur for several reasons, including cellulose-based dialysates [30], surgical gauze [31], blood products (γ-globulin and albumin) [32, 33], certain antibiotics (amoxicillin-clavulanic acid) [34], and drugs containing glucan (e.g., schizophyllan, lentinan, crestin, scleroglucan) [35]. With the Fungitec G-test MK, false-positive results as an effect of turbidity have been noted in patients with hyper-γ-globulinaemia and in patients with jaundice, but methods of pre-treatment are being applied successfully [36]. These false-positive results in particular represent an even greater problem for fields like surgery, emergency medicine, and organ transplantation than for haematology.

6 Preemptive/Presumptive Therapy and Therapeutic Monitoring Using BDG

Detection of a pathogen, regardless of whether it is bacterial or fungal, is usually difficult in cases of febrile neutropenia. The IDSA guidelines for management of these patients recommend empiric therapy with an antifungal agent in instances where febrile neutropenia does not respond to a broad-spectrum antibiotics after 3–5 days [37]. In the author's experience, the effectiveness of an antifungal agent clearly differs depending on the BDG test results at this point, with antifungal agents being

highly effective when high BDG levels are present [38]. In light of such results, Japanese guidelines for the management of patients with febrile neutropenia recommend testing for BDG at the start of empiric antifungal therapy [39]. Insights obtained from this approach reveal that empiric antifungal therapy is usually given in most cases to patients who actually do not have an invasive fungal disease. Therefore, BDG is a promising tool for preemptive antifungal therapy, as has been already demonstrated with GM testing and computed tomography (CT) imaging [40].

In addition to the BDG use in the preemptive setting, testing for BDG may also be attempted to monitor patients' response to antifungal [26, 38]. However, attention must be paid to BDG clearance when this test is used for monitoring patients with IA [41]. Recovery from an IFD is generally associated with the occurrence of symptoms such as fever, which are followed by a drop in other parameters like imaging findings and inflammatory markers (e.g., C-reactive protein). GM testing has been used for this purpose – patients who are improving tend do demonstrate decreased GM levels [42]. The dynamics of BDG reduction will depend on the initial BDG values. In the experience of this author, residual BDG following the disappearance of clinical manifestations does not signify the activity of a fungal disease. Although reductions in BDG levels are important, treatment does not necessarily need be continued until these levels return to normal.

7 Conclusions

This chapter has discussed the utility of BDG testing in patients with IA. In recent years, mould infections have increased in importance in clinical practice. Most of these infections are caused by species of *Aspergillus*, *Fusarium* and the *Zygomycetes*. One of the criticisms about BDG testing is that it does not allow for

Table 3 Differential diagnosis for major mould infections and pneumocystosis. Combining different tests might add significantly to the diagnosis of these conditions, in special if polymerase chain reaction (PCR) test is also used

Diagnosis	β-D-glucan	Galactomannan	Typical chest CT findings
Aspergillosis	+	+	Halo, air crescent and air space consolidation signs; nodules
Fusariosis	+	–	Halo, air crescent and air space consolidation signs; nodules or masses
Zygomycosis	–	–	Halo, air crescent and air space consolidation signs; multiple nodules
Pneumocystosis	+	–	Bilateral diffuse infiltrate with ground glass opacities

Legend: CT, computed tomography.

the differentiation of important fungal pathogens such as *Aspergillus* and *Candida* species. However, discrimination might be achieved when different tests are used in combination, like chest CT images, BDG and GM (Table 3). Although BDG testing might also be positive in patients with *Pneumocystis jirovecii* infection [43], this condition is usually associated with typical imaging results in patients with AIDS, so there is not much overlap with other mould infections. If applied with sufficient understanding for its properties, BDG testing is an useful diagnostic tool for IA.

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Polymerase Chain Reaction (PCR)-Based Tests

P. Lewis White and Rosemary A. Barnes

Abstract PCR to aid in the diagnosis of invasive aspergillosis has been in use for over 15 years but little or no agreement over methodology has limited both widespread clinical use and large scale multi-centre evaluation. Each stage of the assay needs to be evaluated, including specimen type, extraction protocol, PCR amplification and result interpretation. The technical laboratory procedures are the least complicated to assess, although may be specimen-specific and recently efforts have been made to standardise methodology when testing blood specimens. The specimen type influences all downstream applications and will limit assay performance, particularly if the specimen is combined with unsuitable extraction processes. Advances in our knowledge of the disease process and host response may elucidate the optimal specimen but this will vary with disease type and the underlying condition of the host. Indeed, the underlying status of the patient may restrict the use of PCR if testing intrusive specimens is favoured. Large scale clinical evaluations using multiple specimen types and/or the testing of animal models will allow us to define the correct specimen type(s), optimise methodology accordingly and define guidelines for confident result interpretation. Only then will *Aspergillus* PCR gain widespread acceptance and use. This chapter describes the complexities related to each stage of *Aspergillus* PCR and analyses PCR performance based on specimen type, extraction protocol and PCR amplification to explicate what we currently know and reveal what research still needs to be performed.

Keywords DNA extraction · Molecular diagnosis · PCR · PCR amplification · Polymerase chain reaction · Real-time PCR

P.L. White (✉)
NPHS Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff, UK
e-mail: lewis.white@nphs.wales.nhs.uk

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Contents

1 Principles

1.1 PCR – An Overview

The application of polymerase chain reaction (PCR) to aid in the diagnosis of invasive aspergillosis (IA) is not a novel concept. The first manuscripts were published over 15 years ago and new papers on the topic are regularly being presented. Advancing PCR technology, such as real-time PCR, has resulted in a new generation of techniques but conventional methods are still being used and developed due to limitations imposed by the cost of real-time PCR. Despite an abundance of literature on the subject (approximately 200 papers) large multi-centre evaluations have not been performed. As IA is of relatively low prevalence this is essential to contribute a large cohort of proven/probable cases and provide representative performance parameters. Before large-scale evaluation can be performed a consensus regarding an optimal method(s) is needed and without it inclusion in future diagnostic consensus criteria unlikely. Recently, three organisations (The German-Austrian Initiative for *Aspergillus*-PCR standardization, The UK Fungal PCR consensus group and the ISHAM Working group, The European *Aspergillus* PCR Initiative, EAPCRI) have focussed on achieving this goal. Additionally the development of several commercial diagnostic *Aspergillus* PCR kits has minimised concerns regarding standardisation and quality control of reagents but all require extensive validation.

When performing any test it is paramount that correct specimen is obtained and at the correct time. It is essential that thought be given to the origin of infection, the disease process and what specimen is most likely to provide an accurate result at that specific time. However, obtaining certain specimens such as cerebrospinal fluid (CSF) and bronchoalveolar lavage (BAL) may be limited particularly important if high-frequency screening is required. On receiving a specimen a decision must be made as to what is being targeted within that specimen and what nucleic acid extraction technique should be used to efficiently extract this fraction. *Aspergillus* specific or pan-fungal PCR can then be performed but it is essential to combine it with an internal control PCR. An “ideal” PCR test would be able to rule-in and exclude the

presence of infections. Often, this is not possible and a test can only be used to confidently predict one of these. The assay performance will be influenced by its design and how this affects sensitivity/negative predictive value (NPV) and specificity/positive predictive value (PPV). On completing PCR the result must be interpreted within the framework of the assay (extraction, PCR and internal controls) and within the diagnostic guidelines (underlying condition and clinical signs, predisposing risk factors, previous PCR results and results from additional microbiological, radiological and histopathological tests).

This section will focus on each individual stage of molecular diagnostic discussing the principles and limitations of each phase.

1.2 Specimen Type

The ideal specimen for *Aspergillus* PCR is widely debated. If the wrong specimen is taken or extracted using a method unsuitable for that specimen type then the final PCR result may be useless. Hypothetically, the ideal specimen should allow earlier detection, have minimal impact on the patient, be easily processed within the laboratory and generate an interpretable result. In practice this is difficult to achieve. The specimen used should meet the diagnostic strategy. It is unlikely that invasive specimens (e.g., BAL) will be routinely used in a high frequency screening services and would be better applied to a diagnostic test with a high PPV trying to define infection. However, the combined use of different specimen types (occasional BAL and regular blood testing) may provide an optimal approach.

With humans inhaling several hundred conidia per day the site of infection is usually the respiratory tract [1]. Testing respiratory specimens may pre-empt invasive disease and will allow the origin of infection to be targeted providing the earliest opportunity for diagnosis and targeted preemptive therapy. The type of infection (allergic, saprotrophic and invasive) may play a critical role in specimen selection and respiratory specimens may be beneficial when trying to diagnose non-or pre-invasive disease.

The benefits of using respiratory specimens are balanced against contamination, colonisation and the invasive nature of obtaining lower respiratory tract specimens. In patients at risk of haemorrhage (e.g. thrombocytopenic) it is less likely that BAL specimens will be routinely obtained and this may be particularly problematic if high frequency screening is required. The presence of conidia within the respiratory tract does not certify disease. Approximately 50% of *Aspergillus* culture-positive respiratory tract specimens correlate with proven/probable IA and a positive PCR result from a respiratory specimen should carry the same credence as a positive culture [2, 3]. Combining PCR with radiological markers has proved useful. By comparing PCR results from an area of suspected infection (e.g., computed tomography – CT – showing a cavitating nodule with air crescent) to an area thought to be clear of infection, Kawazu et al. [4] showed that the fungal burden in areas of infection was higher than in areas without. The development of quantitative PCR may be useful in distinguishing between infection and contamination.

If invasive disease progresses angioinvasion can lead to the dissemination to other organs and cause systemic infection. Obtaining blood, CSF or deep-tissue specimens may be useful. Unlike the respiratory tract, blood is a sterile environment and the presence of *Aspergillus* (DNA or organism) within the bloodstream is significant but may be limited. *Aspergillus* fungaemia is rarely documented and it is argued that the fungus must be present in a non-viable state [5, 6]. However, viable organism must be present as the infection usually disseminates from the lungs via the blood stream [7, 8]. It is likely that the existence of viable organism in the blood stream is transient and detection could be improved by high frequency large volume screening. Viable hyphal fragments may become phagocytised by neutrophils but targeting this fraction would be limiting in severely neutropenic patients at risk of IA. Additionally platelets can attach to invading hyphae causing direct cell wall damage rendering them non-viable but not phagocytised [1].

The use of non-invasive blood specimens permits high frequency, high volume throughput and is ideal for screening services, but suffers from a possible delay in detection prior to angioinvasion. Their use will be better targeted to invasive disease and PCR positivity rates have been shown to be significantly greater in patients with invasive disease (invasive pulmonary aspergillosis *versus* fungal balls, difference 72%, 95% confidence interval, CI: 29–87, $p = 0.0019$) [9]. Respiratory specimens were not processed in this study.

A large number of publications describe the successful testing of blood specimens but no agreement has been reached as to the optimal fraction (whole blood or serum/plasma). The majority of studies that evaluated PCR test in blood used whole blood, with volumes ranging from 200 μl to 10 ml (usually >3 ml) [10]. In utilising serum only free circulating DNA can be targeted as any fungal cells (free, phagocytosed, viable or not viable) will be lost during the splitting of the blood fractions but it does simplify extraction techniques. In vitro experiments have shown *A. fumigatus* DNA is not released during logarithmic growth and only occurs after mycelial damage suggesting that during infection hyphal damage is required for DNA release [11]. Free DNA found in blood specimens may be released by cell structure damaged caused by antifungal therapy targeting the fungal cell wall or membrane or possibly by the action of platelets and/or neutrophils attached to hyphal fragments.

The sensitivity of PCR blood testing depends on the volume of blood that is analysed. Using whole blood allows large volumes (>4 ml) to be tested, enhancing the opportunity for detection but also increasing the opportunity for contamination and inhibition. For instance, large volumes of blood from patients receiving heparin (e.g., after by-pass surgery) may cause inhibition and vacutainers containing heparin as an anticoagulant have been shown to be inhibitory [12]. Contamination has also been seen in sodium citrate vacutainers [13]. The use of vacutainers containing EDTA is usually preferred for blood testing [4]. Fungal DNA contamination of blood collection tubes has also been described [14], which may potentially lead to false-positive results.

The testing of CSF specimens by *Aspergillus* PCR is extremely limited. The intrusive nature of obtaining the specimens means the few studies that have been

published are generally case studies where PCR has been successfully applied to aid in the diagnosis of already suspected *Aspergillus* CNS infections [15–19]. Therefore a publication bias might exist. With a high mortality rate (>90%) pre-empting this infection by screening processes is paramount. This would not involve high frequency testing of CSF specimens but specimens from the origin of infection, whether this be BAL or nasal swabs/aspirates.

A range of publications have described the use of tissue specimens for the molecular detection of IA. Maxillary sinus, lung, brain, lumbar vertebrate, heart valve and muscle have all been tested by PCR as an aid to diagnosis [19–22].

Table 1 compares the performances of the three main specimen types used for *Aspergillus* PCR. Overall *Aspergillus* PCR using whole blood generated better sensitivities than both serum and BAL specimens (difference with serum = 7.8%, 95% CI: 0.1–14.9%, $p = 0.0378$; difference with BAL = 12.9%, 95% CI: 5.1–20.0, $p = 0.0018$). The opposite was true for specificity (difference with serum = –4.1%, 95% CI: –6.7 to –16.1, $p = 0.0014$; Difference with BAL = –4.6%, 95% CI: –7.5 to –14.9, $p = 0.0068$). Differences in performance between *Aspergillus* PCR using BAL or serum were not statistically significant. Combining the serum and whole blood assays gave the following performance statistics (sensitivity: 78.4%, 95% CI: 74.3–82.0; specificity: 91.1% 95% CI: 89.4–92.5). A meta-analysis of *Aspergillus* PCR on BAL specimens generated similar identical sensitivity (79%) and specificity (94%) values to the results presented in Table 1 [23].

1.3 Extraction Techniques

The principle role of the extraction system is to achieve efficient nucleic acid retrieval. This includes the removal of interfering compounds, such as inhibitors or human DNA and should be specific to the fungal target (free DNA or organism). It is important to know the possible fungal targets within different specimen types and as yet no single extraction method has proved optimal for all specimens. The extraction process will not be able to control source-based contamination but must avoid introducing new sources of contamination (e.g. Zymolase) [24, 25].

By utilising respiratory specimens the target for molecular diagnosis is the fungal cell (conidia or hyphae) and extraction methods can be simplified focussing on lysing the organism with minimal interference from the host. Certain respiratory specimens may require additional processing due to the presence of excessive pulmonary secretions and treatment with SDS and/or proteinase K may help. Bead-beating has been shown to be optimal in comparative study of six extraction methods testing BAL [26]. However, comparing the literature reveals that bead-beating and lyticase methods have similar sensitivities [27].

Whole blood extraction techniques are particularly demanding involving red-cell and white cell lysis prior to fungal lysis and DNA purification. Incorporating multiple steps will affect extraction efficiency which may also vary between users [28]. High frequency PCR screening has generated optimal results and utilising large whole blood volumes (4–10 ml) combined with complex manual extraction methods

Table 1 Comparison of specimen type, target, extraction method and performance

Specimen	Invasive	High frequency screening	Available target	Recommended extraction method	Performance	
					Sensitivity (%)	Specificity (%)
BAL	Yes	Unlikely[a]	Hyphae/conidia	Bead-beating[b]	84.6	87.7
				Lyticase[c]	79.7	94.6
				Both[d]	77.1	94.1
				Other[e]	77.8	94.0
				Overall	78.5	93.7
Serum	No	Yes	Free circulating fungal DNA	Qiamp kit[f]	71.7	93.9
				Proteinase K[g]	73.4	100
				Other[h]	75.3	91.6
				Overall	73.4	94.2

Table 1 (continued)

Specimen	Invasive	High frequency screening	Available target	Recommended extraction method	Performance	
					Sensitivity (%)	Specificity (%)
Circulating viable and non-viable fungal fragments	No	Yes	Free circulating fungal DNA (method dependent), fungal-associated DNA sources (phagocytosed and circulating non-viable fungal fragments)	Lyticase[i]	81.2	90.4
				Zymolase[J]	100	82.8
				Bead-beating[k]	92.3	94.6
				Overall	86.3	89.6

Legend: BAL, bronchoalveolar lavage.

[a]Unlikely in thrombocytopenic patients or other patients at risk of major haemoptysis.

[b]Combined statistical analysis performed on the studies of Melchers et al. [36] and Verweij et al. [37]. Total number of cases of IA/no IA: 13/65.

[c]Combined statistical analysis performed on the studies of Skladny et al. [64], Buchheidt et al. [65], Spiess et al. [66], and Schabereiter-Gurtner et al. [67]. Total number of cases of IA/no IA: 59/242.

[d]Combined statistical analysis performed on the studies of Rantakokko-Jalava et al. [68] and Francesconi et al. [69]. Total number of cases of IA/no IA: 35/102.

[e]Combined statistical analysis performed on the studies of Bretagne et al. [70], Jones et al. [71], Hayette et al. [72], Raad et al. [73], Musher et al. [74], and Sanguinetti et al. [75]. Total number of cases of IA/no IA: 126/546.

[f]Combined statistical analysis performed on the studies of Bretagne et al. [76], Kami et al. [40], Kawazu et al. [77], and Challier et al. [78]. Total number of cases of IA/no IA: 92/262.

[g]Combined Statistical analysis performed on the studies of Yamakami et al. [9, 79], and Kawamura et al. [80]. Total number of cases of IA/no IA: 94/59.

[h]Combined statistical analysis performed on the studies of Williamson et al. [43], Costa et al. [29], Pham et al. [81], and Millon et al. [41]. Total number of cases of IA/no IA: 81/95.

[i]Combined statistical analysis performed on the studies of Van Burik et al. [38], Skladny et al. [64], Loeffler et al. [60, 61], Buchheidt et al. [65], Lass Florl et al. [50, 82], Speiss et al. [66], and Halliday et al. [44]. Total number of cases of IA/no IA: 117/591.

[j]Combined statistical analysis performed on the studies of Einsele et al. [83], Löffler et al. [84], and Hebart [51, 52]. Total number of cases of IA/no IA: 37/174.

[k]Statistical analysis performed on the study of White et al. [34]. Total number of cases of IA/no IA: 14/149.

may deter laboratories from providing a diagnostic fungal PCR service. The development and application of automated nucleic acid extraction systems may alleviate the problem although for whole blood pre-automated processing is still required to cope with the large specimen volumes and to lyse the fungal target [29–34]. To date all published automated methods have utilised the Roche MagNA Pure but with generation of new systems with improved extraction efficiencies continued assessment is required. Indeed, comparison of two automated extraction systems by a real-time quantitative PCR revealed 1–2 log difference in extraction efficiency dependent on the initial fungal burden [32]. Additionally some new generation extraction systems utilising on-board multiple use reservoirs of lysis and wash buffers have suffered from *Aspergillus* contamination (White, unpublished data).

Whole blood specimens may contain both fungal organism and free DNA, but the latter is usually lost during the complicated extraction process. High speed centrifugation combined with lyticase treatment, rather than mechanical disruption may allow both the organism and DNA to be targeted. Alternatively, serum or plasma could enter the extraction system downstream of any fungal cell lysis. A recent study showed that combining serum and whole blood extraction methods resulted in an early diagnosis of IA, in comparison to the isolated use of any of these methods [35].

Methods targeting *Aspergillus* conidia or hyphae (respiratory specimens, whole blood) should utilise bead-beating or recombinant lyticase to lyse fungal cells. Bead-beating is highly efficient in extracting DNA directly from both conidia and hyphae and has been successfully used in clinical studies using whole blood and BAL [28, 30, 32–34, 36–38]. Lyticase has had widespread application in clinical studies using BAL and/or whole blood (Table 1). Performance parameters for lyticase and bead-beating methods in testing blood/BAL are similar with sensitivities of 80.6% and 88.9% (difference: 8.3%; 95% CI: –9.5 to 17.9) and specificities of 91.6% and 92.5% (difference: 0.9%; 95%CI: –4.4 to 3.7) for lyticase and bead-beating, respectively.

Serum extractions focus entirely on free circulating DNA and with no cellular material present only require basic DNA purification and elution/precipitation. PCR performance using serum appears consistent independent of extraction method (Table 1). The application of basic commercial extraction systems has general appeal and may allow *Aspergillus* PCR to gain widespread use but with assay specificity being superior to sensitivity it may be better suited to ruling in rather than excluding disease.

Biopsy specimens must be adequately sliced/homogenised/digested to access and lyse the invading fungal hyphae accordingly. The use of fresh tissue is also beneficial with paraffin embedded specimens needing additional processing reducing the sensitivity of molecular assays [39]. Little validation has been performed on CSF extraction methods. Methods targeting both free DNA and fungal organism have been successfully used and as with whole blood both targets may be present. Extraction methods can be basic as interference from human cells should be minimal but inhibition should be monitored by the use of an internal control PCR.

1.4 PCR Amplification

The original PCR systems were limited to block-based amplification followed by post-amplification analysis. Since 2000 the application of real-time PCR has attained widespread use. This section will not focus on PCR type but discuss some of the general limitations associated with *Aspergillus* PCR. Some of these apply to both conventional and real-time PCR and some apply to fungal PCR as a whole. The benefits and limitations of conventional and real-time PCR will be discussed in Sections 2 and 3, in this chapter.

1.4.1 Oligonucleotide Design

PCR amplification is only as efficient the primers and optimisation will allow. In designing primers secondary structures (e.g., dimers, hairpins) should be avoided. These can be determined by various mathematical modelling programmes. In principle avoiding the formation of secondary structures should be straight-forward. In practice the perfect primers may not exist due to limitations in sequence variation between organisms. This is particularly relevant for highly conserved multi-copy genes such as the ribosomal RNA cluster that is used, as a single fungal genome may contain 10^2 copies. Comparison of the human and *Aspergillus* 18S rRNA genes reveals approximately 75% homology between sequences. This should allow scope for oligonucleotide design if *Aspergillus* species and or even genus level identification is required. If a pan-fungal approach is needed then the available sequence is more limited as variable regions generally differentiate fungi from each other as well as from the human 18S rRNA gene.

As secondary structures are determined by mathematically modelling it should be remembered that these will have limitations and if no alternative oligonucleotide options are available then it may be worth proceeding with the original choice. On reaching a decision regarding oligonucleotides the PCR reaction should be stringently optimised using positive material at a level considered clinically significant and negative material that is likely to be encountered in specimens (e.g., human DNA, DNA from other pathogens). Only then will a true detection limit and false-positivity range be determined.

For a species/genus specific assay single copy genes may be better suited. If a high sensitivity is paramount then multi-copy targets (rRNA or MtDNA) should be used, but the presence of a homologous gene within the human host must be considered. When designing primers, particularly pan-fungal primers, large areas of the conserved regions of the rRNA genes will be both pan fungal and human. In assays utilising specific probes this may lead to a lack of sensitivity as primers bind to and amplify the larger amounts of human DNA over the target *Aspergillus* DNA leading to false-negative results [32]. Conversely, in assays simply relying on PCR amplification (SYBR green or ethidium bromide detection) a lack of specificity will occur and give rise to false-positive results [32]. False-positives may also occur in assays utilising pan-fungal probes that possibly cross react with human DNA.

1.4.2 PCR Platform

Generally conventional block-based PCR assays can be transferred between thermal blocks with minimal re-optimisation. For real-time systems this is not always the case. For assays utilising hybridisation (FRET) probes their use is limited to the Roche Light-Cycler, whereas hydrolysis (Taqman) probes can usually transferred between platforms, although occasionally labelling with a different fluorophore is required.

In 2006 the UK Fungal PCR consensus group highlighted two optimal assays in use within UK centres performing *Aspergillus* PCR [33]. Both assays utilised hydrolysis probes but, one was developed for the Taqman, the other for the Light-Cycler [33, 34, 40]. Within the group most centres used Light-Cyclers so the Taqman assay was evaluated using this platform. PCR amplification of DNA extracted from a range of *Aspergillus* conidia revealed that the Taqman assay demonstrated equal if not more amplification efficiency when compared to the Light-Cycler assay. Conversely, when testing simulated whole blood specimens the Taqman assay displayed a dramatic reduction in detection limit and signal quality. Repeated attempts to re-optimise the assay for use on the Light-Cycler were unsuccessful. Sequence comparison of the oligonucleotides and human DNA revealed the primers shared high similarity with the human 18S gene [32–34]. Interestingly the assay was designed primarily for use with serum/plasma specimens, although was successful using whole blood at all centres using a Taqman platform. The reasons for the Light-Cycler associated difficulties have not been fully resolved.

Additional research evaluated the performance of the Light-Cycler assay on alternative platforms. Using the same running conditions the assay was able to detect 10 conidia/ml of whole blood on every platform tested, although 100% reproducible detection limits were 100, 25 and 10 conidia/ml of whole blood for the Light-Cycler, Corbett Rotorgene and Taqman, respectively [33, 34]. Care should be applied when interpreting this data as more centres used the Light-Cycler and variation in user experience may have had an effect.

1.5 Result Interpretation

A PCR result needs to meet test criteria before it can be interpreted in a clinical perspective. A negative result is only true if positive controls are indeed positive and the internal control PCR confirms amplification. A positive result is only true if negative controls have excluded experimental contamination and PCR positive results should always be confirmed by retesting. However, non-reproducible positive results may be of significance as *Aspergillus* DNA may be present in specimens below 100% reproducibility thresholds [41]. To exclude source-based contamination the definition of an *Aspergillus* PCR positive result is still debated. Some use two or more consecutive PCR positive results, others use two or more PCR positive results within the same period of neutropenia. A higher chance for IA seems to exist when two PCR results are obtained within a 2-week period [32]. Recent research

testing whole blood revealed positive likelihood ratios to be 2.7, 3.8 and 8.3 for single non-reproducible, single reproducible and multiple positive results, respectively with two or more positive PCR results to be used as significant mycological evidence [42]. The authors also proposed a diagnostic strategy incorporating PCR in to a neutropenic fever care pathway to screen for fungal disease and target antifungal therapy [42].

As such single PCR positive results are less convincing but need to be interpreted with care. When using blood specimens the presence of *Aspergillus* target appears transient and single positive result may be representative of disease. Assay sensitivities have suffered when using a preferred PCR criterion of two positive results [43]. A recent meta-analysis [10] showed that a single negative PCR result in blood is sufficient to exclude the diagnosis of IA – in order to improve specificity (from 75% to 87%; $p = 0.027$), two positive samples are required. For invasive specimens (CSF or BAL) a second sample may never arise and the clinician may be forced to interpret a single *Aspergillus* PCR positive result. Halliday et al. proposed interpretative guidelines for PCR diagnosis of IA but without extensive validation their approach is only applicable to their assay [44].

PCR results should be determined according the diagnostic strategy. PCR can be used as adjunct test to aid in the diagnosis in a patient with possible IA or it can be used to screen high-risk populations. For a diagnostic PCR high specificity and PPV are essential whereas for a screening test a high sensitivity and NPV are required. Screening assays allow antifungal therapy to be withheld in cases that are consistently negative but with the possibility of increasing incidence of non-*Aspergillus* mould infections a pan-fungal approach may be required [45].

The lack of an accepted consensus method has limited multi-centre evaluation of a defined system and consequently PCR is not yet included in current diagnostic criteria. However, a PCR result should be interpreted within the clinical, microbiological and radiological findings as defined by the modified EORTC-MSG consensus criteria [46]. PCR positive results lacking supporting clinical and/or microbiological factors may represent contamination. Alternatively it may be an early sign of infection as PCR results precede clinical diagnosis in haematological patients by up to 30 days (mean 14 days) [44]. Screening 83 patients with febrile neutropenia with real-time PCR Cuenca-Estrella et al. demonstrated that serial detection of *Aspergillus* DNA preceded IA diagnosis by computed tomography and galactomannan by an average 21 and 68 days, respectively [47]. Multiple positive PCR results in a high-risk patient should always be considered significant. Importantly, all PCR results should be combined with predisposing factors, other test results (e.g., high resolution CT scan, galactomannan testing and β-D-glucan), current antifungal therapy (including prophylaxis), patients underlying condition, and immune status. In a study in liver transplant recipients, concomitant real-time PCR performed on the first galactomannan-positive sample markedly improved the specificity of the galactomannan assay result [48]. The best strategy for combining these diagnostic tests in different scenarios is still undefined.

A PCR positive respiratory specimen can be used as a prognostic marker to highlight the presence of *Aspergillus* within the patient and is particularly relevant in

high-risk groups where prophylaxis to empiric or pre-emptive therapy may be necessary. Conversely, in the previous meta-analysis of *Aspergillus* PCR on BAL specimens the author suggests that a negative result should not delay therapy as sensitivity was 79% [23]. However, marked heterogeneity was observed in the study. If multiple specimens taken from areas of suspected infection are consistently negative then a high negative predictive value can be expected.

Aspergillus PCR positive results from CSF specimens should be considered significant but it is too early to agree whether PCR negative results can confidently exclude *Aspergillus* CNS infection.

Antifungal therapy may have a deleterious effect on PCR performance and PCR positivity may be reduced post-antifungal therapy [49, 50]. This may represent resolving disease, but occasionally clinical symptoms have persisted despite negative PCR results [51–53]. Initiating antifungal therapy enhances the clearance of fungal components from the blood and the benefit of testing of whole-blood specimens during antifungal therapy may be limited [50]. Conversely antifungal therapy may initiate DNA release and explain why molecular methods targeting free circulating DNA do not produce PCR negative results during therapy whereas methods targeting the organism (removing or destroying free DNA) may not useful in patients on antifungal agents [11]. It may be necessary to have a flexible approach when testing blood specimens using whole blood and serum pre and post treatment, respectively.

2 Conventional PCR

Papers describing *Aspergillus* PCR were first published in the early 1990s. Using conventional block-based thermal amplification post-amplification handling, usually gel electrophoresis, was required to obtain a result. Sensitivities were enhanced by the application of nested PCR, PCR ELISA or Southern hybridisation, the last two also enhancing specificity [54]. Restriction fragment length polymorphism (RFLP) was also used to differentiate between targets [55]. In addition to DNA amplification nucleic acid sequence based amplification (NASBA) technology targeting RNA has been successfully applied [56]. With a highly efficient amplification it is possible that NASBA would provide enhanced sensitivity in comparison to PCR but to date very little clinical evaluation has been performed while the technology has advanced utilising real-time detection using molecular beacons. In comparison to culture techniques conventional molecular techniques represented a major advancement in turn-around times, sensitivity and specificity and PCR has been successfully applied to histological investigations [57].

However, post-amplification handling is labour intensive, may require additional technical skill and increases the opportunity for contamination [54]. This can occur within the same run where cross-contamination may arise when an amplicon is carried-over when a pipette tip is mistakenly reused. For nested PCR protocols this may be particularly problematic as a small volume of first round contamination may

contain massive copy numbers of target but produce results that are representative of a typical positive. The contamination risk associated with nested-PCR methods affects the positive predictive value and they are unlikely to gain widespread diagnostic use [57, 58]. If nested-PCR methods are to be used they should be applied to screening services where high negative predictive values can be used to exclude disease [32–34]. A second type of post-amplification contamination is environmental amplicon contamination where previously amplified products have gained exposure to the working environment during processing and can result in the generation of false-positive results in future experiments. These contaminations may be detectable in the early stages of PCR amplification, distinguishing them from most true positive results but as end-point analysis is usually performed this is not possible. Environmental amplicon contamination can be controlled by the use of separate rooms for PCR set-up and amplification and by applying the guidelines of Kwok and Higuchi [59].

Mean sensitivities for conventional *Aspergillus* PCR methods for BAL, serum and whole blood specimens are shown in Table 2. The overall mean sensitivity and NPV are 79.5% (95% CI: 75.1–83.3) and 94.7% (95% CI: 93.4–95.7), respectively. Mean specificities are high (Table 2) with an overall mean specificity of 90.4% (95% CI: 88.8–91.8) and the PPV is 67.1% (95% CI: 62.6–71.3).

Performance statistics are affected by the low prevalence of disease resulting in a biased population which in this combined study has 4-fold more true negative than true positive patients. Despite the accepted contamination risk associated with conventional PCR, specificities remain high but this value is buffered by the large number of true negative cases in the study. The PPV is lowered possibly as a result of the lower number of true positives or it could represent a contamination effect. Alternatively, it may reflect the current inadequacies in consensus criteria for diagnosing IA, generating PCR false-positive results that are actually correct and currently undiagnosed. Sensitivity and NPV will be affected but assays with high negative predictive values used to exclude disease are better suited to diseases with low prevalence.

Calculating likelihood and Odds ratios (less influenced by prevalence) shows a positive result is approximately 8 times more likely to be seen in a patient with the disease than without and a negative result is approximately 5 times more likely to be seen in patients without IA. Conventional PCR generated a diagnostic Odds ratio of 36.5. A comparison of likelihood and Odds ratios for a selected range of *Aspergillus* PCR methods is shown in Table 3.

As is the case for most fields of molecular diagnosis it was felt that the development of real-time systems (discussed below) would supersede the original molecular technology. Interestingly, since the first application of real-time *Aspergillus* PCR in 2000 the number of papers describing conventional methodology is still greater than that for real-time PCR [27]. This is probably a consequence of the additional expense in obtaining real-time PCR equipment that can be in excess of 10 fold more expensive than the conventional equivalent.

Table 2 Real-time *versus* conventional *Aspergillus* PCR

Specimen	Platform	Sensitivity (%)	Difference (95% CI)	*p* Value	Specificity (%)	Difference (95% CI)	*p* Value
BAL	RT[a]	80.0			96.9		
	Con[b]	77.8	2.2% (–8.8 to 13.1)	0.74	92.8	*4.1% (0.3 to 6.8)*	*0.03*
Serum	RT[c]	73.9			93.7		
	Con [d]	72.9	1.0% (–9.5 to 11.6)	0.89	95.9	2.2% (–6.2 to 4.2)	0.619
WB	RT[e]	83.3			97.2		
	Con[f]	86.9	3.6% (–21.1 to 7.8)	0.567	86.9	*10.3% (6.6 to 13.4)*	*0.0001*
Combined	RT	77.3			95.7		
	Con	79.5	2.2% (–8.7 to 4.2)	0.561	90.4	*5.3% (3.1 to 7.3)*	*0.0001*

Legend: BAL, bronchoalveolar lavage; CI, confidence interval; Conv, conventional PCR; RT, real-time PCR; WB, whole blood.

[a]Combined statistical analysis performed on the studies of Rantakokko-Jalava et al. [68], Sanguinetti et al. [75], Speiss et al. [66], Musher et al. [74], and Schabereiter-Gurtner et al. [67]. Total number of cases of IA/no IA: 105/194.

[b]Combined statistical analysis performed on the studies of Tang et al. [85], Melchers et al. [36], Verweij et al. [37], Bretagne et al. [70], Jones et al. [71], Skladny et al. [64], Buchheidt et al. [65], Hayette et al. [72], and Raad et al. [73]. Total number of cases of IA/no IA: 108/760.

[c]Combined statistical analysis performed on the studies of Kami et al. [40], Costa et al. [29], Pham et al. [81], Kawazu et al. [77], Challier et al. [78], and Millon et al. [41]. Total number of cases of IA/no IA: 138/319.

[d]Combined statistical analysis performed on the studies of Yamakami et al. [9, 79], Bretagne et al. [76], Kawamura et al. [80], and Williamson et al. [43]. Total number of cases of IA/no IA: 129/97.

[e]Combined statistical analysis performed on the studies of Loeffler et al. [60, 61], Speiss et al. [66], and White et al. [34]. Total number of cases of IA/no IA: 30/249

[f]Combined statistical analysis performed on the studies of Einsele et al. [83], Löffler et al. [84], Van Burik et al. [86], Skladny et al. [64], Hebart et al. [51, 52], Buchheidt et al. [65], Lass-Florl et al. [50, 82], and Halliday et al. [44]. Total number of cases of IA/no IA: 138/665.

Table 3 Likelihood ratios and diagnostic odds ratios for a selected range of published *Aspergillus* PCR methods (Results kindly provided by C. Mengoli and M. Cruciani)

References	Number of samples needed for a positive PCR test	LR+	LR–	DOR
Hebart et al. [52]	1	9.74	0.01	874.50
Hebart et al. [51]	1	4.60	0.01	360.64
Williamson et al. [43]	2	18.46	0.08	228.00
Williamson et al. [43]	1	4.70	0.01	371.25
Buchheidt et al. [65]	1	4.91	0.10	47.88
Raad et al. [73]	1	72.73	0.28	264.00
Ferns et al. [53]	2	0.83	1.11	0.75
Ferns et al. [53]	1	1.11	0.83	1.33
Buchheidt et al. [49]	2	4.73	0.69	6.86
Buchheidt et al. [49]	1	1.74	0.57	3.04
Kawazu et al. [77]	2	4.97	0.42	11.92
Kawazu et al. [77]	1	1.60	0.21	7.61
Lass-Florl et al. [50]	1	1.25	0.88	1.43
Jordanides et al. [62]	2	2.74	0.34	7.97
Scotter et al. [63]	1	6.60	0.01	561.00
El-Mahallawy et al. [87]	1	9.19	0.27	33.75
Florent et al. 2006	2	6.15	0.41	15.17
Halliday et al. [44]	2	4.03	0.01	303.60
Halliday et al. [44]	1	1.89	0.02	89.72
Stenghele et al. 2006	2	5.31	0.43	12.50
Stenghele et al. 2006	1	1.96	0.23	8.66
White et al. 2006 [34]	2	17.19	0.08	211.50

Legend: DOR, diagnostic odds ratios; LR+, likelihood ratio positive; LR–, likelihood ratio negative.

3 Real-Time PCR

Real-time PCR allows rapid (1–2 h) PCR product detection during the amplification process. In its most simple form this involves the use of the fluorescent dye SYBR green that binds to double stranded DNA. As amplification occurs, the amount of bound SYBR green increases and when this passes the background level a signal is detected and this point is known as the crossing point (Ct Value). The Ct value is dependent on the starting amount of DNA target and larger initial DNA levels will have earlier Ct values. The major issue with SYBR green detection is a lack of specificity as it will bind to any form of double stranded DNA including non-specific amplification and oligonucleotide dimers. This can be resolved by melt-curve analysis where the target amplicon should have a markedly different melt temperature [57].

To enhance sensitivity and specificity real-time PCR allows the use of fluorescently labelled probes. Two main probe designs are usually used. Hydrolysis (Taqman) probes are dual-labelled oligonucleoties with a reporter dye at the 5’ end and quencher at the 3’ end. The probe is designed to have a very high melt

temperature (≥10°C above anneal-extension temperature) that permits a very tight binding. The system works by the probe binding to the target prior to PCR extension and as the *Taq* polymerase extends from the primer it is unable to dislodge the probe from DNA and fragments the oligonucleotide separating the reporter and quencher dyes releasing a signal. As it is necessary to destroy the probe no melt-curve analysis can be performed and it is essential that all hydrolysis probes be accurately designed to avoid non-specific results. An alternative is the use of hybridisation (FRET) probes. This involves the use of two fluorescently labelled oligonucleotides that will be bind to the target sequence in a head to tail arrangement no more than a few base-pairs apart. During anneal the probes bind and the oligonucleotide labelled with 3' fluorescein donates excitation energy to the second oligonucleotide labelled with 5' LC red 640 and a fluorescent signal is emitted [60, 61]. As the probes are dislodged during PCR extension they can be reused and it is possible to perform melt-curve analysis.

For both systems the Ct value is dependent on initial target DNA concentrations and so can be used to quantify PCR reactions. Unfortunately, hybridisation probe have a limited labelling range and are restricted to use on the Roche LightCycler, whereas hydrolysis probes can be used on most real-time systems. Additionally, hybridisation systems are more expensive, requiring the design of two specific oligonucleotides. Their use may be restricted by limited sequence variation, although, in principle only one of the pair needs to be target specific. Melt curves analysis will allow sequence variation to be differentiated and this can be used to distinguish between species/genera or even detect single nucleotide polymorphisms (SNPs) that may be responsible for antifungal resistance [60–62].

With no requirement for post-amplification handling neither intra-run cross-contamination nor environmental source contamination is likely. The additional use of uracyl-DNA-glycosylase (UDG) to inactivate previously amplified PCR products controlling accidental amplicon release should all but eradicate post-extraction contamination and real-time PCR results should provide greater specificity over its conventional counterpart.

Mean performance statistics for real-time *Aspergillus* PCR methods for BAL, serum and whole blood specimens are shown in Table 2. Within the study population there were approximately 3 fold more true negatives than positives. The overall mean sensitivity and NPV are 77.3% (95% CI: 72.0–81.9) and 92.2% (95% CI: 90.1–93.8), respectively. Mean specificities are high (Table 2) with an overall mean specificity of 95.7% (95% CI: 94.0–96.9) and the PPV is 86.5% (95% CI: 81.6–90.2). The likelihood ratio positive/negative for real-time PCR was 18.0 and 0.24, generating an odds ratio of 75.

Comparison between real-time and conventional PCR show that the additional contamination controls used in real-time PCR are taking effect and specificity is enhanced when using real-time techniques (Table 2). Sensitivities are not statistically different and previous research comparing PCR with alternative non-culture diagnostic tests (galactomannan ELISA) indicated that assays using conventional two step (nested PCR, PCR-ELISA) analysis generated better performance [32, 63]. The negative predictive value was slightly better for conventional PCR methodology (difference: 2.5, 95% CI: 0.4–4.9; $p = 0.0216$) and expectedly PPV favoured

real-time PCR (difference: 19.4%, 95% CI: 12.9–25.2; $p = 0.0001$). Caution is needed when comparing the two populations as despite both studies containing many more true negatives than true positives the differences in prevalence are statistically different (results not shown) and differences between likelihood and diagnostic odd ratios will be more representative (Table 3).

4 Concluding Remarks

Aspergillus PCR has been in existence for approaching two decades but widespread acceptance of diagnostic strategies has not been achieved. This is principally a result of a lack of consensus preventing large scale multi-centre evaluation. Galactomannan ELISA and β-D-glucan testing have been included in consensus criteria as they benefit from standardised methodology and a quality controlled commercial production allows evaluation from multiple centres to be collated and analysed providing a representative demonstration of assay performance. Only recently have commercial *Aspergillus* PCR systems been released and all suffer from the same limited evaluation as "in-house" methodology.

In theory the optimal specimen for PCR should be decided first, but in practice this is difficult to attain due to limitations of the current diagnostic approaches, patient requirements and incomplete understanding of the disease process. Reaching agreement on optimal molecular methodology before applying it to large scale studies may be more feasible and allow the decision regarding specimen to be deciphered retrospectively. This is the approach of the EAPCRI who are currently in the final stages of evaluating different PCR methods in use across Europe and Australia with the aim of applying the optimal method to large-scale clinical trials, which will not only evaluate assay performance but generate guidelines for accurate result interpretation. Additional evaluation of PCR testing will come from the Invasive Aspergillosis Animal Model (IAAM) strategy. A National Institutes of Health study (NIH, USA) to develop a mouse/guinea pig model to standardise biomarker assays for IA aimed at developing a Food and Drug Administration (FDA, USA) approved test for early diagnosis and treatment. Also the *Aspergillus* Technology Consortium (ASTEC) aim to link clinical specimens with laboratory tests to develop a cost-effective commercial assay.

This work is of paramount importance if PCR is to gain acceptance and inclusion in both consensus criteria and clinical management of patients. Its necessity has been highlighted by recent systematic reviews utilising meta-analysis where the heterogeneity of methods affected performance [10, 23] and without it the value of molecular diagnosis of IA may be underestimated.

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Aspergillus Precipitins and Serology

Richard C. Barton

Abstract Precipitating antibodies to *Aspergillus* have been measured as a diagnostic marker in aspergillosis since 1959. Methods of detecting precipitins have included passive diffusion, immunoelectrophoresis and enzyme-linked immunosorbent assays (ELISA), the latter aiming to detect IgG antibodies thought to make up the bulk of precipitins. There is increasing interest in the use of single *A. fumigatus* gene product antigens in *Aspergillus* ELISA in order to improve specificity. Traditionally, *Aspergillus* precipitin and IgG testing has been useful in the diagnosis of aspergillosis in immunocompetent patients such as those with fungal balls or allergic bronchopulmonary aspergillosis. However research has suggested antibody testing in patients with haematological malignancies prior to their becoming immunocompromised may be useful in targeting therapy. Developments in *Aspergillus* antibody testing and the increased scope of its use mean that this test will continue to contribute to aspergillosis patient management in the future.

Keywords Aspergillosis · Counter immunoelectrophoresis · Diagnosis · Double diffusion method · IgG · Precipitins · Serology

Contents

R.C. Barton (✉)
Department of Microbiology, Mycology Reference Centre, Leeds Teaching Hospitals Trust, Leeds, UK
e-mail: Richard.Barton@leedsth.nhs.uk

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1 Historical Perspective

In 1948 when Orjan Ouchterlony first published his research on precipitating reactions between antibodies and antigens the manuscript was initially rejected from a well known international journal as describing an artefact or phenomenon of little interest [1]. Happily, this did not stop Ouchterlony's gel diffusion method for the detection of antibodies from becoming an immensely important method, which is still employed to detect antibodies to *Aspergillus* species today. The earliest report of precipitin testing in aspergillosis in humans is almost 50 years ago [2]. It is interesting to note that as early as 1963 Stallybrass [3] was discussing issues such as a multiplex approach to diagnosis of aspergillosis – clinical, radiological, mycological and serological; the relative importance to the diagnosis of precipitating antibodies to *A. fumigatus* antigens and sputum culture; and the potential value of titres of these antibodies both for diagnosis and the monitoring of treatment response. It is perhaps a sobering thought that many of these issues and questions continue to exercise the medical mycology community today. This chapter will focus on precipitin reactions to *Aspergillus* antigens, specifically IgG antibodies, in particular their role in diagnosis.

2 Methods for Detecting Precipitins and IgG Antibodies to *Aspergillus*

When antibodies diffuse through an agar or agarose gel from one well and meet antigens diffusing from an adjacent well and bind with reasonable avidity, the resulting antibody-antigen complex will precipitate and appear as visible line in the gel between the two wells. This passive, immuno- or double diffusion method was a popular approach to detecting antibody responses to *Aspergillus* for many years following Pepys et al. initial observations in 1959 [2]. Early on, there was emphasis on the standardisation of the antigen extracts used and the observation that responses to antigens from various species of *Aspergillus* were often seen where the aetiology of disease was unknown, though precipitins to *A. fumigatus* were commonest [4]. There was debate surrounding the significance of a titre as opposed to or in addition to counting the number of precipitin lines observed between the two wells [4]. Philpot and Mackenzie [5] showed that there was little difference between strains or between antigens extracted from *A. fumigatus* grown under different conditions or using different formats of wells in the diffusion assay. Though later studies were to

show significant differences between the antigens from *A. fumigatus* grown under different conditions. The immunoglobulin molecules participating in a precipitin reaction against *Aspergillus* antigens have been assumed in most cases to represent IgG molecules, though the involvement of IgE against *A. fumigatus antigens* in precipitin reactions has also been demonstrated [6].

There are numerous variations on the double diffusion (DD) or Ouchterlony precipitin reaction. Many involve the enhancing of a passive diffusion by electophoresis. Counter immunoelectrophoresis (CIE) involves the adding of antigen and serum to adjacent wells in an agarose slab, but then exposing the agarose gel to an electric field [7]. By using a buffer with a pH matching the pI of immunoglobulins, 8.2, and by making up the agarose gel with half the running buffer strength, most antigens migrate to the anode, while antibodies are carried by endoosmosis toward the cathode (Fig. 1). Numerous studies indicated that CIE was more sensitive, produced equivalent results quicker and generated larger numbers of precipitin bands than DD in patients with some form of aspergillosis [5].

The need for more sensitive and quantitative methods lead to the development of solid phase radioimmunoassays (RIA) for the detection of specific IgG antibodies to *Aspergillus* [6]. However, methods using radio-labelled reagents are less safe and have technical drawbacks and in 1975 the first use of an enzyme linked immunosorbent assay (ELISA) for the detection of both IgG and E antibodies to *A. fumigatus* antigens was described [8] representing a sensitive, quantitative but operationally simple method that continues to be widely used today. Sepulveda et al. [8] showed that *A. fumigatus* antigens, both polysaccharide and protein could be adsorbed to the surface of plastic microtitre plates, patient sera allowed to bind to fixed antigen, and be detected by secondary antibodies to human IgG or E conjugated to an alkaline phosphatase enzyme whose presence could be assayed by the addition of an appropriate substrate. There was broad correlation between the number of DD precipitin

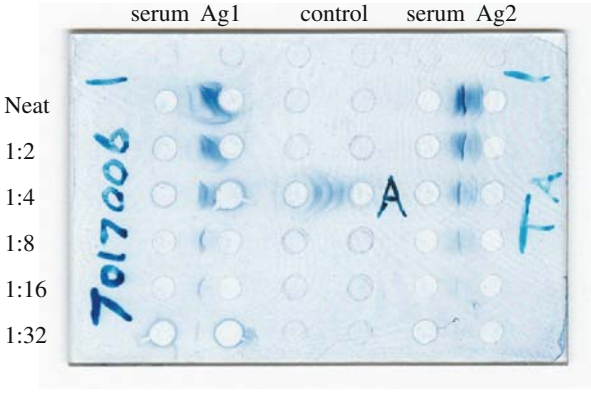

Fig. 1 A counter immunoelectrophoresis slide showing a titration of a patient serum (left hand well of pairs of wells) against 2 different antigens (right hand well of pairs of wells) somatic (*left*) and culture filtrate (*right*) from *Aspergillus fumigatus.* Sera from a patient with a fungal ball have been titrated giving a titre for Ag1 of at least 1:32 and for Ag2 of 1:16

lines and the IgG ELISA reading and interesting differences in the reactions of sera from different patient groups to the protein and polysaccharide antigens. ELISA was shown to be more sensitive than CIE [9]. There is relatively high level of cross-reactivity between sera from patients from whom different species of *Aspergillus* have been cultured and antigens prepared from different species, though there is not always a straight forward correlation between the two [10]. Most early work with ELISA used complex whole cell antigen extracts, an approach that has been criticised by others looking at IgE responses to *Aspergillus* in ABPA [11]. However in recent years there has been an interest in using ELISA to look at patient responses to purified antigens [12–15]. In many cases this follows use of the technique of Western blotting where the protein profile of *Aspergillus* cells are separated by electrophoresis, blotted to a membrane which is then probed with sera from patients with various forms of aspergillosis [16] in order to determine which antigens are immunodominant. In an effort to standardise the measurement of IgG to *A. fumigatus,* there is much interest in commercial automated immuno assay systems such as ImmunoCAP which generates absolute concentrations of antibody, as opposed to optical density measurements [17, 18]. The advantages to such standardised methods include the ease of monitoring patient IgG responses to *Aspergillus* over time (Fig. 2) as well as enabling reliable comparisons of results between different laboratories.

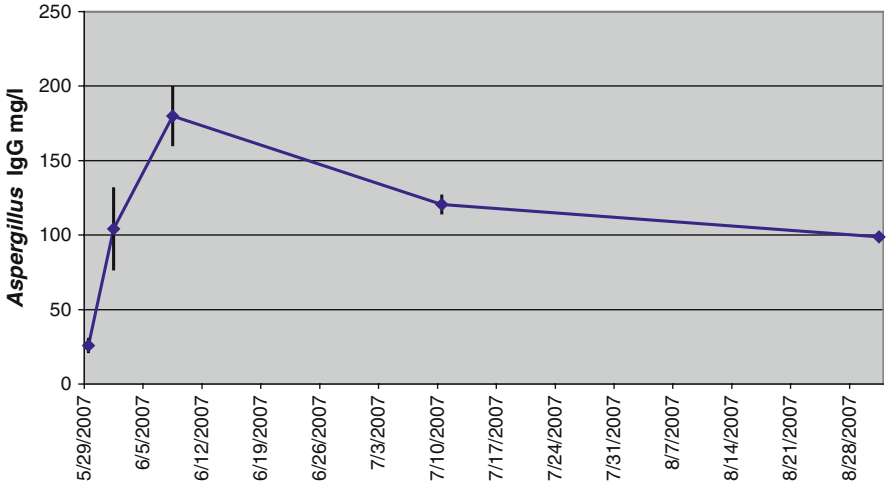

Fig. 2 A plot of the levels of IgG to *Aspergillus fumigatus* over time in a patient with aspergillosis measured by ImmunoCAP. Note the rapid increase and slow decline in levels of IgG

3 IgG Responses to *Aspergillus*

While *Aspergillus* species, particularly *A. fumigatus* are ubiquitous in the environment and virtually all humans are regularly exposed to and inhale airborne *Aspergillus* conidia, it was shown early on that not everyone has detectable

precipitins to these fungi. Thus, Pepys et al. 1959 [2], first proposed that precipitin (effectively IgG) antibodies to *Aspergillus* may be an approach to determine significant, exposure to *Aspergillus*, and thus differentiate between the transient or non-pathogenic presence of this fungus, and a true infection. In general, it is considered that while IgG antibodies readily form against *A. fumigatus* in people significantly exposed to this fungus, they are not protective. However, Holmberg et al. [9] considered that in a group of immunocompromised patients with invasive aspergillosis (IA), a rise in antibody levels to *A. fumigatus* detected by ELISA correlated with histologically documented eradication of infection. In contrast, Forsyth et al. [19] showed that levels of IgG against *A. fumigatus* correlated with disease state in patients with cystic fibrosis, suggesting that in this patient group *Aspergillus fumigatus* caused pulmonary damage by mechanisms mediated by IgG.

4 Diagnosis of Allergic Bronchopulmonary Aspergillosis (ABPA) in Cystic Fibrosis (CF)

4.1 Precipitins and IgG to Diagnose ABPA in CF

An estimated 1–15% of people with CF suffer from ABPA which is frequently difficult to diagnose as the symptoms overlap those of CF itself [20]. As early as 1967 Mearns et al. [21] found they could detect precipitating antibodies to *A. fumigatus* in a relatively high proportion (31%) of 112 people with CF, compared to patients with asthma (7%) or patients with non-respiratory disease (3%). Furthermore, they found that the incidence of precipitins was higher in patients with more severe pulmonary signs and symptoms. Subsequent studies essentially replicated this finding consolidating the view that in CF, ABPA was characterised by precipitating antibodies or IgG to *A. fumigatus*, along with positive skin tests to the same antigen, raised total IgE and specific IgE antibodies. Nelson et al. [22] defined a set of seven criteria – "Nelson's criteria" for the diagnosis of ABPA including the presence of precipitating antibodies to *A. fumigatus.* Hutcheson et al. [23] followed a group of 118 people with CF defined in part by *Aspergillus* precipitins and in addition measured IgG responses by ELISA to *A. fumigatus* and identified only 6 subjects with strictly defined ABPA. The focus of this paper was that amongst the 112 people with CF without ABPA there was a high frequency of both precipitating antibodies (42%) and IgG to *A. fumigatus* (61%). Furthermore, they showed extensive fluctuations in various immunological parameters over time, including precipitins and *Aspergillus* IgG, appearing and diminishing without intervention [23]. In an attempt to improve the sensitivity and specificity of *Aspergillus* IgG testing for ABPA in CF, Skov et al. [24] looked at subclass specific responses to *A. fumigatus* in relation to the presence of ABPA. While there were differences between the subtypes in IgG responses to *A. fumigatus* in ABPA, for example IgG_3 levels were low, and IgG_4 antibodies to *A. fumigatus* gave the highest specificity for the diagnosis of ABPA, this approach did not lead to a single diagnostic test. Skov et al. [20] also calculated that precipitins to *A. fumigatus* with titres >2 would provide a positive predictive value

(PPV) of 80%, though a very limited negative predictive value of 50% for the diagnosis of ABPA in CF. In 2003 a consensus conference recognising the limited value of precipitins and IgG, set one of the four minimum diagnostic criteria for ABPA in CF as either precipitins or IgG to *A. fumigatus* or abnormalities on chest radiography [25]. In a recent sophisticated analysis of serological parameters Kraemer et al. [26] found that while elevated IgG responses to *A. fumigatus* provided a high sensitivity (100%) and surprisingly high specificity (86%) for the diagnosis of ABPA in CF, using binary logistic regression analysis to predict ABPA, specific IgG was not a significant marker, compared to specific IgE or even total IgE.

4.2 Diagnosis of ABPA in CF Using Purified, Recombinant Antigens

A criticism of many of the serological tests to detect immunoglobulin responses to *Aspergillus* is that antigen reagents prepared from total cell protein are not standardised, or are difficult to standardise. Much of the interest and success in improving the diagnosis of ABPA in CF has focussed on specific IgE responses to purified antigens, identified by screening cDNA libraries from *A. fumigatus* with sera from patients with ABPA, cloning and expressing the antigen proteins in order to dissect the humoral response to this fungus [11]. Kurup et al. [15] have recently examined levels of IgE, IgA and IgG and IgG subtypes to total *A. fumigatus* antigen and to five different recombinant antigens in CF patients with ABPA. Though they concluded that no one antigen-antibody combination was able to cleanly differentiate patients with ABPA from others, by combining results identified as important by discriminant analysis, the classification of CF patients with ABPA could be significantly enhanced [15]. This approach opens up the possibility of using scores made up of responses with different immunoglobulin types, including IgG to different recombinant antigens to refine the diagnosis of ABPA in CF. Sarfati et al. [13] noted temporal changes in levels of antibodies to purified antigens in ABPA-CF patients. There was variation between patients in terms of which antigen they reacted to most, but interestingly there was an association of elevated levels of antibodies generally during periods where patients fulfilled the ABPA criteria, and declines during periods of clinical improvement.

5 Diagnosis of ABPA in Asthma Patients

It is well recognised that diagnosing ABPA in an asthma patient without CF is much more straightforward. In this patient group, Greenberger [27] has suggested that either elevated specific IgE or IgG to *A. fumigatus* should be required, but not necessarily serum precipitins. Van Hoeyveld et al. [17], using the commercial automated fluorescent immuno assay system (ImmunoCAP) found a median of 70 mg/l *A. fumigatus* IgG in patients with non-CF patients ABPA, compared to 13.7 mg/l in healthy controls, and 18 mg/l in patients with asthma. Receiver operator

characteristic (ROC) curve analysis was performed on a group combining ABPA and other forms of aspergillosis, and versus the "diseased" control group generating a cut-off of 35.2 mg/l. When this was applied to ABPA against patients with asthma this gave a sensitivity of 90% and specificity of 75% [17].

6 Diagnosis of Aspergillosis in Chronic Cavitary Pulmonary Aspergillosis

Denning et al. [28] have described a series of immunocompetent patients with chronic cavitating and fibrosing pulmonary aspergillosis and proposed that the presence of precipitating antibodies to *Aspergillus* are part of a minimal set of diagnostic criteria. In certain patient groups at risk of invasive forms of pulmonary aspergillosis, antibody testing is also useful, such as those with chronic granulomatous disease [29], Job's disease and pulmonary histiocytosis X (R. Barton, unpublished observations). Monitoring of precipitin titres or *A. fumigatus* IgG levels to may also be of value to follow responses to treatment [28]. Most patients with chronic cavitary pulmonary aspergillosis however will remain positive for *Aspergillus* precipitins, despite antifungal treatment (Dr Pasqualotto, personal observations).

Chu et al. [14] have focussed on antibody tests to examine the aetiology of haemoptysis in patients previously treated for tuberculosis but without evident fungal ball (aspergilloma) and in patients with bronchiectasis. They have prepared antigen from the product of the gene for galactomannoprotein from *A. fumigatus* and *A. flavus* and found that many patients in this group, none of whom had positive cultures, had high levels of antibody, particularly to *A. flavus*. The authors propose that in patients with significantly high levels of IgG antibody to these antigens, *Aspergillus* sp. may be the causal agent for their haemoptysis [14]. There is increasing interest in pulmonary aspergillosis in patients with chronic obstructive pulmonary disease (COPD). A recent proposal is for serum precipitins or other positive serology to be included in the criteria for defining proven and probable invasive pulmonary aspergillosis (IPA) in COPD [30].

7 Diagnosis of Fungal Ball

The serodiagnosis of a fungal ball caused by *Aspergillus* is perhaps the application for precipitin or IgG testing that has repeatedly shown to be most valuable. Coleman and Kaufman [4] found 93% of patients with aspergilloma to be positive for preciptins and observed that on radiological resolution in one patient, precipitating antibodies to *A. fumigatus* disappeared. Aspergilloma patients frequently exhibit higher levels of antibody to *Aspergillus* than any other form of aspergillosis. Using an ELISA to detect IgG, IgM and IgA to a purified *A. fumigatus* antigen mitogillin Weig et al. [12] found 100% of 13 aspergilloma patients had high levels of IgG against mitogillin and a specificity of 95%. A discussion following that publication is illuminating. Woo et al. [31] query the use of the cut-off proposed by Weig

et al. [12] of 3 standard deviations (SD) above the mean of 307 donor serum and suggested aiming for a higher specificity of 99% and using 10 SD. In their reply, Weig et al. [32] proposed receiver operator characteristic analysis to balance specificity and sensitivity in defined manner, and make the important point that cut-offs are best defined in distinct patient groups. Sarfati et al. [13] looked at IgG reactions to a range of purified antigens in aspergilloma patients and while alone, none had a sensitivity >81%, in combination this could be increased to 95%. *Aspergillus* IgG or precipitin testing continues to be the investigation of choice to confirm a clinical diagnosis of fungal balls in patients with mobile masses in pulmonary cavities.

8 Diagnosis of Invasive Aspergillosis in Immunocompromised Patients

In a study in 1973, Young and Bennett were unable to detect precipitating antibodies to *A. fumigatus* in immunocompromised patients with IA [33]. This established the paradigm that detection of serum antigens or DNA would be more productive for the diagnosis of IA in the immunocompromised. Despite this, many studies have sought to challenge this view. Using the technically laborious approach of isoelectric focussing and Western blotting, Hearn et al. [16] found that sera from 11/13 patients with proven or probable IA were present and reacted with one or more *A. fumigatus* antigens. Weig et al. [12] used an IgG ELISA to mitogillin and could detect 64% of patients with IPA, and slightly fewer (60%) with disseminated disease. Herbrecht et al. [34] found 40% of patients with definite IA had antibodies to *A. fumigatus*, and furthermore that the galactomannan (*Aspergillus* antigen) test was a significantly less sensitive in patients who were antibody positive suggesting this might be a reason for false-negatives. This suggests greater potential for antibody testing for the diagnosis of IA than originally thought. Furthermore, Sarfati et al. [13] have taken the approach of using IgG to purified *A. fumigatus* antigens to predict IA in patients prior to the initiation of immunosuppressive treatment regimes. Examining sera over time, Sarfati et al. [13] found that about 50% of patients who developed IA during immunosuppressive therapy exhibited sustained, significantly high levels of IgG antibodies to certain purified *A. fumigatus* antigens, particularly catalase. The potential utility of screening patients with haematological malignancies for the presence of existing, often occult, *Aspergillus* infection by detection of IgG and targeting prophylaxis merits further investigation.

9 Diagnosis of Other Forms of Aspergillosis

Diagnosis of chronic rhinosinusitis is complex and controversial. Pant et al. [35] examined serum IgG, IgM, IgA and IgE responses to *A. fumigatus* and *Alternaria alternata* in patients with chronic rhinosinusitis and eosinophilic mucin and found that both IgG and IgA, but not IgE to *A. fumigatus* and *A. alternata* were significant elevated compared to controls. While *Aspergillus* endocarditis is rare, one

might expect, as with *Candida* endocarditis, antibody detection would be a useful approach. In one of the few systematic studies Raoult et al. [36] used a haeagglutination screening test to identify *Aspergillus* spp. as the causative agent in 1 of 427 patients with definitive infective endocarditis.

10 Discussion

In the midst of developments in molecular diagnosis and antigen detection, antibody testing appears to be undergoing a renaissance Ouchterlony would have been proud of. The usefulness or otherwise of precipitin and IgG tests for *Aspergillus* frequently hinges on either case definitions or technical parameters. As the Weig et al. [12] discussion revealed, where to set the cut-off for a test is of critical importance. The almost self-evident aim in many of the above studies is to use a formal diagnosis of aspergillosis to include as may patients in a necessarily arbitrary diagnostic box in order to focus attention on management. In aspergillosis, where exposure is almost universal and disease is a function of host susceptibility with all its complexity and variation, precipitin and IgG responses to *Aspergillus* spp. in health, disease and formally diagnosed aspergillosis will form a continuum. Knutsen et al. [37] described a case study of ABPA in CF where slavish adherence to the limits of diagnostic criteria would have prevented an intervention (steroids and itraconazole) which lead to symptom resolution. Increasingly, laboratory scientists are being encouraged to relate their investigations to patient outcomes. Future developments in the use of *Aspergillus* diagnostic IgG testing are likely to include the use of purified antigens to enhance specificity and commercial automated platforms to increase intra and inter-laboratory reproducibility. However, whichever technical approaches are taken, assays will need to carefully consider which patients are tested and how to interpret results in order to identify as many patients as possible who will respond to treatment.

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Specific IgE Testing (RAST)

Reto Crameri

Abstract The diagnosis of allergic diseases is based on a combination of clinical history, physical examination, in vivo challenges, and in vitro test methods for the detection of allergen-specific IgE antibodies in the serum of patients. In spite of the fact that the determination of allergen-specific IgE in serum is widely used in daily clinical practice, one should be aware that such determinations, although useful for routine screening of possible allergies, are not sufficient to diagnose the disease. Clinical history and provocation tests should always be considered to confirm an allergy indicated by elevated allergen-specific IgE levels in serum. This specially applies to the diagnosis of mould allergy in general, and of *Aspergillus fumigatus*-related allergic complications in particular.

Keywords *Aspergillus* · Allergy · Diagnosis · Fungal allergens · Serum IgE

Contents

R. Crameri (✉)
Head Molecular Allergology Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland
e-mail: crameri@siaf.uzh.ch

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1 Introduction

The first documented clinical history of an allergy was reported by Dr. John Bostock which recorded his own case history as first complete description of *catarrus aestivus* or hay fever in 1819 [1], followed by the description of pollen grains as causative agents for allergic reactions by Charles Blackley in 1873 [2]. The term "allergy" is derived from the Greek words *allos* (other) and *ergon* (reaction) and was proposed by the Viennese paediatrician Clemens von Pirquet in 1906. He described allergy as "an altered capacity of the body to react to a foreign substance" [3]. In 1921 Prausnitz and Küstner discovered a transferable tissue-sensitising factor in serum [4], which was then identified as a self-protein representing a new immunoglobulin subclass (immunoglobulin E or IgE) by Johansen and Bennich [5] and Ishizaka et al. in 1967 [6]. The pivotal discovery of IgE as a mediator of allergic disease marks the beginning of modern allergology.

Like other immunoglobulins, IgE consists of two light and two heavy chains (ε-isotype) and can be produced in two forms by alternative splicing: a secreted and a membrane-bound form [7]. In sensitised atopic individuals, allergen exposure induces cross-linking of high-affinity FcεRI receptor-bound IgE on effector cells and, thus, immediate release of anaphylactogenic mediators [8]. Normally, IgE is a tissue-bound molecule which is, however, also present in low concentration in serum, in equilibrium with that bound to effector cells [9]. The soluble form of human IgE consists of two identical light chains (either kappa or lambda) that are indistinguishable from the light chains of the other human immunoglobulins. In contrast, the two heavy ϵ-chains contain a variable domain (VH) and four isotype-specific structural domains (Cϵ1, Cϵ2, Cϵ3, and Cϵ4) [10]. The structural differences between the IgE constant domains and the constant domains of all other immunoglobulin classes, allows the development of highly specific tests for the detection of serum IgE.

2 Assays for the In Vitro Determination of Specific IgE Antibodies

The prototype for the in vitro measurement of the serum IgE concentration was the radioallergosorbent test (RAST), first described in 1967 [11]. The original RAST employed a paper disc as solid phase to covalently immobilize the allergen (allergosorbent), used to capture allergen-specific antibodies of all isotypes (mainly IgG, IgA, and IgE) from serum samples. Following different washing procedures to remove unbound serum proteins and antibodies, bound IgE was detected with ^{125}I-labelled polyclonal anti-human IgE. Results were reported in classes or in arbitrary units (PRU/ml) by interpolation from a reference standard curve [12]. Although not quantitative, the so-called Phadebas-RAST commercially introduced in 1974 by Pharmacia (Uppsala, Sweden) under the trademark "RAST", represented a milestone for the laboratory diagnosis of atopy. Whilst the terms "RAST" and "RAST class" are still widely used in clinical practice to refer to the presence of

allergen-specific IgE in serum, the original radioallergosorbent test has been made obsolete by advances in assay technology.

Modern immunoassays for the detection of allergen-specific IgE have undergone relevant improvements, including substitution of the radio-labelled polyclonal antibodies with enzyme-labelled monoclonal detection antibodies [13], the development of solid phase systems with higher allergen binding capacities [14], and the introduction of fully automated devices [15]. Most of the currently used IgE detection systems are calibrated against the World Health Organization (WHO) Standard 72/502, and can be considered "quantitative" assays [12]. Results of quantitative assays are expressed in U_A or kU_A/l where 1 U_A IgE antibody corresponds to 1 IU IgE directly convertible to 2.42 ng of IgE [16]. However, the quantity of allergen-specific IgE measured in serum depends on many factors, especially from the quality of the extract immobilised to the solid phase (see below), and might be subject to huge variations between different test systems.

3 Problems Related to Allergen Extracts and Mould Extracts in Particular

Allergen extracts prepared from natural sources are complex mixtures containing allergenic and non-allergenic proteins. In the normal case the single allergens will constitute a minor component of the extract compared to the non allergenic proteins, because only a minority of the proteins are able to induce a switch to IgE production [17]. These extracts are subject to batch-to-batch variation due to different reasons like extraction procedure used, biochemical stability of the allergens, protease content, and storage [18]. Many efforts have been undertaken to standardize allergen extracts [19] but only a few allergen extract preparations, out of hundreds commercially available, have been approved by the WHO for the use as international standards [20]. Recently, the WHO-IUIS Allergen Standardization Committee has taken an initiative, founded by the European Union, to develop certified reference materials based on purified natural and recombinant allergens [21]. The aim of the project is to develop certified reference materials to be used to standardize allergen extracts. Such material would definitively help to calibrate the internal reference preparations used in different laboratories of manufacturers, research groups, and control authorities against a commonly accepted reference extract.

Special problems have been encountered in the standardisation of fungal extracts. The raw material used to prepare fungal extracts derives from fungal cultures grown in complex media. Depending on strain used, culture conditions and time, huge variations in the allergenic potency between different commercial products have been detected [22, 23]. The problem is best exemplified by a study which analysed different commercial fungal extract for their major allergen content using highly specific monoclonal antibodies [24]. The concentration of the major allergen Alt a 1 in *Alternaria alternata* extracts from 8 companies ranged from less than 0.01 to 6.09 μg/ml. *A. fumigatus* extracts showed even a much greater variability in the concentration of the major allergen Asp f1 within extracts from 8 companies ranging

from less than 0.1 to 64 μg/ml. Obviously serologic and skin test investigations with different extracts showing such a high variability even for the content of major allergens will automatically generate discordant results. This was impressively shown by a skin prick test study with four different commercial *A. fumigatus* extracts [25]. None of the four commercial skin prick test extracts was able to detect all of the 31 *A. fumigatus*-sensitised patients enrolled in the study.

Amongst the systems routinely used in the daily clinical practice, ImmunoCAP is considered the "golden standard" for the in vitro determination of IgE [15]. Unfortunately the extracts immobilised on ImmunoCAPs are not available as skin test solution, hampering a direct correlation between in vitro and in vivo diagnostic outcomes.

The most common systems routinely used for the determination of allergen-specific IgE are the already mentioned ImmunoCAP system (Phadia, Uppsala, Sweden), Immulite (DPC Los Angeles, USA), and ADVIA Centaur (Bayer Healthcare, Tarrytwon, NY, USA). Comparative studies of the performance of these systems have been reported and partly correlated with skin test outcomes [26–28]. Considering the clinical diagnosis of allergy as reference, sensitivity and specificity of all in vitro IgE determination systems give comparable results. However both, sensitivity and specificity varies between allergen extracts tested in all systems. Notably, mould extract panels show identical sensitivity problems (>40%) for all IgE assay systems [28]. The discrepant results between skin test, clinical history, and mould-specific IgE is mainly due to the quality of the mould extracts used provided by different manufacturers [29].

4 Clinical Relevance of Serum IgE

Despite the fact that allergen-specific IgE immunoassays are promoted as tests for "allergy diagnosis", the only information provided by the tests relates to the presence or absence of allergen-specific IgE in serum. It is commonly agreed that the presence of allergen-specific IgE is a prerequisite to develop allergic symptoms against a given environmental allergenic source. However, up to more than 40% of the subject with serum IgE to inhalant allergens did not present (respiratory) symptoms [30, 31] and therefore the in vitro determination of allergen-specific IgE is not sufficient for a clinical diagnosis of allergy. The most reliable criterion for a clear diagnosis of an allergy is still a clear correlation between exposure and symptoms that can be derived, in many cases, from the clinical history. Obviously, the patient has to provide the information to the clinician. This might be a relatively simple story in the case of seasonal allergy like hay fewer, or exposure to animal dander. The situation is not so easy in the case of mould allergy where the patient might or might not be aware of the fact that he is exposed to fungal allergens and therefore unable to provide useful information to the clinician.

Although the synthesis of allergen-specific IgE is required for the development of allergic diseases we should not forget that the biologically active form of IgE is not those present as soluble IgE in serum. Whether the presence of allergen-specific IgE translates into clinical allergy depends on a complex interplay of multiple factors

recently reviewed [31]. As stated in this review, there are many unanswered important questions that need to be addressed in order to better understand how IgE sensitisation translates into clinical allergy.

5 Conclusions

Whilst a clinical history is sufficient to identify causes and judge the severity of most allergic diseases, sometimes the history is complex and, especially in polysensitised patients many allergens may be involved. To identify the causative allergens, assessments can be performed in vivo by skin tests or in vitro by measuring allergen-specific serum IgE. Both methods strongly depend from the quality of the extracts used and in this regard recombinant allergens can contribute to substantially improve the diagnostic outcomes [32]. Compared to in vivo tests, in vitro determination of allergen-specific IgE has the advantage to avoid allergen challenges which might elicit severe side effects or even induce a de novo sensitisation. Modern methods for the determination of serum IgE are reliable, reproducible, and quantitative. They represent useful and suitable methods to screen large numbers of patients for potential allergies in clinical routine assessments. However, one should be aware of the fact that, although propagated as methods for "allergy diagnosis", serum IgE-detection systems does not provide a direct evidence for an allergy which always needs to be confirmed by clinical history, skin and/ or provocation tests.

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Molecular Typing of *Aspergillus fumigatus* Isolates

Corné H.W. Klaassen

Abstract Microsatellites (or short tandem repeats) offer unprecedented discriminatory power and many other advantages compared to other strain typing methods. They have shown to be excellent tools for discriminating between *Aspergillus fumigatus* isolates from various origins. Microsatellites are easily amplified by PCR using high stringency conditions. Amplified products are separated according to size using high-resolution equipment such as a DNA sequencer. If properly performed, microsatellite data are straightforward to analyze. However, under certain experimental conditions, well-known PCR artifacts and equipment related issues may complicate interpretation of microsatellite based data and could lead to wrong interpretation of the data. This chapter provides the necessary technical background that needs to be taken into consideration in order to adequately reproduce and interpret a microsatellite based typing assay for *A. fumigatus*.

Keywords *Aspergillus fumigatus* · Microsatellite · Typing

Contents

C.H.W. Klaassen (✉)
Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
e-mail: c.klaassen@cwz.nl

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1 Introduction

Strain typing, or the ability to distinguish between clonally-unrelated isolates, is an indispensable tool in the clinical microbiology laboratory and for hospital epidemiology. Some of the most important reasons to perform strain typing in the mycology field are patient monitoring and treatment follow-up, outbreak analysis, environmental monitoring and the study of local and global epidemiology of fungal pathogens. *Aspergillus fumigatus* has been an important target of many molecular fingerprinting methods that have been developed in the fungal kingdom and numerous different methods have been reported to study the epidemiology of this organism [1–5]. This interest is best explained by its clinical relevance and increasing prevalence as number one fungal pathogen in immunocompromised patients. At least a dozen different methods have been reported for typing *A. fumigatus* isolates. These methods and uses thereof have been reviewed by Varga et al. [6] and more recently by de Valk et al. [7]. Unfortunately, the vast majority of typing methods yield fingerprints that consist of complex banding patterns that are difficult to describe and are probably impossible to reproduce in different settings. Only few fingerprinting methods yield typing data that can be described in an unambiguous format and that can be reproduced in different laboratories. Multilocus sequence typing (MLST), a method based on the detection of single nucleotide polymorphisms (SNPs), lacks discriminatory power for typing organisms with low levels of genetic diversity such as *A. fumigatus* [8]. Therefore, markers that have higher mutation rates are required for these isolates.

Microsatellites are genomic sequences consisting of tandemly repeated short motifs of 2–6 nucleotides. Mutations occur at a high frequency in these sites approx. 10^{-2}–10^{-6} per generation, which compare with 10^{-9} for point mutations (as detected by MLST) [9]. Mutations in microsatellites usually result in changes in the numbers of repeat units. At the moment, typing assays for *A. fumigatus* based on microsatellites (or short tandem repeats, STR's) offer the most advantages in terms of speed, reproducibility, discriminatory power and costs and do not suffer from the drawbacks mentioned for previous typing methods. Clonally-unrelated isolates can be identified on the basis of differences in repeat numbers of these microsatellite markers between such isolates. Bart-Delabesse et al. initially developed a dinucleotide-based microsatellite typing method that was found to suffer from intense occurrence of "stutter peaks", which limited test interpretation (more detail on topic 3.1, below) [3]. Later on, de Valk et al. showed the results of a study evaluating a novel panel of 9 microsatellite markers that were analysed in three separate multiplex multicolour

polymerase chain reactions (PCR). This method targets 3 dinucleotide repeat markers (M2), 3 trinucleotide repeat markers (M3) and 3 tetranucleotide repeat markers (M4), respectively [10]. This assay is currently referred to as the STR*Af* assay (*STR* for *A. fumigatus*). Each of the markers in the STR*Af* assay has at least 20, and the majority even more than 40, allelic variants (Table 1). The sizes of the PCR-amplified products are determined on a high-resolution electrophoresis platform and are converted into repeat numbers by comparison to fragments with established repeat numbers (e.g., by DNA sequence analysis). The STR*Af* assay has been shown to have an enormous discriminatory power: based on the allelic variants, no less than 3×10^{13} different genotypes can be discriminated [10]. A Simpson's index of diversity of 0.9992 was obtained by combining markers M3 and M4 (0.9994 if all markers are used in combination). The repeat numbers of all nine markers constitute the genotype of an isolate. These numerical genotypes are suitable for storage, exchange between laboratories and are easily compared to each other. This assay has withstood the comparison to other high-resolution fingerprinting methods that are based on complex banding patterns such as amplified fragment length polymorphism (AFLP) analysis, restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, and/or retrotransposon insertion-site context (RISC) analysis [11–17]. It has been shown to be a very robust assay that yields highly reproducible genotypes [18]. Furthermore, the STR*Af* assay has proven to be a very useful instrument in the identification of epidemiologically

Table 1 Characteristics of the microsatellite markers constituting the STR*Af* assay

STR*Af* marker	Label	Labeled primer sequence (5'–3')	Unlabeled primer sequence (5'–3')	Repeat units	No. of Alleles
2A	FAM	AAGGGTTATG GCCATTAGGG	GACCTCCAGG CAAAATGAGA	GA	20
2B	HEX	TATTGGATC TGCTCCCAAGC	GAGATCATGC CCAAGGATGT	AG	25
2C	TET	TCGGAGTAGTT GCAGGAAGG	AACGCGTCCTA GAATGTTGC	CA	35
3A	FAM	GCTTCGTAGA GCGGAATCAC	GTACCGCTGC AAAGGACAGT	TCT	106
3B	HEX	CAACTTGGTGT CAGCGAAGA	GAGGTACCACA ACACAGCACA	AAG	78
3C	TET	GGTTACATGGC TTGGAGCAT	GTACACAAAGG GTGGGATGG	TAG	51
4A	FAM	TTGTTGGCCGC TTTTACTTC	GACCCAGCGCC TATAAATCA	TTCT	35
4B	HEX	CGTAGTGACC TGAGCCTTCA	GGAAGGCTGTA CCGTCAATCT	CTAT	47
4C	TET	CATATTGGG AAACCCACTCG	ACCAACCCATC CAATTCGTAA[a]	ATGT	41

[a]The underlined residue is not a match to the genomic reference sequence based on the Af293 strain. This mismatch was deliberately introduced to minimize formation of minus-A peaks (see text for details).

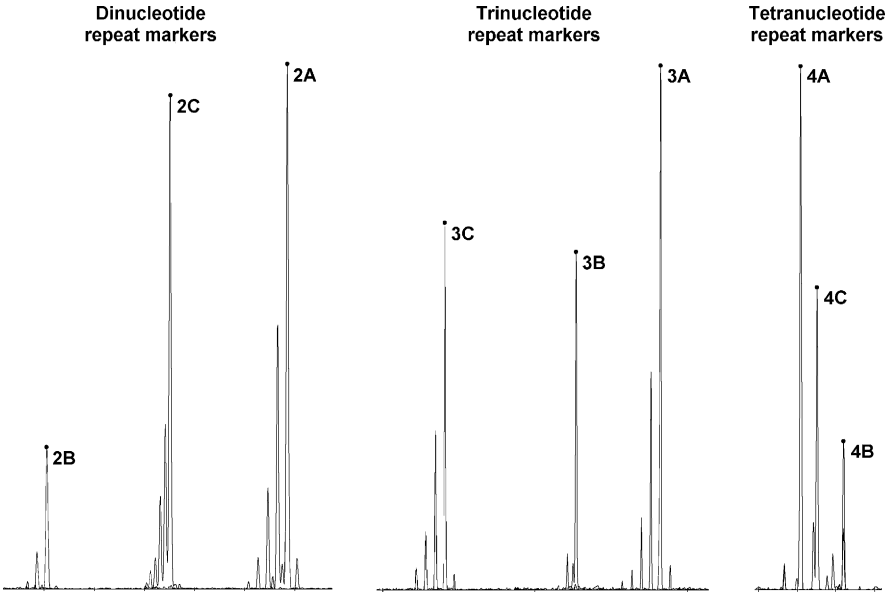

Fig. 1 Typical result obtained with the STR*Af* assay. Electropherogram displaying representative results obtained with the STR*Af* assay. Each of three multiplex panels amplifies 3 different markers. The individual marker in each panel can be identified based on the fluorescent label (where A = FAM, B = HEX and C = TET, respectively). Usually, the tallest peak in each marker is the one that should be assigned (as indicated with a *dot*, on the *top* of the peak). Additional low intensity peaks in each marker are the result of strand slippage during polymerase chain reaction (PCR) amplification of the DNA fragments. This leads to the so-called formation of stutter peaks. See text for further details

related isolates, i.e. during an outbreak [19] and to distinguish between clonally-unrelated isolates [10].

The STR*Af* assay seems to fulfil all key ingredients for global standardization of *A. fumigatus* typing. If properly performed, it should be quite obvious which peak to assign in the resulting electropherograms (Fig. 1). However, as with any other molecular typing method, certain practical aspects of the assay require special attention for reproducing this assay elsewhere [20]. Here, I will discuss some of the major practical aspects that need to be taken into consideration upon successful implementation of the STR*Af* assay in a different laboratory.

2 Practical Procedures

2.1 DNA Isolation

Prior to the analysis of microsatellite markers, DNA needs to be isolated from the isolates in question. Since the STR*Af* assay has been optimised with the use of 1 ng of DNA, isolation of nucleic acids can be performed on a relative low scale. DNA

can be isolated from fresh cultures using either the conidia or mycelium as starting material using a variety of either manual or automated purification methods. Many different protocols for isolation of DNA from fungi have been reported in the literature and basically any protocol will be suitable as long as the isolated DNA is free from PCR inhibitors. Mechanical disruption of the mycelia or conidia will greatly improve recovery of nucleic acids and can be performed by bead-beating using for instance a MagNA lyser instrument (Roche Diagnostics) or FastPrep instrument (Bio101 systems). Alternatively, the cell wall can be weakened by enzymatic treatment with, for instance, lyticase (Sigma). Subsequent suitable procedures for manual DNA extraction include but are not limited to QIAamp (QIAgen) or Chromaspin kits (Clontech) and phenol/chloroform extraction. Automated platforms for DNA isolation include the MagNA Pure LC instrument (Roche Diagnostics). It is recommended that the DNA yield is estimated on agarose gels or preferably even better, quantitated by UV absorbance measurements taking into account that an $Abs_{260\,nm}$ of 1 corresponds to ~50 μg/ml. Pure genomic DNA should have an $Abs_{260\,nm}/Abs_{280\,nm}$ ratio of ~1.8. If this ratio is substantially lower, the calculated DNA concentration will be overestimated. Alternatively, DNA concentrations can be measured by fluorescence measurements using highly sensitive intercalating compounds (such as picrogreen (Invitrogen)) but these usually provide no information about the purity of the DNA.

2.2 Multiplex PCR Amplification

Microsatellite markers are easily and reproducibly amplified under high stringency conditions using PCR. With the aim of increasing sample and marker throughput, the STR*Af* assay was designed as a multiplex assay. The most important aspect about multiplex PCR reactions is that the primers are designed in such a way that formation of primer-dimers and/or other unwanted products is minimised. The ability of primers to cross-react or to form stable structures with each other can be predicted using a variety of software programs or can simply be tested in a PCR reaction containing no template DNA. Ideally, this should yield no detectable amplification products. Another important aspect about multiplex amplifications is that each of the separate amplification reactions is equally efficient in order to prevent predominant amplification of the most efficient amplified marker. However, this is also influenced by the capillary electrophoresis platform that is used. Because of different light sources and different detector formats, different capillary platforms may detect the different fluorescent labels with different efficiencies. Thus, even if all amplification products are amplified in equimolar amounts, this could lead to a significant unbalance between peaks in the final electropherogram. If such an unbalance between the individual amplification products is observed, the following strategy could be adopted: first, perform amplification reactions with all primers at 0.5 μM. If one of the amplification products predominates the results, reduce the primer concentrations for this marker stepwise to 0.1 μM (e.g., 0.5, 0.3, 0.2 and 0.1 μM) and determine which concentration works best. This is best judged

directly on the capillary instrument instead of on agarose gels. Second, if one of the amplified markers is still present in too low concentrations, increase the primer concentrations for this marker stepwise up to 1.0 μM (e.g., 0.5, 0.6, 0.8 and 1.0 μM). There is usually no point in increasing the primer concentration above 1.0 μM. Instead, try to reduce the other primer concentrations further in order to obtain a balanced result. If proper primers concentrations have been established prepare a 10x concentrated stock solution (in sufficient amounts for at least 100 amplifications) with the required concentrations. Store at –20°C. For the STR*Af* panels, using equimolar amounts of all primers in a single amplification reaction has been shown to work well under various conditions (Fig. 1) [18]. Suitable amplification conditions for each of the 3 multiplex reactions are as follows: Combine 1 ng of genomic DNA, 0.5 μM of all primers, 0.2 mM of each dNTP, 3 mM $MgCl_2$ and 1 U FastStart Taq DNA polymerase (Roche diagnostics) in 25 μl of 1x reaction buffer. Cycling conditions are 10 min activation/denaturation at 94°C, 30 cycles of 30s 94°C, 30s 60°C and 1 min 72°C. Next, a 10 min incubation at 72°C is performed. After that, cool to ambient temperature. Store the amplification products at 4°C in the dark if the sample is analysed within days; alternatively, store at –20°C.

2.3 Capillary Electrophoresis

Each of the three STR*Af* panels amplifies 3 different microsatellite markers (e.g., M2, M3 and M4). In each panel, the distinction between the 3 markers is made using different fluorescent labels. The STR*Af* assay was designed using FAM, HEX and TET as fluorescent labels since these are available from multiple suppliers at competitive prices. A fourth colour that needs to be detected is for the internal lane standard that needs to be added to each sample in order to determine the relative mobility of the DNA fragments. This could be any colour that is spectrally different from the above labels but often involves ROX or TAMRA. Each of the labels is detected in a different channel on the capillary instrument. It is very important that the platform is properly calibrated to the combination of fluorescent labels that is used. The proper procedure to establish this may be different for each capillary platform.

Capillary electrophoresis platforms use electrokinetic injection to load the samples into the capillaries. This requires excess salt to be removed since the anions will compete with the DNA molecules for entering the capillaries. An efficient way to establish this is simply by diluting the sample in distilled water. Prior to running the samples, the internal lane standard needs to be added. The following procedure works well in most laboratories. First, dilute the amplification products 10-fold with distilled water. Next, combine 1 μl of diluted products with the recommended amount of internal lane standard and distilled water into a final volume of 10 μl. Use this for injection. Recommended injection and running conditions may vary between instruments.

3 Data Analysis and Interpretation

Once the samples have been run, most platforms come with software that will interpret the results and may produce a list of peaks that are present in the sample. In a multicentre study aimed to evaluate the reproducibility of the STR*Af* typing assay [20], participants who produced identical typing results had prior experience with this type of analysis and an in-house capillary electrophoresis platform (and software) that ensured correct detection. For various reasons outlined below I would recommend that the obtained electropherograms are always inspected visually as well to check the automated assignment of the obtained peaks. In doing so, the following artefacts should be taken into consideration.

3.1 Stutter Peaks

PCR amplification of sequences containing microsatellites leads to the well-known phenomenon of so-called stutter peaks. These are additional PCR-products differing in size by one or more repeat units from the actual number of repeats. These stutter peaks primarily contain fewer repeat units instead of additional repeat units and as such are usually easily recognised in the electropherogram as additional lower intensity peaks (Fig. 1). The amount of stutter peaks is influenced by the size as well as by the number of repeat units in the amplified fragment. Shorter repeat units give rise to a greater proportion of stutter peaks, so amplification of dinucleotide repeats is particularly prone to stuttering (Fig. 2) [3, 9]. Also, fragments with a high number of repetitions produce more stutter peaks than fragments with low or moderate numbers of repetitions.

3.2 Minus-A Peaks

Another well-known PCR artifact is the addition, by Taq DNA polymerase, of an extra A residue to the newly synthesised strand. This is the result of the template-independent polymerase activity (extendase activity) of this enzyme. Reaction conditions for the STR*Af* assay have been optimised for maximal addition of this extra A residue. Incomplete addition of this extra A will lead to minus-A peaks. These are extra peaks in the electropherogram which are exactly 1 nucleotide shorter than the principal product (Fig. 3). Obviously, this phenomenon will only be detected in the strand containing the fluorescent label. Excessive formation of minus-A peaks will occur under suboptimal amplification conditions such as an improper $MgCl_2$ concentration, too much template DNA and/or insufficient enzyme activity, visualised in reference [18] and may complicate data interpretation. Knowledge about minus-A peaks is essential for proper interpretation of the electropherograms and for the identification of the principal peak in a sample that is produced under suboptimal conditions. Furthermore, this template independent polymerase activity is strongly

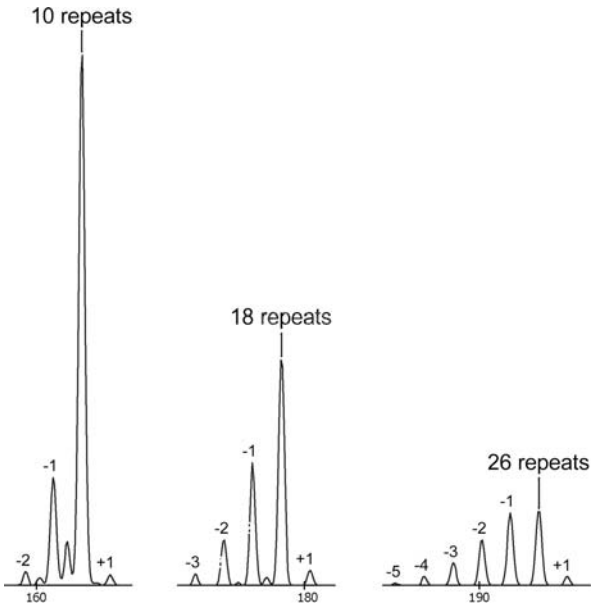

Fig. 2 Formation of stutter peaks upon amplification of microsatellite markers. The number and proportion of stutter peaks that are formed depend on the number of repetitions of the microsatellite. Examples are shown for a dinucleotide repeat marker. A sample with only 10 repeat numbers yields a –1 and –2 stutter peak whereas in a sample with a repeat number of 18, 3 stutter peaks are visible (–1, –2 and –3, respectively). In addition, the relative intensity of the stutter peaks compared to the principal peak in this sample have significantly increased compared to the relative intensity of the stutter peaks in the sample with only 10 repeat numbers. In a sample with 26 repeat numbers, the –1 stutter peak is almost as intense as the principal peak. In this sample at least 5 stutter peaks are visible (–1, –2, –3, –4 and a low intensity –5 stutter peak, respectively). Please note that the relative intensity of the +1 stutter peak is much less affected by the repeat number in all samples. As the number and relative intensity of the stutter peaks increases, the intensity of the principal peak in each sample markedly decreases

influenced by the last residue in the template strand (consequently, the first residue of the unlabeled PCR primer). If this last residue is a pyrimidine (C or T), this extendase activity is maximal. All unlabeled PCR primers were therefore designed to begin with a purine (G or A), even if this would result in a mismatch to the target sequence (Table 1).

3.3 Spectral Cross-Talk

One of the most disturbing artifacts is seen when the capillary platform has not been properly calibrated to the combination of fluorescent labels that is used. This however, is easily recognised by the superposition of peaks (at the exact same positions) with different fluorescent labels (Fig. 4). Since it is virtually impossible that

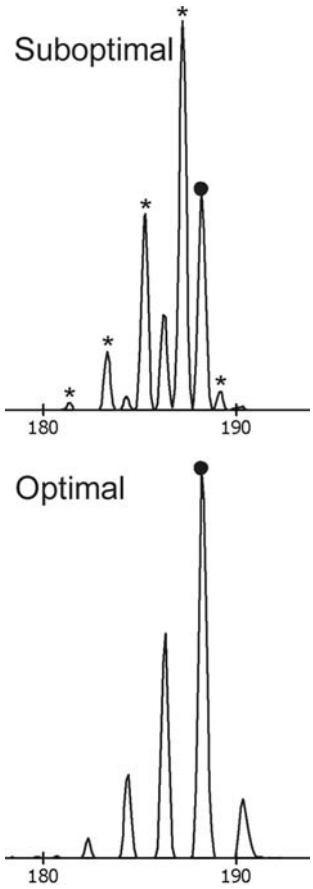

Fig. 3 Formation of minus-A peaks under suboptimal amplification conditions. Suboptimal amplification conditions may lead to excessive formation of additional minus-A peaks visualised as additional peaks that are exactly 1 nucleotide shorter than the expected fragments (indicated with *asterisks*). Excessive minus-A peaks may complicate data interpretation. In this example of a dinucleotide repeat marker, the peak indicated with a *dot* should be assigned instead of automatically choosing the tallest peak in the electropherogram. Under optimal conditions, minus-A peaks are completely absent and only stutter peaks appear as amplification artefacts

this will occur with a real sample, it must be the result of improper spectral calibration and thus, one should recalibrate the instrument till this cross-talk is reduced to acceptable levels. Improper spectral calibration could even erase peaks from the electropherogram (Fig. 4). Some electrophoretic platforms allow post-run spectral calibration since they also store the raw data (like the MegaBACE platforms from GE Healthcare). With other platforms, the samples may need to be re-run after the spectral calibration settings have been adjusted.

3.4 Allele Assignment

Crucial in the analysis of microsatellites and in the exchangeability of data between laboratories is the establishment of proper reference values for each allele. It is important to bear in mind that allele size obtained by capillary electrophoresis does

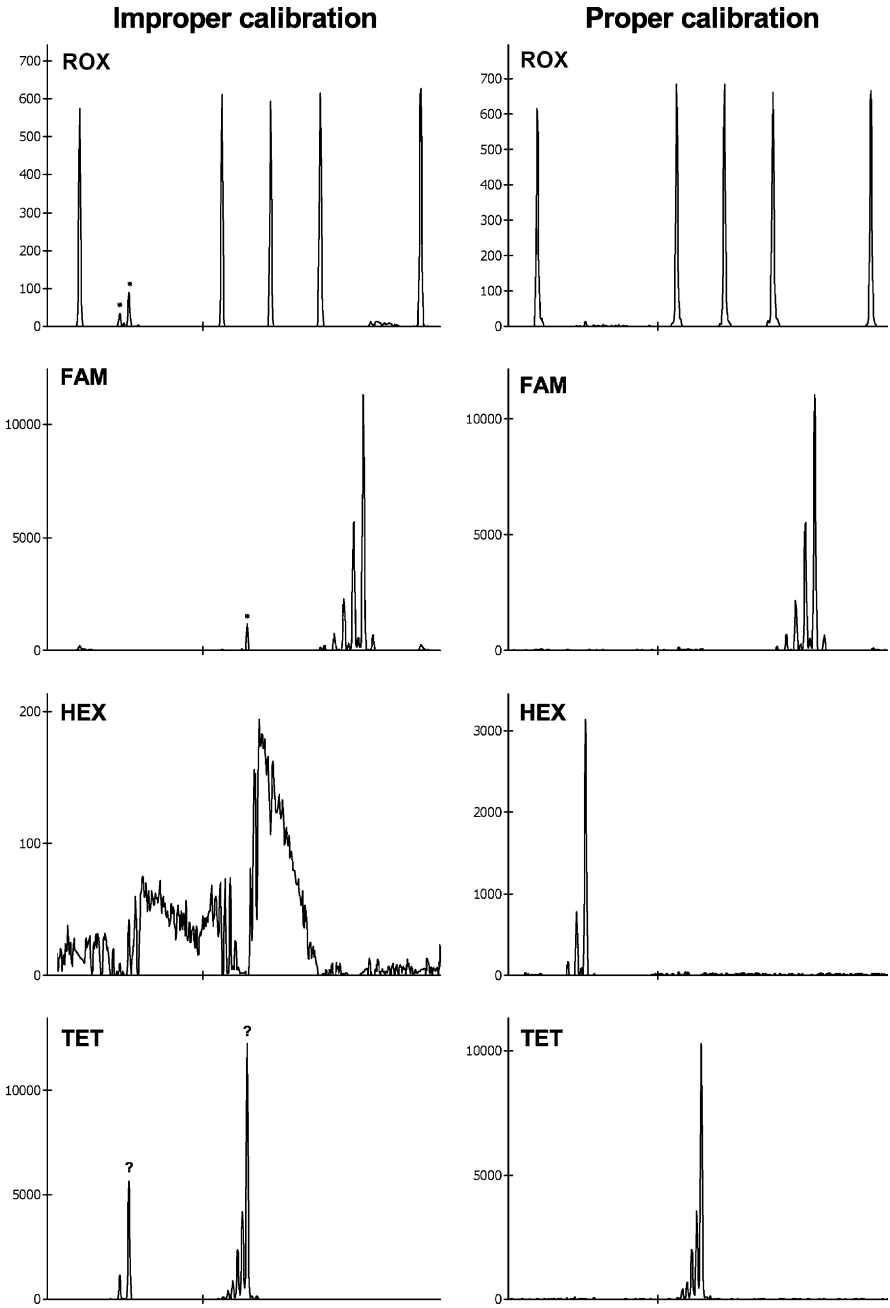

Fig. 4 Effect of improper spectral calibration. In **Panel A**, improper spectral calibration leads to cross-talk (i.e., the presence of peaks at exactly the same position in different channels). This phenomenon may lead to unintended extra peaks, (indicated with *asterisks*), but may also eliminate peaks from the electropherogram. In this example, no peak is visible in the TET channel, but two

not exactly correspond to the actual PCR fragment size, as determined by sequencing [9]. The main problem here is that the electrophoretic mobility of any DNA fragment will differ under different electrophoretic conditions and on different capillary platforms [9, 18, 21, 22]. On a single platform and using identical experimental conditions, the relative size of an allele can usually be determined with a precision of $\pm$ 0.2 nucleotide. Thus, if an allele in a given marker with 20 repeat units yields a relative size of 160.1 $\pm$ 0.2 nucleotide in laboratory A and a relative size of 165.8 $\pm$ 0.2 nucleotide in laboratory B, how do they explain to each other that they are referring to the same allele? The best solution to this problem comes from the field of human forensics in the form of allelic ladders. An allelic ladder is a mixture of characterised and well-defined alleles with established repeat numbers. By running an allelic ladder under the same experimental conditions as used with actual samples, reference sizing values for every allele can be produced. These reference values are then used for the interpretation of actual data. An interlaboratory study aimed at providing the proof of principle for such allelic ladders in microbial microsatellite based typing schemes has recently been described by de Valk et al. [20].

3.5 Non-integer Alleles

The high resolution and precision of capillary electrophoresis allows adequate separation of fragments differing in size by 1 nucleotide. Theoretically, one would only expect to find alleles that differ in length by multiples of 1 repeat unit. However, alleles have indeed been observed that differ in size from the expected reference values by 1 or more nucleotide (Fig. 5) [10]. These are the result of insertion or deletion of one or more nucleotides in the repeat itself or in its flanking sequence. The exact nature of the underlying cause can only be established by DNA sequence analysis. However, if an insertion or deletion occurs somewhere in the middle of the stretch of repeats, this can be probably be recognised by the reduction of the proportion of stutter peaks that is formed with that allele relative to an allele of the same size containing only uninterrupted repeat elements. Analogous to the nomenclature of such alleles in the human forensic community, such alleles are named n.x, indicating that the size of the allele corresponds to the size of a fragment with n repeats + x additional nucleotides. Obviously, x is smaller than the size of the repeat unit. Insertions or deletions in the flanking sequence of the repeats matching the size of the repeat unit may not be recognised. In this light, the genomic sequence of Af293 shows the presence of 20 repeat units in the STR*Af* 3B marker. According to the STR*Af*

Fig. 4 peaks appear in the HEX channel. Without additional information, it may be difficult to determine which of these two peaks should be assigned. Once properly calibrated, the proper peaks reappear in the TET channel and the amount of cross-talk is great reduced (**Panel B**) allowing unambiguous analysis of marker peaks. Note the differences in relative fluorescence units on the *Y*-axes

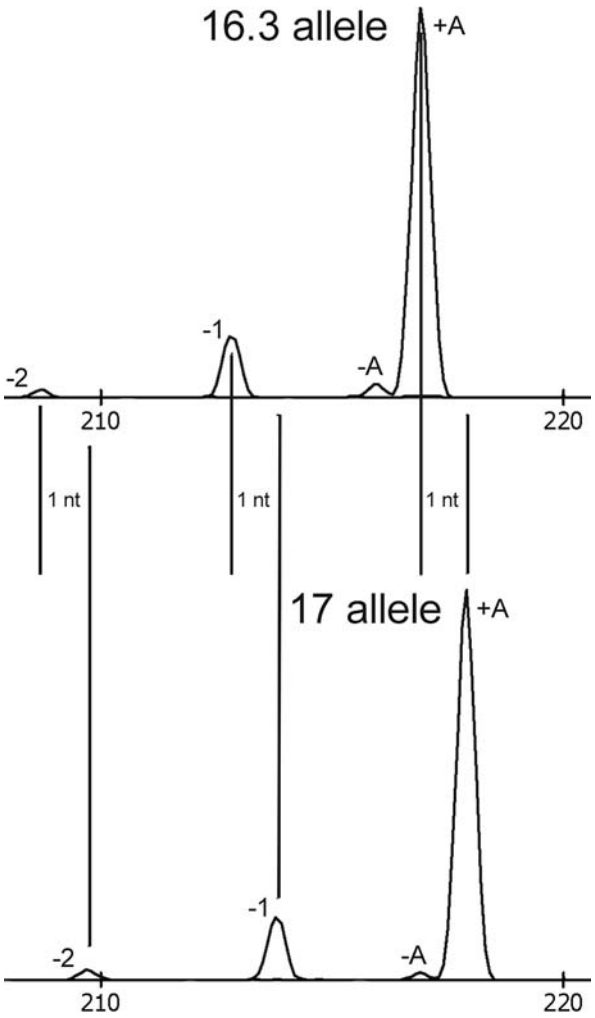

Fig. 5 Example of a marker with a non-integer repeat number. Insertion or deletion of bases in the repeat or in its flanking sequence may result in the formation of non-integer repeat numbers leading to unusual nomenclature. For the markers in the STR*Af* assay, this is most often observed in the 4A marker. In this example, *all* peaks in the sample have shifted exactly 1 nucleotide to the left relative to the same peaks in an allele with 17 repeats. The size of this allele corresponds to the size of a "16" allele + 3 additional nucleotides, hence the nomenclature "16.3". DNA sequence analysis of this allele showed a 1 base deletion in the flanking sequence of the repeat

assay, 21 repeat units are obtained. The difference between the two is an insertion of 3 bp in the flanking sequence of this marker in Af293 that has not been observed in at least 15 other randomly selected *A. fumigatus* isolates (CHW Klaassen and HA de Valk, unpublished observations) nor by others [9]. Non-integer repeat numbers are quite frequently observed in the STR*Af* 4A marker. Approximately 10% of all

alleles in this marker are an n.3 allele. DNA sequence analysis of a random selection of these alleles shows that it is the result of a deletion of 1 base pair in the flanking sequence of this marker. No non-integer repeat numbers have been observed so far in a collection of close to 2,000 isolates for any of the di-, or trinucleotide repeat markers. Very rarely (<1%), n.2 alleles are observed in the STRAf 4A and STRAf 4C markers.

3.6 Double Alleles or Absence of Alleles

The STR*Af* assay has a unique feature in that it is able to identify samples that are composed of a mixture of isolates with different genotypes. These are easily identified by the presence of more than one allele for multiple markers. The ratio of fluorescent intensity between these alleles is a rough denominator for the relative contribution of each genotype. However, if both alleles have peaks of about the same fluorescence intensity, it is impossible to determine the genotype of each of the individual isolates anymore unless they can be isolated from each other by sub culturing. Mixed isolates are most often observed in environmental samples as well as in patients with fungal balls.

Incidentally, use of the STR*Af* assay yields none of the typical peak patterns in any of the microsatellite panels. If it can be excluded that this is the result of poor-quality DNA, pipetting errors, faulty reagents, some kind of technical malfunction or otherwise, the best alternative explanation for this is that the isolate in question is simply not an *A. fumigatus*. Several other species belonging to the *Fumigati group* of *Aspergilli* are morphologically very similar and can easily be mistaken for an *A. fumigatus*. The STR*Af* assay will not produce the characteristic peak patterns with such isolates.

4 Concluding Remarks

Strain typing data is most useful if it can be exchanged between labs so that the distribution and dissemination of specific genotypes can be monitored on a global scale. This requires typing assays to be highly reproducible in different settings and that the typing data can be described in an unambiguous format. Microsatellites offer unprecedented discriminatory power in strain typing, and because of its compact and numerical nature, microsatellite data are perfectly suitable for long-term storage and exchange. For these reasons, the human forensic community also heavily relies on microsatellite markers for many years now. With the guidelines described in this chapter, it should be possible to adopt and implement the microsatellite based STR*Af* assay for *A. fumigatus*. The next step would be to centralize and collect these data in a global database which could bring our understanding of the epidemiological behaviour of this important pathogenic fungus to a next level.

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Antifungal Susceptibility Tests of *Aspergillus* Species

Arnaldo Lopes Colombo, Viviane Reis, and Patricio Godoy

Abstract Although different methods are now available to assess the susceptibility of *Aspergillus* species to antifungal agents, there are still limited data correlating in vitro resistance with meaningful clinical endpoints. Moreover, there is no consensus on the breakpoints to define resistance/susceptibility to different antifungal agents. This chapter reviews the technical issues related to antifungal susceptibility tests for *Aspergillus* species, including the use of control strains, inoculum preparation, culture media, and incubation conditions.

Keywords Antifungal susceptibility · Antifungal resistance · Triazoles · Echinocandins · Etest

Contents

A.L. Colombo (✉)
Special Mycology Laboratory, Division of Infectious Diseases, Federal University of São Paulo (UNIFESP), São Paulo, Brazil
e-mail: colomboal@terra.com.br

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1 Introduction

The development of in vitro susceptibility tests goes back to the advances of antibacterial chemotherapy. The use of in vitro susceptibility testing including principles of agar-based methods as well as broth dilution methods was initially performed by Fleming, during his evaluation of the therapeutic potential of penicillin. Soon after, with the rise of new antimicrobial agents, in addition to the discovery of penicillin-resistant bacteria, several microbiology laboratories started conducting susceptibility testing to antimicrobial agents [1].

Until recently, chemotherapy employed to fungal invasive diseases presented large disparity in relation to antibacterial therapy. Up to the development of azole antifungal agents on the 1970s, only amphotericin B and 5-fluorocytosine (5-FC) were available for treating systemic mycoses. Currently, several antifungal drugs for systemic use are available in the market, including different formulations of amphotericin B, 5-FC, ketoconazole, several triazoles (itraconazole, fluconazole, voriconazole and posaconazole), and three new echinocandins (anidulafungin, caspofungin and micafungin) [2–4].

The increased incidence of systemic fungal infections on susceptible populations and the development of new therapeutic alternatives have led to ever greater need and interest from the medical-scientific community to develop and standardise antifungal susceptibility tests. In light of such a need, initially the Clinical and Laboratory Standards Institute (CLSI) and later the European Committee on Antimicrobial Susceptibility Testing (EUCAST) created task forces with the purpose of standardising antifungal susceptibility testing of yeasts and, later on, filamentous fungi. As a result, there are now several well standardised methods available for the evaluation of in vitro antifungal activity, including broth-based methods (CLSI and EUCAST) and agar-based methods (CLSI Disk-diffusion method and Etest®) [5, 6].

Although different methods to assess the *Aspergillus* spp. susceptibility to antifungal agents are available, we still have limited information of the clinical relevance

of the in vitro data generated by different methodologies. So far, there is no consensus on the breakpoints related to resistance or susceptibility to different antifungal agents [5, 7]. In this context, *Aspergillus* susceptibility testing is mostly used in assessing the in vitro therapeutic potential of new drugs, as well as in characterising potential geographic or temporal trends in terms of susceptibility patterns to different antifungal drugs. Data currently available on the in vitro and in vivo correlation still does not support the routine clinical use of antifungal susceptibility test results for driving antifungal therapy for individual cases of invasive aspergillosis [5, 6].

Regardless of limitations of the clinical relevance of minimum inhibitory concentration (MIC) results generated by antifungal susceptibility tests with moulds, several authors have recorded the occurrence of mutant isolates of *Aspergillus* spp. that show low in vitro susceptibility to triazoles. Such samples may be isolated after in vitro induction of mutations by sequential exposition of the pathogen to sub inhibitory concentrations of antifungal drugs [8], or even may be isolated from patients with aspergillosis who were exposed to itraconazole during long periods of time [9], particularly in the presence of low itraconazole serum levels. In these two scenarios, several molecular mechanisms of resistance *of Aspergillus fumigatus* to triazoles have been proposed, including efflux pumps and changes in the 14-steroldemethylase enzyme secondary to cyp51A and cyp51B gene mutations, or the over expression of the protein product secondary to mutation in the promoter region of cyp51A [10–13].

There are still few longitudinal studies recording the evaluation of the in vitro antifungal susceptibility of clinical isolates obtained from patients colonised and/or infected with *Aspergillus* who have been exposed to antifungal agents for long periods. Such studies are essential to understand the potential of *Aspergillus* spp. for developing antifungal resistance to different drugs.

2 Standardisation of Broth Microdilution Method by the Clinical and Laboratory Standards Institute (CLSI)

The standardisation of antifungal susceptibility testing with filamentous fungi followed similar steps and parameters successfully used during the standardisation of the CLSI broth dilution method for yeasts. In this regard, several parameters have been adjusted such as inoculum size, culture medium, incubation period and temperature, which have been applied to standardise antifungal susceptibility tests with yeasts to achieve internal consistency and reproducibility in the assays involving mostly the following filamentous fungi: *Aspergillus* spp., *Fusarium* spp., *Scedosporium apiospermum, Sporothrix schenckii* and *Rhizopus* spp. (M38-A, document) [14].

2.1 Inoculum Preparation

Antifungal susceptibility testing with moulds use viable conidia in the preparation of the standard inoculum solution for assays, instead of using hyphal elements that

usually represents the only fungal elements found in the tissues of infected patients. Such adaptation is essential for obtaining consistent and reproducible results of in vivo assays of filamentous fungi, in view of the great difficulty in preparing homogeneous solutions with hyphal elements. Some authors have suggested that antifungal MICs generated with inocula prepared with hyphal elements are significantly higher compared to those obtained from assays with conidial suspensions [15–17].

Espinel-Ingroff and Kerkering [18] performed the first study on the standardisation of a method for preparing inoculum suspensions for antifungal susceptibility testing with moulds. This study evaluated the use of spectrophotometer for preparing conidia suspensions with different transmittance levels at 530 nm, establishing the optimal parameters for obtaining different sizes of inoculum with different fungi. Table 1 shows the transmittance range necessary to prepare the inoculum suspensions containing adequate counting of colonies for testing the following agents: *Aspergillus* spp., *Pseudoallescheria boydii*, *Sporothrix schenckii*, *Mucor* spp. and *Rhizopus* spp.

Table 1 Spectrophotometer transmittance ranges (530 nm) and corresponding inoculum sizes (UFCs/ml) of inocula suspensions of moulds

Fungal species	Transmittance change (%)	Change (CFU/ml)
Aspergillus flavus	78–81	$1–3.8 \times 10^6$
Aspergillus fumigatus	78–82	$1–4 \times 10^6$
Mucor spp.	69–71	$8 \times 10^5–2 \times 10^6$
Rhizopus spp.	68–72	$0.9–2.9 \times 10^6$
Pseudoallescheria boydii	68–71	$1–2 \times 10^6$
Sporothrix schenckii	78–82	$1–5 \times 10^6$

Legend: CFU, colony forming unit.

The internal consistency and reproducibility of tests using spectrophotometer for preparing standard inoculum suspensions with 5 filamentous fungi was evaluated by a multicentre study involving 6 different laboratories. That study found a high rate (>90%) of intra- and inter-laboratory reproducibility of inoculum sizes prepared by spectrophotometer when tests were performed by different centres [19].

A recent study aimed to validate quality control isolates for antifungal susceptibility tests with moulds has confirmed that 92–96% of inoculum sizes prepared by spectrophotometer, as originally proposed by Espinel-Ingroff and Kerkering [18], have reproducible quantification of fungal elements within the target range [20].

2.2 Selection of Culture Medium and Incubation Conditions

Selecting a culture medium to conduct antifungal susceptibility testing is a critical step to allow proper fungal growth under testing conditions, as well as for preventing undesirable interactions between the antifungal agent and components of the proper culture medium [21]. Another aspect to advocate the use of fully defined synthetic media, without the presence of macromolecules, is to prevent possible interactions

between molecules of the media (e.g., proteins) and the antimicrobial drug to be tested. Finally, fully defined synthetic media do not present differences of medium composition between different batches.

Espinel-Ingroff et al. [19] conducted a study performing macro- and microdilution tests with 5 different moulds adapting the same parameters suggested by the M27-P document of the CLSI (formerly NCCLS,1992) for testing yeasts [22], including the use of RPMI -1640 with L-glutamine, without bicarbonate and buffered with 3-(N-morpholino)-propanesulfonic acid (MOPS) at pH of 7.0; preparation of inoculum from 0.5 to 2.5 $\times$ 10^3 colony forming units per ml (CFU/ml) using spectrophotometry, with readings after 48–72 h of incubation at 35°C,. It is worth mentioning that inoculum suspensions of moulds were prepared with the aid of spectrophotometer at transmittance ranges needed to achieve the target range for different genera of fungi (Table 1). The RPMI-1640 culture medium supported properly the growth of all filamentous fungi tested and allowed sufficient growth of colonies after 24 h of incubation for *Aspergillus* and *Rhizopus* species, and after 3–4 days for isolates of *Pseudoallescheria boydii* and *Sporothrix schenckii.* These results suggest that this broth dilution method has a potential for testing of filamentous fungi.

In a multicentre study involving 11 different laboratories, coordinated by Espinel-Ingroff et al. [23], 6,552 values of MICs were generated for amphotericin B and 6,410 for itraconazole, based on the assessment of 30 moulds tested in duplicate. Such study showed that the methodology proposed by the CLSI, including the use of RPMI-1640 medium, buffered with MOPS, inoculum prepared using the spectrophotometer with assays incubated at 35°C generated reproducible results when readings were performed after 48–72 h (93 to 97.7%, respectively).

2.3 Quality Control: The Use of Reference Strains

The use of quality control isolates in routine laboratories is essential to ensuring the following aspects: quality of reagents, culture media, to control the accuracy in the preparation of antifungal drug dilutions and the reproducibility of tests.

For quality control proposal, antifungal susceptibility testing should include at least one reference strain whose MIC limits against different antifungal agents are easy to read, reproducible and accepted by the international community. Control organisms or strains are acceptable when they generate MIC results for different antifungal agents within a defined target variation in more than 95% of tests, as previously documented by multicenter studies [20, 24].

Following this principle, Espinel-Ingroff et al. [20] performed the first multicentre study involving 8 laboratories to test the inter- and intra-laboratory variability of MICs generated by the CLSI microdilution method performed with 12 filamentous fungi against triazoles and amphotericin B, in order to validate quality control strains (QC) for such tests. After tests have been performed, only 7 out of 12 strains tested showed $\geq$95% of the MIC values within the expected range for such organism. Table 2 summarises the strains that can be used as control organisms and

Table 2 Recommend MIC limits for quality control (QC) and reference strains tested against several antifungal agents by the CLSI microbroth dilution method (Document M38-A2)

Isolate	Purpose	Agent	MIC range (μg/ml) (% within range)
P. variotii ATCC MYA-3630	QC	Amphotericin B	1–4 (100)
		Itraconazole	0.06–0.5 (100)
		Posaconazole	0.03–0.25 (99.5)
		Voriconazole	0.015–0.12 (100)
A. flavus ATCC MYA – 3631	Reference	Amphotericin B	2–8 (98.8)
		Posaconazole	0.12–1 (97.1)
		Voriconazole	0.5–2 (98.3)
A. fumigatus ATCC MYA- 3626	Reference	Amphotericin B	0.5–4 (98.7)
		Posaconazole	0.25–2 (95.7)
		Voriconazole	0.25–1 (100)
A. fumigatus ATCC MYA – 3627	Reference	Amphotericin B	0.5–4 (99.2)
		Itraconazole	≥16 (95)
		Posaconazole	0.25–2 (98.3)
		Voriconazole	0.25–1 (99.2)
A. terreus ATCC MYA – 3633	Reference	Amphotericin B	2–8 (98.3)
		Voriconazole	0.25–1 (99.2)
F. moniliforme ATCC MYA – 3629	Reference	Amphotericin B	2–8 (99.6)
		Itraconazole	≥16 (97.9)
		Posaconazole	0.5–2 (98.1)
		Voriconazole	1–4 (100)
S. apiospermum ATCC MYA – 3635	Reference	Amphotericin B	4–16 (98.9)
		Posaconazole	1–4 (98.3)
		Voriconazole	0.5–2 (100)

Legend: CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

the expected MIC changes for the different drugs. It is worth mentioning that *Paecilomyces variotii* was the isolate which presented the best performance as control organism for all drugs tested.

In CLSI M38-A2 document [25], in view of the studies conducted by Espinel-Ingroff et al. [20] and Pfaller et al. [26], the following strains were included as quality control strains for antifungal susceptibility tests with moulds: *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *Paecilomyces variotii* ATCC MYA-3630. All other strains were listed as reference strains, including the following species of *Aspergillus: A. flavus* ATCC 204304, *A. fumigatus* ATCC MYA-3627, *A. flavus* ATCC MYA-3631 and *A. terreus* ATCC MYA-3633.

2.4 Reading Results and Breakpoints for Different Antifungal Drugs

2.4.1 Amphotericin B

The end point definition for reading tests with amphotericin B is the lowest concentration of the drug which fully prevents any visual growth of the inoculum tested for that drug.

Only a few clinical isolates have been described as truly resistant to amphotericin B, which complicates the definition of breakpoints based on clinical data. Although there is no consensus regarding this issue, initial data suggest that lower treatment success rates are achieved with amphotericin B for infections caused by a wide variety of filamentous exhibiting MIC results of $\geq$2.0 μg/ml [6].

2.4.2 Triazoles

The end point definition for reading in vitro assays with triazoles is the lowest drug concentration capable of fully inhibiting the growth of the isolates tested. Of note, this parameter is different from that used in antifungal tests performed with yeasts where trailing growth may occur. Thus, the concept of trailing phenomenon, as discussed in tests with *Candida*, should not be used in tests with filamentous fungi [27].

Although there is no consensus on breakpoints for different triazoles, initial data suggest that lower treatment success rates for infections caused by *Aspergillus* have been observed with patients infected with organisms exhibiting MICs of $\geq$2.0 μg/ml for itraconazole, voriconazole and posaconazole [6, 28].

2.4.3 Echinocandins

Document NCCLS M38-A [14] has set forth the parameters for susceptibility testing of filamentous fungi involving amphotericin B and triazoles. However, since echinocandins present mechanisms of antifungal activity completely different from the drugs mentioned earlier, several authors have suggested that drugs of this therapeutic class should be evaluated under different parameters. In this context, it has been suggested to define the in vitro activity of echinocandins based on the detection of hyphal morphology changes of filamentous fungi induced by the exposition to the drug, a finding called as "minimum effective concentration" (MEC), which is nowadays regarded as the most appropriate parameter for in vitro testing of echinocandins [29, 30].

To determine the MEC values, small amounts of inoculum exposed to different concentrations of echinocandins are observed on the microscope to identify the minimum concentration of the drug which triggers morphological changes of the hyphae. The lowest drug concentration which triggers aberrant hyphae with atypical behaviour, when compared with the control well, is determined as being the MEC. The visual reading of the MEC can be determined by identifying the first well in which round microcolonies are present [6].

Although there is no consensus on breakpoints for echinocandins, initial data suggest that clinical resistance has been observed with patients infected by strains exhibiting MICs of $\geq$4.0 μg/ml [6].

Table 3 summarises the parameters proposed by the CLSI (document M38-A, 2002) [14] for performing the microdilution test of filamentous fungi against different antifungal drugs.

Table 3 CLSI parameters for conducting antifungal susceptibility tests with moulds belonging to genera *Fusarium* spp., *Aspergillus* spp., *Rhizopus* spp., *Scedosporium apiospermum* and *Sporothrix schenckii*

Features	CLSI document M38-A2, 2008
Preparation of inoculum	Conidial solution – use of spectrophotometry
Size of inoculum	0.4–5 $\times$ 10^4 UFC/ml
Medium	RPMI 1640 with L-glutamine
Buffer	3-(N-morpholino)propanesulfonic acid (MOPS)
Format	Microdilution in broth
Incubation temperature	35°C
Incubation time	48 h (24 h for *Rhizopus* spp.)
Endpoints	
AmB and Triazoles	Growth fully inhibited
Echinocandins	MEC value

Legend: AmB, amphotericin B; CLSI, Clinical and Laboratory Standards Institute; MEC value, the lowest drug concentration which triggers aberrant hyphae with atypical behaviour when compared with the control well.

3 Standardisation of Broth Dilution by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

Parallel to the efforts of the CLSI for improving antifungal susceptibility tests, the European scientific community organised studies to optimise in vitro tests with yeasts and moulds. Recently, the entity responsible for standardizing all clinical laboratory methods used in European heath institutions, the European Committee on Antimicrobial Susceptibility Testing (EUCAST), appointed a subcommittee to draft an alternative proposal on the standardisation of the broth microdilution assay for testing fermenting yeasts. The EUCAST proposal on susceptibility testing for moulds, particularly for *Aspergillus* species, relies markedly on the methodology proposed by the CLSI (M38-A document) for filamentous fungi and the CLSI document M27-A for yeasts [14, 31].

The EUCAST proposal introduced few modifications for testing moulds when compared to the CLSI standard documents: (i) use of a higher inoculum size of *Aspergillus* spp. prepared by haemocytometer (instead of spectrophotometer) and (ii) RPMI medium with a 2% concentration of glucose [32].

3.1 Inoculum Preparation

Denning et al. [33] performed the first study using an inoculum concentration different from that proposed by Espinel-Ingroff et al. [19] for conducting susceptibility testing with moulds. In such study the authors used an inoculum solution with a concentration of 2 $\times$ 10^6 conidia/ml prepared by using a haemocytometer instead of a spectrophotometer.

Petrikkou et al. [34] and Aberkane et al. [35] compared the use of a haemocytometer versus spectrophotometer in preparing inoculum solutions of filamentous fungi. The target sizes of inoculum suspensions prepared by both methods were

highly reproducible and there was a clear correlation between data generated by them. Despite the good agreement between methods, some authors advocate that the use of haemocytometer may be more appropriate once that they noticed that the natural colour variability exhibited by some conidia of specific moulds may cause interference in the adjustment of inoculum suspension by spectrophotometry.

Additional validation of using haemocytometer for preparing inoculum suspensions of moulds came from a multicentre study performed with the collaboration of 3 laboratories, which analyzed 40 isolates representative of 4 *Aspergillus* species. The study showed a good intra- and inter-laboratory agreement of data generated by this method and 89.2% of experiments recorded inoculum sizes within the target range from 1×10^6 to 5×10^6 [36].

3.2 Selection of Culture Medium and Incubation Conditions

A study conducted by Denning et al. [33] assessed the impact of different parameters of in vitro tests on MIC generated by microdilution assays conducted on 5 different conditions to test itraconazole against *Aspergillus fumigatus* strains. It is worth mentioning that amongst the strains of *Aspergillus* tested were included isolates exhibiting in vitro and in vivo resistance to amphotericin B and itraconazole. After testing the enrolled clinical isolates, the parameters of the test which generated best in vitro versus in vivo correlation and higher reproducibility of MIC results were: RPMI culture medium with L-glutamine and 2% glucose, inoculum size of 10^6 conidia/100 μl, incubation temperature at 35°C and readings performed after 48 h of incubation. This study, along with the reports previously mentioned validating the use of haemocytometer in the preparation of inoculum solutions, provided the cornerstones parameters for the conduction of the EUCAST broth microdilution assay for *Aspergillus* spp. as summarised in Table 4.

Table 4 EUCAST parameters for conducting antifungal susceptibility tests with moulds

Features	EUCAST – Moulds
Appropriate	*Aspergillus* and mould species
Inoculum	$2–5 \times 10^5$ CFU ml^{-1}
Inoculum standardisation	Haemocytometer
Medium	RPMI 2% glucose
Buffer	3-(N-morpholino)-propanesulfonic acid (MOPS)
Format	Microdilution
Temperature	35°C
Incubation time	48 h
Endpoints	
AmB and triazoles	MIC with growth fully inhibited
Echinocandins	MIC or MEC value

Legend: AmB, amphotericin B; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MEC value, the lowest drug concentration which triggers aberrant hyphae with atypical behaviour when compared with the control well; MIC, minimum inhibitory concentration.

Recently, Tudela et al. [37] conducted a study evaluating the in vitro activity of triazoles and amphotericin B against a panel of 183 *Aspergillus fumigatus* isolates, including 150 strains identified as "sensitive" and 33 itraconazole-resistant strains. The authors used the EUCAST broth microdilution method for testing all *Aspergillus* strains and MIC readings were performed after 24 and 48 h readings. The authors corroborated the good performance of the parameters suggested by EUCAST for in vitro susceptibility testing, and observed that 48 h readings enabled better discrimination of the 33 itraconazole-resistant strains tested. Too many false negative results in resistant strains were recorded if readings were performed only after 24 h incubation.

3.3 Quality Control

The validation of control strains for antifungal susceptibility tests with filamentous fungi, following the EUCAST broth microdilution method was initially conducted by Cuenca-Estrella et al. [38] that performed a multicentre study involving 7 laboratories, assessing the inter- and intra-laboratory variability of antifungal MICs generated by 12 fungal isolates. After the determination of 18,144 MIC values for amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole and posaconazole against 6 *Candida* spp. strains and 6 *Aspergillus* spp. isolates, the observed rates of intra-laboratory and inter-laboratory agreement ranging from 90 to 95% of the results. Only 2 out of 12 strains tested were excluded from the pool of potential control strains because they exhibited difficult reading end point due to the presence of "trailing". Table 5 summarises the list of reference strains which can be used as quality control organisms and the expected range of MIC results for antifungal drugs generated by the EUCAST broth microdilution method.

3.4 Reading Results and Breakpoints for Different Antifungal Drugs

The reading end point for antifungal susceptibility testing using triazoles and amphotericin B shall be performed at the lowest drug concentration that fully prevents any discernible growth of the isolate tested. Regarding tests with echinocandins, the EUCAST document does not clarify whether the best reading criteria is identifying the MIC or the MEC [39].

Interpretative breakpoints for MICs generated by the EUCAST methodology still are not well established. The clinical relevance of testing moulds remains uncertain [39].

The testing conditions pursuant to EUCAST are listed in Table 4. The standard broth microdilution method described by the EUCAST is supposed to be a valid method for testing different conidia forming moulds. However, most of the

Table 5 Acceptable MIC ranges (μg/ml) for quality control strains of *Aspergillus* spp. when tested against four antifungal drugs by using the EUCAST broth microdilution method

Isolate	Purpose	Agent	MIC range (μg/ml) (% within range)
A. flavus ATCC 204304	Reference	Amphotericin B	0.5–2 (97.2)
		Itraconazole	0.12–0.5 (100)
		Posaconazole	0.12–0.5 (91.7)
		Voriconazole	0.5–2 (91.7)
A. fumigatus ATCC 204305	Reference	Amphotericin B	0.25–1 (100)
		Itraconazole	0.12–0.5 (100)
		Posaconazole	0.03–0.25 (90.3)
		Voriconazole	0.5–2 (94.4)
A. fumigatus CNM-CM 237	Reference	Amphotericin B	0.25–1 (99.1)
		Itraconazole	0.12– 0.5 (96.3)
		Posaconazole	0.015–0.25 (88)
		Voriconazole	0.25–1 (91.6)
A.fumigatus CNM-CM 2159	Reference	Amphotericin B	0.25–1 (87.1)
		Itraconazole	8 to >16 (98.1)
		Posaconazole	0.5–2 (100)
		Voriconazole	0.5–2 (96.3)
A terreus CNM-CM 2524	Reference	Amphotericin B	1–8 (90.7)
		Itraconazole	0.12–0.5 (86.2)
		Posaconazole	0.06–0.25 (87.1)
		Voriconazole	0.5–2 (100)
A. flavus CNM-CM 1813	Reference	Amphotericin B	1–4 (91.7)
		Itraconazole	0.12–0.5 (100)
		Posaconazole	0.12–0.5 (85.3)
		Voriconazole	0.5–2 (97.2)

Legend: EUCAST, European Committee on Antimicrobial Susceptibility Testing; MIC, minimum inhibitory concentration.

information available is derived by tests performed with *Aspergillus* spp. strains [32–38].

4 Agar-Based Diffusion Susceptibility Testing

Broth dilution methods performed according to the standards suggested by the CLSI and EUCAST are considered gold standard tests for the conduction of in vitro antifungal susceptibility assays. However, these methods are labour intensive and time consuming, which prevent its regular use by routine microbiology laboratories. Accordingly, alternative and more suitable-for-routine-laboratory methods have been developed in the last years, initially for testing yeasts and, more recently, for testing filamentous fungi against antifungal drugs [27, 40–44].

This chapter presents the advances made in the standardisation of three agar-based systems: Etest® (AB Biodisk, Solna, Sweden), disk diffusion pursuant to the CLSI standards (CLSI M44-A) [45] and disk diffusion performed with

antifungal disks manufactured by Rosco Diagnostica (Taastrup, Denmark), identified as Neosensitabs®.

4.1 Etest®

Etest® (AB Biodisk, Solna, Sweden) is a test based on agar diffusion, where different concentrations of antifungal agents incorporated into plastic strips provides quantitative results of the in vitro susceptibility of fungal isolates to different drugs. The outward diffusion of the drug from the strip toward agar takes place steadily, keeping a continuous gradient of antifungal concentrations through the extension of the strip, inhibiting the growth of the microorganism inoculated in the agar plate, rendering an ellipse-shaped inhibition area. The MIC is read as the drug concentration at which the border of the elliptical inhibition zone intersects the scale on the Etest® antifungal test strip. Consequently, this method combines the benefits of simple execution of agar-based diffusion testing with the quantitative information provided by broth dilution-based assays [40, 46]. Results from several studies attest that Etest® results present significant concordance with data generated by the CLSI standard method for yeasts [40, 48]. Based on those findings, such test has been approved by the Food and Drug Administration (FDA) for use in routine laboratories in the USA.

One of the first studies using the Etest® method for testing filamentous fungi was performed by Székely et al. [41], by comparing the MIC results generated by this technique with values rendered by the CLSI microdilution broth method [31]. Ninety strains of filamentous fungi were selected, including 40 samples of *Aspergillus* spp. and tested against amphotericin B and itraconazole. Concordance of results between methods was approximately 100% of MIC results generated with *Aspergillus* strains tested against both drugs. It is worth mentioning that the Etest® was able to identify the three samples resistant to itraconazole included in the study. Etest® strips containing triazoles when testing moulds may exhibit growth of microcolonies inside the elliptical growth inhibition zone. This finding should be interpreted as trailing and be ignored in the reading, as per the susceptibility testing of azoles with yeasts [49, 50].

Espinel-Ingroff and Rezusta [46] tested 107 *Aspergillus* spp. isolates against triazoles and amphotericin B assessed by Etest® and CLSI broth microdilution. As to the triazoles, the correlation between the results of MICs generated by both methods ranged from 82.3 to 96.3%. In relation to amphotericin B, test correlation was 91.7% for readings taken after 24 h of incubation, and 64.5% for results after incubation for 48 h. The authors remarked that despite the 24 h reading gives results more consistent with data rendered by the CLSI methodology, there still remains unclear which incubation time would provide the most clinically relevant MIC result.

Pfaller et al. [44] conducted a comparative study between Etest® and CLSI broth microdilution by testing a panel of 376 *Aspergillus* spp. isolates against itraconazole and voriconazole. Agreement of MIC results generated by the two methods with both triazoles topped 95%. When differences between results rendered by the

two methods existed, Etest® MICs for voriconazole tended to be lower than those rendered by the broth microdilution, and the opposite trend was observed for itraconazole.

Guinea et al. [51] evaluated the correlation between MIC results rendered by CLSI broth microdilution tests and Etest® with antifungal drugs. Strips of amphotericin B, voriconazole and itraconazole were used to test a panel of 283 *Aspergillus fumigatus* isolates. The correlation of both methods in identifying strains considered susceptible or resistant to antifungal agents was excellent for tests involving voriconazole and itraconazole, which were 100% concordant. However, assays with amphotericin B generated substantial discrepancies of MIC results between tests and 23% of strains were incorrectly classified by Etest® when compared to the standard method.

By taken together data generated by all mentioned papers, we may suggest that the correlation between methods was higher in assays performed with *Aspergillus* spp. tested against triazoles, with significant discrepancies documented between methods in tests involving amphotericin B. It is essential to carry out in vitro versus in vivo correlation studies to evaluate critically which methodology provide the most clinically relevant MIC results with different antifungal drugs.

In view of the data suggesting that Etest® has a good performance when compared to standard broth methods and is simple to perform, this methodology has been extensively used in multicentre studies to monitor resistance to antifungal agents, allowing the evaluation of large sampling amounts [44, 52].

4.2 Antifungal Disk Diffusion Tests Performed According to CLSI Standards

In the 1970s and 1980s, many authors worked with agar-based disk-diffusion methods with disks containing 5-FC, amphotericin B, clotrimazole and miconazole. However, studies were conducted using different methodologies and their results are hard to compare. Similarly, the authors did not attempt to standardise different parameters and test conditions to ensure consistency and reproducibility of results [53–56].

The CLSI conducted several studies in order to set the standardisation of the antifungal disc-diffusion method summarised in the document M44-A [45] for testing yeasts suggesting the following parameters: Müeller-Hinton medium supplemented with dextrose and methylene blue, inoculum size of 0.5–2.5 $\times$ 10^3 CFU/ml prepared by means of spectrophotometry, incubation temperature at 35ºC and reading at 24 h. Several studies showed good correlation between in vitro results rendered by disk diffusion with triazoles, when compared with broth microdilution, pursuant to the CLSI standards [57, 58].

Serrano et al. [50] evaluated the susceptibility profile of 77 *Aspergillus* spp. strains tested against voriconazole by using three different methods: broth microdilution, Etest® and agar-diffusion with disks of 1 μg of voriconazole (Difco, Oxoid). This was the first study to assess the correlation between voriconazole disk-diffusion

tests, performed according to standards suggested by the CLSI in document M44-P [59], and broth microdilution tests (CLSI M38-A document) [14]. The *Aspergillus* spp. strains tested exhibited good growth under test conditions and results of voriconazole disk diffusion tests after 24 h reading presented good correlation with data rendered by the reference method.

Espinel-Ingroff et al. [43] conducted a multicentre study with the purpose of determining the ideal conditions for conducting agar-based disk-diffusion assays with filamentous fungi, comparing the results with the data obtained with the CLSI microdilution test [14]. A total of 555 mould isolates, including 203 strains of the *Aspergillus* genus were assessed in a disk-diffusion assay performed with two different media supplemented Müeller-Hinton with 2% dextrose and 0.5 μg/ml versus plain Müeller-Hinton, three incubation times (16–24, 48 and 72 h), and tested with seven disks of antifungal agents: amphotericin B and itraconazole (10 μg), voriconazole (concentrations of 1 and 10 μg), caspofungin (5 μg BBL and 5 μg Oxoid) and posaconazole (5 μg). The best correlation between the disk-diffusion and the standard method results rendered was obtained when tests were conducted in plain Mueller-Hinton (MH) agar, with incubation periods from 16 to 24 h for zygomycetes, 24 h for species of *Aspergillus* and 48 h for other fungus species. It is worth mentioning that the correlation between the standard broth microdilution and disk-diffusion tests was way higher in assays involving triazoles, when compared to tests with amphotericin B. The study also showed that disks of voriconazole at a concentration of 1 μg presents better results in assays with *Aspergillus* spp. samples. Regarding the assays with caspofungin, the 5 μg disks manufactured by BBL (USA) presented better performance vis-à-vis disks manufactured by Oxoid (England).

4.3 Disk Diffusion with Antifungal Tablets Manufactured by Rosco Diagnostica (Neo-Sensitabs®)

Disk-diffusion tests can be performed using 9 mm tablets impregnated with single concentrations of different antifungal drugs as the "Neo-Sensitabs® Tablets" manufactured by Rosco Diagnostica SA, Denmark.

Espinell Ingroff [60] conducted a study evaluating the disk-diffusion method with antifungal tablets manufactured by Rosco Diagnostica (Neo-Sensitabs®) containing single concentrations of amphotericin B (10 mg) and posaconazole (5 g). A total of 126 *Aspergillus* spp. strains were tested by using Müeller-Hinton medium, incubated at 35°C with readings taken after 24 and 48 h. Results were further compared with MICs rendered by the CLSI microdilution standard method. There was sufficient growth of *Aspergillus* strains tested under the abovementioned conditions, and the 24 h readings presented better correlation with results generated by the CLSI reference method.

Neo-Sensitabs® Tablets with antifungal drugs were further evaluated in a study conducted by Espinel-Ingroff and Canton [61] where a panel of 183 filamentous fungi strains, including species of *Aspergillus*, *Fusarium*, *Scedosporium*, zygomycetes, and dematiaceous fungi were tested against tablets of amphotericin

B (10 μg), itraconazole (8 μg), posaconazole (5 μg), voriconazole (1 μg) and caspofungin (5 μg). The authors obtained good correlation between methods when the susceptibility results of moulds were tested with triazoles, mainly voriconazole. Significant discrepancies were observed between both methods when testing moulds against amphotericin B.

By taking together the in vitro results generated by agar-based methods performed with the CLSI disk-diffusion method and Neo-Sensitabs® Tablets with antifungal drugs as a whole, it becomes evident that the correlation of both systems with the CLSI broth microdilution standard method is higher for assays performed with triazoles than for tests with amphotericin B.

5 Commercial Panels for Broth Microdilution Assays with Chromogenic Media

The Sensititre YeastOne (AccuMed International, Westlake, Ohio) colorimetric antifungal panel is a commercial system that provides a series of different concentrations of five dehydrated antifungal agents (amphotericin B, fluconazole, 5-FC, itraconazole, ketoconazole) dispensed on individual wells of a microdilution disposable tray. Besides different concentrations of drugs, each individual wells contain a colour indicator, Alamar blue dye (AccuMed International), which generates a visually detectable colour change pending on the inhibition or proliferation of fungal cells. This colorimetric system triggers a redox reaction with subsequent change from blue to red or purple in the wells where inoculum growth is under course [62].

The pioneer study comparing the Sensititre YeastOne colorimetric system with MIC results generated by the CLSI broth microdilution method was conducted by Pfaller et al. [63], initially testing only yeasts. These authors assessed the susceptibility results of 300 clinical isolates of *Candida* spp. against that were tested against five azoles. Concordance of results generated by both methods evaluated topped 90% for all drugs tested, showing the great potential value of the Sensititre YeastOne colorimetric system for use in the clinical laboratory for the antifungal susceptibility testing.

The first evaluation of the Sensititre YeastOne colorimetric method for susceptibility testing with moulds was developed by Castro et al. [64]. In their study, the authors evaluated the susceptibility profile of 63 samples of *Aspergillus* spp. tested against voriconazole, on a comparative basis, by the colorimetric system and the CLSI reference test [14]. Concordance of MIC results generated by both methods was 83% for assays with *Aspergillus* spp., ranging from 62 to 100% of results related to other agents tested.

Linares et al. [65] have also compared the Sensititre YeastOne colorimetric system with the microdilution broth proposed by the CLSI (M38-A document) [14] by testing 244 filamentous fungal isolates, including 223 species of the genus *Aspergillus*. Although the panel of Sensititre YeastOne includes 6 different antifungal drugs (amphotericin B, fluconazole, 5-FC, itraconazole, ketoconazole, voriconazole), only voriconazole MICs were evaluated by both methods. Isolates of

Aspergillus selected for this study exhibited minimum inhibitory concentration values for 50% of the isolates tested (MIC_{50}) of ≤ 1 μg/ml, indicating the good in vitro activity of voriconazole against this pathogen. Concordance of results generated by both methods ranged from 97 to 99% of the results.

Considering the in vitro data generated by comparative studies of Sensititre YeastOne system with a CLSI reference method by testing filamentous fungi as a whole, we may suggest that the broth microdilution colorimetric system presents good potential as an alternative to evaluate the susceptibility of *Aspergillus* strains to voriconazole.

6 Other Methods

In recent years, different strategies for monitoring the growth and/or inhibition of microorganisms for conducting in vitro antimicrobial susceptibility testing have been adapted to assays with filamentous fungi. The purpose of incorporating into antifungal susceptibility tests new approaches for monitoring of microorganism growth is basically three: (i) rendering clearer the definition of "end point" readings making easier the processes and increasing the reproducibility of results; (ii) contributing to the development of systems with great potential for automated reading; and (iii) reducing the assay incubation time, to provide to the clinician early test results. The costs related to most of these assays is still very high to be incorporated into routine laboratories, but they have great potential to be used in reference laboratories, or they can even represent a further step toward the formatting of new technologies. In the near future, some of these methods should evolve into less-complex systems, easy to use, and offering better cost-benefit relations. Promising results have been obtained by the following techniques:

6.1 Evaluation of Fungal Biomass Through Metabolic Activity Measurement

Biomass of fungal elements exposed to different concentrations of antifungal drugs may be determined by colorimetric methods using colour indicator which are reduced by microorganisms under increasing metabolic activity [66, 67].

Salt 2,3-Bis-{2-methoxy-4-nitro-5-[(sulfenylamino) carnonyl] 2H-tetrazolium-hydroxide}, known as XTT, is a yellow-coloured tetrazolium salt which is converted by mitochondrial dehydrogenases of viable fungi to a orange formazan product whose colour is orange and which is soluble in water [68, 69]. The basic premise of using this colour indicator in antifungal susceptibility assays is that the increase of any organism's biomass is preceded by a substantial increase of their metabolic activity. Consequently, any change in the total biomass of fungal elements can be measured by means of the reduction of XTT to formazan looking for colour change from yellow to orange, easily detected by reading the optical density of assays with the help of a spectrophotometer.

Antachopoulos et al. [70] developed an assay to evaluate the susceptibility of 39 *Aspergillus* spp. strains tested against two antifungal agents (amphotericin B and voriconazole), by measuring the metabolic activity (through XTT) of strains exposed to different concentrations of antifungal drugs. The results obtained by assays with XTT were compared with amphotericin B and voriconazole MIC values rendered by the CLSI microdilution broth standard method (Document M38-A) [14]. The authors used a high-inoculum biomass to early acquire signals from assays with XTT. The study showed the feasibility of reading tests performed with *A. fumigatus* and *A. flavus* after incubation periods of 6–8 h for assays with inoculum of 10^6 conidia of these agents, and 12 h of incubation for trials with inoculum of *A. terreus* strains. The study also demonstrated that assays with the assessment of biomass by metabolic activity generated MIC values with excellent reproducibility (97–100%) and good correlation with results generated by the CLSI reference method (91–100%).

6.2 Evaluation of Fungal Biomass Through the Measurement of Oxygen Consumption

Araujo et al. [71] have developed a methodology for measuring modifications of biomass of fungal elements secondary to its exposition to different concentrations of antifungal drugs by detecting modifications on the oxygen consumption rates by colonies present in the assays. The principle of such methodology is the assumption that the total fungal biomass size is closely related to the oxygen consumption rate. The antifungal susceptibility assays were prepared in test tubes (broth macrodilution) containing 1.5 ml of a conidia inoculum suspension in RPMI medium, exposed to two different concentrations of antifungal drug (0.25 and 2.0 mg/l), that were incubated at 37°C for 12 h. The assays were read with the aid of a biological oxygen consumption monitor (YSI model 5300, YSI Inc., Yellow Springs, Ohio, USA) as suggested by the manufacturer. In this study, a total of 20 clinical isolates of *Aspergillus fumigatus* were tested against amphotericin B, itraconazole, voriconazole and posaconazole by two different methods: CLSI broth microdilution test and the new broth macrodilution method with oxygen consumption rates monitored by the mentioned automated system. The new macrobroth method exhibited good reproducibility of MIC values (87% of results) and satisfactory agreement (85% of MICs) with results generated by the reference test.

6.3 Employment of the Biocell-Tracer® System in Susceptibility Tests with Aspergillus spp.

The Biocell-Tracer® automated system (Hidan Co., Ltd, Chiban, Japan) also comes in handy as a new tool for recording the dynamics of formation, growth and development of hyphae, pseudo-hyphae and germination of conidia linked or not to the exposure to antifungal agents. The BCT® system monitors hyphal growth

continuously, and it is expected that when an antifungal agent is added, the hyphae growth curve will be changed if the fungus is sensitive to the exposed antifungal agent. This system is comprised by a base for storage of plates containing the fungal inoculum, microscope, image scanner and a computer with software that allows the automatic monitoring of the growth of the hyphal extremities.

This microfilming and recording system of hyphal formation and growth dynamics has been successfully applied to the study of antifungal susceptibility of *Aspergillus* spp. to antifungal agents in only two published papers [72, 73]. This system's features are incompatible with the needs of a routine laboratory but represent a tool with good potential in the investigation of biological activity of new drugs, or even in the characterisation of potential synergy or antagonism of combinations between antifungal drugs.

6.4 Employment of the Flow Cytometry System in Susceptibility Tests with Aspergillus *spp.*

Flow cytometry has also been used to monitor the development of fungal structures, physiological changes and to analyze the viability of conidia. The advantage of flow cytometry is the possibility of analyzing a large amount of cells quickly and objectively, with results rendered by fluorescence. The choice of fluorochromes for flow cytometry assays is one of the major steps in this process since such compounds may increase their fluorescence after binding to specific cell compounds (proteins, lipids, etc.), or after crossing through damaged cell membranes and binding to nucleic acids, or even depending on physiological parameters such as changes in pH, membrane potential, enzyme activity [74].

Specifically for conducting antifungal susceptibility tests, the choice of propidium iodide (PI) fluorochromes is an interesting option because this compound's fluorescence level increases when it passes through damaged cell membranes and binds to nucleic acids, and they are PI positive cells indicating cell dysfunction and/or death.

Ramani et al. [75] have applied successfully flow cytometry with PI fluorochromes to assess the susceptibility of fourteen strains *Aspergillus fumigatus* against three antifungal drugs: amphotericin B, itraconazole and voriconazole. The results obtained were compared with MICs generated by the CLSI microdilution broth method. The overall concordance of results generated by both methods was 93%. The flow cytometry-based method enabled reading assays within 4 h, with results exhibiting good correlation with the reference method (93% of results with the 3 drugs).

6.5 Real Time PCR

Garcia-Effron et al. [76] recently published the results of a comprehensive study in which a multiplex real time polymerase chain reaction (PCR) assay using molecular beacons was used to detect resistance to triazoles in *Aspergillus* isolates. The

study targeted specific mutations known to affect the cyp51A gene. The assay was designed in a two-tier format. The first tier consisted of a probe panel that distinguished triazole-susceptible from triazole-resistant isolates. The second tier panel provided possible therapeutic options depending on the mutation detected. The assay was shown to be rapid (3–4 h turnaround time) and highly efficient in the detection of triazoles resistance associated with cyp51A mutations. Since new mutations are likely to be detected in the clinical practice, frequent updated might be needed. This approach is also limited by costs. Moreover, it does not detect triazoles resistance not related to cyp51A mutations.

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Antifungal Therapeutic Drug Monitoring for Invasive Aspergillosis

Susan J. Howard and William W. Hope

Abstract Invasive aspergillosis is a leading cause of morbidity and mortality in immunocompromised patients. Therapeutic drug monitoring (TDM) is a strategy to optimise the outcome for a wide range of drug-disease combinations. Accumulating evidence suggests that TDM of the triazoles is required to optimise outcome of patients with invasive aspergillosis. TMD is also required for treatment with 5-flucytosine. Currently, TDM is not routinely used for the polyenes or the echinocandins. This chapter describes the indications and approach to TDM for patients receiving antifungal therapy for invasive aspergillosis.

Keywords Antifungal · Pharmacokinetics · Therapeutic drug monitoring · Triazole

Contents

Susan J. Howard (✉)
The University of Manchester, Manchester Academic Health Science Centre, Manchester, UK
e-mail: susan.j.howard@manchester.ac.uk

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1 Introduction

Invasive aspergillosis is a significant public health problem. The overall mortality is approximately 50%, and this syndrome represents a major obstacle to the achievement of optimal clinical outcomes for immunocompromised patients [1]. The three main antifungal classes that are currently used for treatment are the triazoles, polyenes and echinocandins. The goal of therapeutic drug monitoring (TDM) is to maximise the probability of successful outcome, while minimising the probability of adverse events.

The optimal use of available antifungal compounds is especially important for a syndrome for which there are relatively few therapeutic options. TDM is one aspect of optimal anti-infective therapy. TDM is not routinely performed for the echinocandins and the polyenes because of their complicated pharmacology and a limited understanding of exposure-response relationships. In contrast however, increasing data suggests that TDM for the triazoles is required for most members of this class of agents.

The dominant reasons for performing TDM for any compound are as follows: (i) established exposure-effect relationships; (ii) established exposure-toxicity relationships; (iii) significant inter-individual pharmacological variability leading to a large proportion of a population at risk of either subtherapeutic or toxic drug exposures. The most commonly used measure of drug exposure is the trough concentration. This has the advantage of relative simplicity, but it is a somewhat crude measure of drug exposure that may not provide robust estimates of total drug exposure in individual patients. Average concentrations have also been used in drug development programs for voriconazole and posaconazole and are also a rather cumbersome measure of drug exposure.

2 Itraconazole

2.1 Pharmacology and Pharmacokinetics

Itraconazole is a highly lipophilic drug that is poorly soluble at physiological pH. Itraconazole is available in both a capsule formulation and as an oral and IV solution in which the active moiety is solubilised using a hydroxypropyl-B-cyclodextrin excipient. Itraconazole exhibits Michaelis-Menten pharmacokinetics (PK) (i.e. nonlinear PK) [2]. In early studies conducted in healthy volunteers, steady state was not achieved until day 15, which is much longer than predicted from the terminal

half-life (~24 h). The use of the intravenous (IV) formulation enables patients to achieve therapeutic concentrations in the initial 24–48 h [3, 4]. Higher weight-relative based dosages are required in smaller children because of disproportionably higher rates of clearance.

The major limitation of capsules is relatively poor oral bioavailability [5, 6]. Solubility (and hence absorption) is improved in an acidic environment. Consequently, itraconazole capsules should be administered with food or an acidic drink [5, 7]. Histamine antagonists and proton pump inhibitors decrease absorption via their effect on gastric pH. Hypochlorohydria (or achlorhydria) is frequently present in critically ill patients, and may result in incomplete absorption and subtherapeutic levels. Itraconazole solution has higher oral bioavailability, and leads to higher systemic exposure than capsules [8]. The incidence of gastrointestinal intolerance is higher because of the osmotic effect of the cyclodextrin excipient. This is frequently an obstacle to the long term usage of this formulation.

Itraconazole undergoes oxidative metabolism via the CYP P450 enzyme 3A4. This process produces an active metabolite, hydroxy-itraconazole that has antifungal activity comparable to the parent [9]. Consequently, antifungal activity is greater than predicted from concentrations of itraconazole alone. Itraconazole has a number of important drug interactions [10, 11]. Compounds such as rifampicin, phenytoin and carbamezepine induce levels of CYP P450 that results in accelerated clearance of itraconazole and potentially subtherapeutic levels [12].

Both formulations of itraconazole are associated with significant inter-individual variability. Some of this variability can be understood in the terms of erratic oral bioavailability. Importantly, however, even when this is considered, there is still a significant proportion of residual (or unexplained) variance. Pharmacokinetic variability (from whatever cause) has an impact upon the achievement of effective and toxic serum levels in a suitably high proportion of the population. This is one of the reasons that TDM is required for the optimal use of itraconazole.

2.2 Therapeutic Targets Associated with Success

Several studies have sought to identify serum trough levels of itraconazole that are associated with a high probability of successful outcome. An early study suggested that a trough level of 0.25 mg/l (measured by high performance liquid chromatography, HPLC) in patients with neutropenia prevented invasive fungal infections [13]. A subsequent study suggested that a trough level of 0.5 mg/l (determined by HPLC) is required, and this target is now widely used for TDM [14]. This level equates to approximately 5 mg/l when measured using bioassay, since bioassay also detects hydroxy-itraconazole [15, 16]. Importantly, however, there are a number of caveats for the use of these therapeutic targets: (i) this target level was determined in the setting of prophylaxis, rather than the treatment of established disease; therapeutic levels for the latter are not definitively known, but may well be higher; (ii) this level was determined using both *Candida* and *Aspergillus* infections as the endpoint; the therapeutic level is likely to vary from pathogen to pathogen; (iii) effective trough levels for other fungal pathogens, such as agents causing endemic mycoses,

are completely unknown; (iv) effective trough levels to prevent the emergence of itraconazole resistance in chronic forms of invasive aspergillosis are not known; (v) the applicability of this level for the broad range of entities that comprise the syndrome of invasive aspergillosis is not known.

2.3 Therapeutic Targets Associated with Toxicity

Itraconazole is associated with a number of adverse events including fluid retention, hepatotoxicity, electrolyte disturbance, confusion and gastrointestinal tolerance [5]. The toxicodynamics of itraconazole are not well characterised. Anecdotally, at least, some adverse events appear to be related to high itraconazole levels, although further study is required to define the relationship between drug exposure and probability of toxicity.

2.4 Measurement of Itraconazole

Itraconazole is most commonly measured by HPLC or bioassay. The results are discordant because the bioassay detects both the parent compound and active metabolite; consequently levels of itraconazole appear higher [9, 17, 18]. A number of seed organisms can be used in the assay – the original bioassay used *Candida kefyr*. The limit of detection for bioassays ranges from 0.16 to 0.63 mg/l [19]. Plasma levels above the working range of the assay require dilution and reanalysis. The bioassay can be performed in routine clinical microbiology laboratories because of its relative simplicity. The principal disadvantage is accurate determination of itraconazole concentrations in the context of combination antifungal therapy (although seed organisms that are resistant to the second drug are available for most combinations). The limit of detection for HPLC is 0.005 mg/l [19, 20]. The primary disadvantage of HPLC is the relatively high equipment and operational costs.

2.5 Indications for TDM

All patients receiving itraconazole should have TDM performed. A level should be measured in the first 5–7 days of therapy [21], and repeat levels taken to ensure that effective and non-toxic levels are being maintained. The time to steady state is 2 weeks, meaning that continuous sampling and interpretation of levels is required to ensure that the level can be confidently interpreted. If levels are low, then factors affecting poor absorption or possible drug interactions should be sought and rectified. If levels are persistently low, then an increase in capsule dosage may be considered or capsules changed to suspension. The appropriate management of a patient with high levels is more difficult in the absence of a better understanding of the toxicodynamics of itraconazole. If a patient has signs or symptoms of toxicity,

then the drug should be temporarily stopped or the dosage reduced. TDM can be used to ensure that dosage reduction does not result in levels that become inadvertently subtherapeutic [22].

3 Voriconazole

Voriconazole is a structural congener of fluconazole which was specifically engineered for anti-*Aspergillus* activity. Voriconazole is a first-line agent for the treatment of invasive aspergillosis [23, 24]. Increasing data suggest that voriconazole should be monitored to ensure effective and non-toxic concentrations, although considerable uncertainty remains regarding a concentration range which is safe and effective. The reasons for monitoring voriconazole levels are: (1) clinically relevant exposure-effect relationships; (2) clinically relevant exposure-toxicity relationships; and (3) marked inter-individual variability following fixed dosing.

Voriconazole is available as an oral and IV formulation. Oral bioavailability is approximately 96% in normal volunteers, which facilitates the use of voriconazole as consolidation therapy in ambulatory settings. The IV preparation contains a cyclodextrin excipient to achieve solubilisation. In adults, voriconazole exhibits classical Michaelis-Menten PK, which manifests as disproportionate increases in drug exposure following dosage escalation. The pharmacokinetics in children are pseudolinear, and much higher weight-based dosages are required to achieve comparable exposures to those observed in adults [25, 26]. Voriconazole levels vary as much as 100-fold in populations receiving fixed dosages [25]. Voriconazole is a substrate for the CYP P450 enzymes 2C9, 2C19 and 3A4. Polymorphisms within CYP 2C19 account for a relatively large portion of inter-individual variance. Patients with poor metaboliser phenotype have levels 4–5 times higher than extensive metabolisers. The incidence of the poor metaboliser phenotype varies according to the ethnicity of the population; the incidence is 3–5% in Caucasians, but 15–20% in individuals from South East Asia. Patients with an ultra-rapid metaboliser genotype (CYP2C19*17) have lower voriconazole levels [26]. The prevalence of this genotype is 4% in Chinese people, but as high as 20% in Swedes [26]. This may partly account for the findings in a recent study in which 15% of patients receiving standard dosages had undetectable serum levels [27].

3.1 Therapeutic Targets Associated with Success

There have been extensive efforts to identify the minimum voriconazole concentration associated with a high probability of a successful therapeutic outcome. This is difficult because of the multitude of additional factors that have an impact upon therapeutic response in clinical settings (e.g. underlying disease, neutrophil recovery). An increasing number of studies suggest voriconazole exhibits clinically relevant concentration-effect relationships. Patients with invasive aspergillosis who have random voriconazole levels <0.25 μg/ml have a higher probability to fail treatment

[28]. In a more recent study, a positive clinical response was observed in 100% (10/10) of patients with random voriconazole concentrations of >2.05 μg/ml, whilst disease progressed (and patients died) in 8/18 patients with concentrations below that level [28]. Pascual et al. [29] reported that trough concentrations of 1 mg/l are associated with a 70% probability of a successful outcome. Higher troughs only result in a marginal increase in the probability of a response. Importantly, however, levels <1 mg/l were associated with a precipitous decline in the probability of a successful outcome [29]. Until further data are obtained, a trough concentration of ≥1 mg/l is a reasonable therapeutic target.

3.2 Therapeutic Targets Associated with Toxicity

The relationship between trough concentration and toxicity is relatively well defined. There is a association between mean levels of voriconazole and the probability of elevated alkaline phosphatase, aspartate aminotransferase and bilirubins, but not alanine aminotransferase (ALT) [30]. As the average concentration increases, there is modest increase in the probability of enzyme elevation. Furthermore, there is a link between voriconazole levels and the probability of photopsia [30], although this is less important than other toxicities, because cessation of therapy is rarely required. More recently, high voriconazole levels have been associated with central nervous system toxicity, which manifests as confusion and hallucinations. There are a number of case reports of other adverse events, such as hypoglycaemia and pulmonary toxicity occurring in the context of very high voriconazole levels, although cause-effect relationships have been difficult to establish [31].

3.3 Measurement of Voriconazole

Voriconazole can be measured using bioassay, HPLC and tandem mass spectrometry (HPLC-MS/MS). The limit of detection of these assays is ~0.1 [32, 33], ~0.03 [32–34] and ~0.01 mg/l [32], respectively.

3.4 Indications for TDM

There are probably adequate grounds to consider TDM in the majority of patients receiving voriconazole, although this is somewhat contentious. Since invasive aspergillosis is a rapidly progressive and frequently fatal infection, ensuring drug exposures that maximise the probability of a clinical response is an important component of routine care. A significant proportion of patients may have undetectable levels. This is a clear reason to at least check one level in each patient. TDM should certainly be considered for patients receiving oral therapy without intravenous loading, and for patients failing therapy or those with poor prognostic disease (e.g. central nervous system aspergillosis). TDM is indicated in particular populations where there may be uncertainty regarding the pharmacokinetics (e.g. morbidly obese patients, hepatic impairment). In certain circumstances drug interactions may

be unavoidable, and TDM may be required to ensure voriconazole levels are both therapeutic and non-toxic.

The use of TDM to prevent toxicity is not well established, because toxicities (including deranged liver function, photopsia, and central nervous system toxicity) are clinically obvious or can be readily identified with routine biochemical testing. Furthermore, toxicity is usually not life-threatening, and resolves with the cessation of drug or with dose reduction. Some clinicians choose to increase the dosage of voriconazole until an adverse event is encountered, then reduce the dose from this point.

The optimal timing for TDM is not known and difficult to predict in individual patients. Since voriconazole exhibits non-linear PK, the time to steady state is variable. Determination of the first level in the first 5–7 days of therapy is probably reasonable. More than one level is required to ensure patients are at steady state; this enables confident clinical interpretation of the drug level. If levels are low, the dosage can be increased, and repeat levels taken to ensure that the desired target has been achieved. The current license enables an increase from 200 to 300 mg bid, and TDM provides a way in which this can be achieved safely and efficiently. If TDM is not available, some clinicians may prefer to start with a higher voriconazole dose (i.e., 300 mg twice daily PO instead of 200 mg twice daily, as this will results in better drug exposure). Such an attempt, however, may also result in more toxic effects.

If levels are high, voriconazole can be temporarily stopped or the dosage reduced. The co-administration of omeprazole is associated with higher voriconazole levels; it may be appropriate to temporarily stop the former if this is possible. Assiduous monitoring of voriconazole levels is then required to ensure that levels do not become inadvertently subtherapeutic.

4 Posaconazole

4.1 Pharmacology and Pharmacokinetics

Posaconazole is structurally related to itraconazole and can be used for prophylaxis and for salvage therapy in patients with invasive aspergillosis who are intolerant or failing other agents [35–37]. Posaconazole is only available as an oral formulation – an IV formulation is being developed.

Posaconazole is a substrate for P-glycoprotein. The absorption is not acid-dependant, but increases with co-administration with food and especially fatty food [38]. Absorption is saturable and systemic drug exposure does not appear to increase for dosages >800 mg/day [39]. Systemic exposure is higher with fractionated regimens (e.g. 200 mg 6 hourly compared with 800 mg daily) [40]. Posaconazole has fewer CYP interactions than either itraconazole and voriconazole. The pharmacokinetics are linear. The half-life is approximately 24 h and the time to steady state is 1 week. The extent of inter-individual variability is not as well defined as is the case for the other triazoles. There are no covariates that can be used to predict drug exposures for individual patients receiving fixed dosages.

4.2 Measurement of Posaconazole

Posaconazole concentrations can be determined using bioassay, HPLC and tandem mass spectrometry. The limits of detection are 0.125 [41], 0.05 [42] and 0.005 mg/l [43] respectively.

4.3 Therapeutic Targets Associated with Therapeutic Success

There appears to be a relationship between average posaconazole concentrations and the probability of therapeutic success in the setting of both prophylaxis and salvage therapy for invasive aspergillosis. In the setting of prophylaxis, an average (Cav) concentration of >0.71 mg/l is required for a 75% probability of successful outcome (the latter being defined as a composite endpoint consisting of proven/probable invasive fungal infections, receipt of more than 5 days of empiric therapy with another systemic antifungal agent, discontinuation of study drug or patients lost to follow-up) [44]. After considering estimated rates of drug accumulation, this figure corresponds to a level of 0.35 mg/l 3–5 h post-dose on day 2 [44]. A similar relationship is seen with the use of posaconazole for salvage therapy for invasive aspergillosis [37]. An average concentration of 1.25 mg/l is associated with a 75% response rate, suggesting that a higher drug exposure is required to treat patients with established disease.

4.4 Therapeutic Targets Associated with Toxicity

Posaconazole may cause gastrointestinal intolerance, hypokalaemia, rash and elevations in liver enzymes. There does not appear to be a relationship between average posaconazole concentrations and adverse events [44].

4.5 Indications for TDM

The indications for routine TDM of posaconazole are less clearly defined than for the other triazoles at present. TDM should be considered in patients with poor prognostic disease, progressive disease, in patients in whom absorption may be compromised and in special populations (e.g. paediatric and obese patients). Patients with low levels should be questioned about compliance. TDM is generally not considered for patients receiving posaconazole for prophylaxis, although this may well be a reasonable strategy to ensure optimal therapy.

For patients with a potentially subtherapeutic level, an increase in dosage may be considered, but the benefits of increasing the dose to >800 mg/day are not clear, since absorption appears saturable. Fractionating the total dosage may improve absorption, although this may have a detrimental impact upon compliance in ambulatory settings. Posaconazole should be administered with food or nutritional supplements. Critically ill patients are frequently intolerant of fatty foods, but full-fat ice-cream may be palatable and a useful way to improve absorption.

5 5-Flucytosine

Another antifungal drug for which TDM is recommended is 5-flucytosine (5-FC). Although this agent is not usually not used for the treatment of invasive aspergillosis (more detail in the chapter by Drs. Mouton and Verweij), 5-FC may be useful in combination therapy, particularly in situations where tissue penetration of the other agent is poor. Liver and haematological toxicity can be minimised with careful maintenance of 5-FC levels <100 mg/l, [45]. In one study, persistence of 5-FC levels above 100 mg/l for more than 2 weeks increased the risk for haematological or hepatic toxicity [46]. On the other hand, low 5-FC levels (particularly if <25 mg/l) may increase risk for secondary resistance [48]. Studies performed in reference laboratories in the United Kingdom have shown that only 17–21% of 5-FC levels are within the expected therapeutic range [45, 48]. Patients receiving 5-FC in combination with deoxycholate amphotericin B are at higher risk for high 5-FC levels, if renal failure occurs [46].

6 Conclusions

TDM is an increasingly important component of care of patients with a wide range of drugs and diseases. TDM is particularly necessary to optimise the use of the triazoles, since these agents display clinically relevant exposure-response relationships and considerable pharmacokinetic variability. More work is required to define the precise therapeutic targets, achieve those targets in individual patients and explore the cost-benefits of monitoring drug levels.

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Part IB
Antifungal Drug Profiles

Azoles

George R. Thompson III and Thomas F. Patterson

Abstract The treatment of invasive aspergillosis remains difficult and until recently only polyenes and itraconazole were viable treatment options. However the development of newer triazoles (voriconazole, posaconazole) has been a welcome addition to the antifungal armamentarium. The proven efficacy of prophylactic posaconazole in high-risk patients, and the use of voriconazole during treatment of invasive aspergillosis have largely replaced amphotericin B and itraconazole use for these indications. Other extended-spectrum triazoles in preclinical development (ravuconazole, isavuconazole, and albaconazole) offer additional pharmacodynamic/pharmacokinetic advantages and will also be reviewed.

Keywords Albaconazole · Isavuconazole · Itraconazole · Posaconazole · Triazoles · Voriconazole · Ravuconazole

Contents

G.R. Thompson (✉)
Division of Infectious Diseases, The Department of Internal Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA
e-mail: thompsong2@uthscsa.edu

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1 Introduction

Azoles, characterised by a core 5-member azole ring, consist of two classes: the imidazoles which contain 2 nitrogen atoms within the azole ring, and the triazoles which contain 3 nitrogen atoms within the same ring. The imidazoles, including ketoconazole, were the initial azoles available, however lacking activity against *Aspergillus* species, these agents are limited primarily to topical treatment of superficial and mucosal mycotic infections due to the lack of enzyme specificity and resultant side effect profile. Ketoconazole was followed by the development of the first triazole antifungal, fluconazole, which has no activity against *Aspergillus* species – and itraconazole which has excellent in vitro activity against *Aspergillus* species, but whose clinical use for aspergillosis is limited due to poor bioavailability and side effect profile. More recently, voriconazole and posaconazole were developed specifically to target *Aspergillus* species [1]. These extended spectrum triazoles have become the recommended agents for the treatment and prophylaxis of invasive aspergillosis (IA), respectively [2]. Additional triazole antifungals with activity against *Aspergillus* species remain in pre-clinical development (ravuconazole, isavuconazole, and albaconazole) [3]. This chapter will focus on triazoles that have efficacy against *Aspergillus* spp., their pharmacokinetic and pharmacodynamic principles, reported mechanisms of resistance, adverse effects and drug interactions, and the clinical evidence for their use.

2 Mechanism of Action

The triazoles exert their effects within the fungal cell membrane. The inhibition of cytochrome P450 (CYP)-dependent 14-α-demethylase prevents the conversion of lanosterol to ergosterol. This mechanism results in the accumulation of toxic methylsterols and resultant inhibition of fungal cell growth and replication (Fig. 1). Itraconazole, voriconazole, posaconazole and the newer triazoles (ravuconazole, isavuconazole, and albaconazole) demonstrate fungicidal activity against *Aspergillus* spp. Azoles differ in their affinity for the 14-α-demethylase enzyme and this difference is largely responsible for their varying antifungal potency and spectrum of activity. However cross-inhibition of several human CYP-dependent enzymes is responsible for the majority of the clinical side effects and drug interaction profiles that have also been described with these agents.

This direct antifungal mechanism of triazoles has been well described, but their indirect immunomodulatory effects are poorly understood but appear to substantially contribute to their antifungal activity. These effects are of obvious significance given the underlying immune deficits observed in the majority of patients afflicted with invasive mycoses [4]. The interaction of triazoles and phagocytic cells remains complex. Evidence suggests that ergosterol depletion increases fungal cell vulnerability to phagocytic oxidative damage [5] and voriconazole has been shown to induce the expression of toll-like receptor 2 (TLR2), nuclear factor-kappa-B (NF-κB), and tumour necrosis factor alpha (TNF-α) [4]. Further understanding of

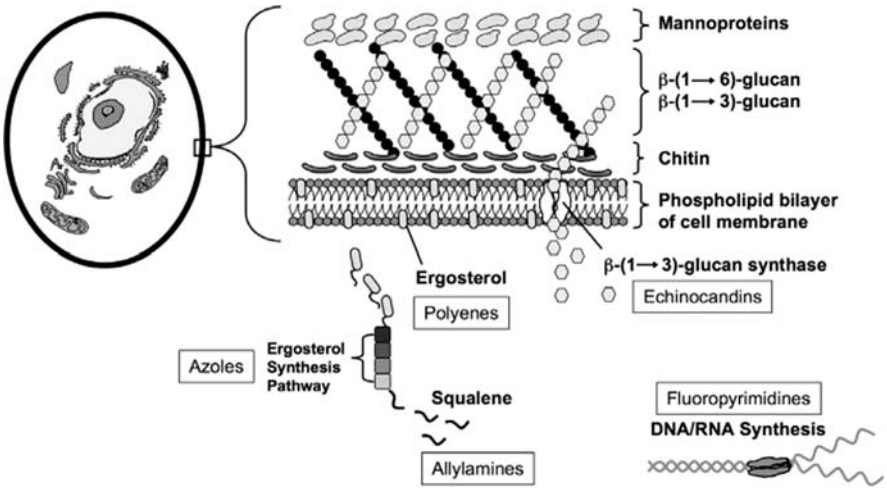

Fig. 1 Main targets of systemic antifungal agents

these processes may result in new drug targets and the potential addition of other effective agents to the antifungal armamentarium.

3 Mechanisms of Resistance

Resistance to triazoles, with the exception of fluconazole, which lacks activity against *Aspergillus* species, is not common among *Aspergillus* species but is well recognised. Previous reports have described the presence of efflux pumps found to convey itraconazole resistance [6]. The most common mechanism of resistance relies upon modification of the 14α-sterol demethylase enzyme encoded by the *cyp51A* and *cyp51B* genes. An amino acid substitution at position M220 invokes resistance to all triazoles, whilst a substitution at position G54 confers only itraconazole and posaconazole resistance, rendering voriconazole still effective [7, 8]. A new mechanism of resistance has recently been discovered, however both a tandem repeat within the *cyp51A* promoter and an L98H substitution within the *cyp51A* gene are both required for triazole resistance to develop [9]. Although felt to be uncommon, exact rates of azole resistance in *Aspergillus* species are not well established. A recent survey detected voriconazole or posaconazole minimum inhibitory concentration (MIC) of >2 μg/ml in <1% of 771 clinical *Aspergillus* spp. isolates [10], yet others have reported a rising incidence of resistance and continued surveillance with the increased use of new-generation azoles seems prudent [11]. A recent survey from a reference mycology laboratory in the United Kingdom reported that amongst 519 prospectively collected *A. fumigatus* isolates 7% showed resistance to azoles [12]. The authors reported that the frequency of azole resistance in *Aspergillus* isolates markedly increased since the year 2004 in their centre.

4 *Aspergillus*-Active Azoles

4.1 Itraconazole

Itraconazole is a high molecular weight, highly hydrophobic and water-insoluble triazole. Although structurally similar to ketoconazole, itraconazole exhibits a broader range of antifungal activity, including activity against most *Aspergillus* spp.

4.1.1 Pharmacodynamics

Itraconazole has demonstrated both species- and strain-dependent fungistatic or fungicidal activity in vitro. Against many *Aspergillus* spp. itraconazole is fungicidal, and its activity is both time- and concentration-dependent [13]. Within 24 h of drug exposure inoculum concentrations are reduced up to 97%. However which of these pharmacodynamic parameters best predicts antifungal efficacy remains to be determined [14].

4.1.2 Pharmacokinetics

Itraconazole is currently available as both capsules and an oral solution suspended in hydroxypropyl-β-cyclodextrin (HPCD). Unfortunately the intravenous (IV) preparation of itraconazole is no longer commercially available.

Itraconazole capsules are dependent upon an acidic environment for maximal absorption, and the concomitant administration of H_2-receptor antagonists, proton pump inhibitors, or antacids causes erratic and unpredictable drug absorption. Subtherapeutic serum drug concentrations have also been reported in HIV-infected, neutropenic cancer, and bone marrow transplant patients [15, 16], as well as in patients taking generic itraconazole formulations [17]. It is thus recommended that itraconazole capsules be taken with food or a cola beverage for better absorption [18, 19].

Itraconazole solution confers enhanced oral bioavailability in comparison to the capsule formulation. The area under the curve (AUC) and peak concentrations are both increased by 30% when itraconazole solution is taken in the fasting state [20, 21]. The cyclodextrin carrier has minimal absorption and no systemic side effects have been attributed to its use in the oral formulation. The osmotic effects of the carrier however may cause gastrointestinal irritation and/or diarrhoea [22].

Peak itraconazole serum levels are observed within 1–4 h after administration. With once daily dosing, steady state is reached in 7–14 days although oral loading (200 mg three times daily for 3 days) allows for more rapid attainment of therapeutic serum levels [23].

Regardless of formulation administered, itraconazole exhibits dose-dependent pharmacokinetics. Saturable metabolic processes are suggested by the hyperproportional increases in AUC with escalating drug dose [24, 25]. Itraconazole is also highly protein bound with <1% available as free drug and has a relatively high volume of distribution accumulating preferentially in skin, fat, liver, bone marrow, kidney and lungs (Table 1). Of note, itraconazole has very poor central nervous system

Table 1 Comparative pharmacokinetics of the triazoles: itraconazole, voriconazole and posaconazole [1]

	Antifungal agent		
	Itraconazole[a]	Voriconazole	Posaconazole
Pharmacokinetic parameter			
Oral bioavailability, %	50	96	No data
Food effect	Empty stomach	Empty stomach	Food
Distribution			
Total C_{max} μg/ml	11	4.6	7.8
AUC, mg x h/l	29.2	20.3	8.9
Protein binding, %	99.8	58	99
CSF penetration, %	<10	60	No data
Vitreal penetration, %	10[b]	38[b]	26[b,c]
Urine penetration, %[d]	1–10	<2	<2
Metabolism	Hepatic	Hepatic	Hepatic
Elimination	Hepatic	Renal	Faeces
Half-life, h	24	6	25

Legend: AUC, area under the concentration curve; C_{max} peak drug concentration; CSF, cerebrospinal fluid.
[a]Data are for oral solution
[b]Human data
[c]Animal data
[d]Percentage for active drug or metabolites

(CNS) penetration and low levels within brain tissue have been reported [26]. The discordance between brain itraconazole levels to those present in other tissue has been attributed to active efflux of itraconazole from the brain by para-glycoprotein (P-glycoprotein) [27].

Itraconazole is extensively metabolised by the liver and the inactive metabolites are excreted predominately in the bile and urine [25]. Itraconazole's major metabolite, hydroxy-itraconazole, does possesses antifungal activity similar to that of the parent drug. After reaching steady state, the plasma concentrations of hydroxyl-itraconazole are 1.5–2 times higher than those of itraconazole [23]. Despite similar antifungal efficacy, hydroxyl-itraconazole is not measured during serum drug level determination by high performance liquid chromatography (HPLC) although the active metabolite is detected by bioassay [28].

4.1.3 Dosing

The recommended dosage of oral itraconazole in adults is 400 mg/day (capsules) and 2.5 mg/kg twice daily (HPCD solution) [2]. In paediatric patients over 5 years of age, itraconazole HPCD solution has been recommended at 2.5 mg/kg twice daily [29]. Considerable concern remains regarding adequate oral absorption (see Section 4.1.2) and oral itraconazole is not recommended in seriously ill or patients with life-threatening disease, and if used during the treatment of IA loading with 600 mg/day

for 3 days, followed by maintenance dosing of 400 mg/day with serum drug monitoring is recommended [2]. Higher doses are associated with significant toxicity.

Dose adjustment is not indicated when the oral formulations of itraconazole is used in patients with renal insufficiency or those receiving haemodialysis/continuous ambulatory peritoneal dialysis (CAPD). The cyclodextrin component of IV itraconazole has caused concerns about vehicle accumulation in renal insufficiency or dialysis dependence, but the lack of availability of the IV formulation has negated this concern. The half-life of itraconazole is prolonged in patients with hepatic dysfunction and drug dose adjustment, liver function testing, and drug interactions need to be carefully assessed [30].

Itraconazole has been found embryotoxic and teratogenic in rodents, and is thus contraindicated during pregnancy unless no alternative therapeutic agent exists [26]. Itraconazole is also excreted into breast milk and other therapeutic options should be considered before exposing the infant to the potential, but largely unknown, risks [31].

4.1.4 Therapeutic Drug Monitoring

Itraconazole drug levels should be routinely monitored in patients receiving itraconazole for both prophylactic and therapeutic purposes. This topic is discussed in more detail elsewhere in this book.

4.1.5 Adverse Effects Profile

Itraconazole is usually well tolerated and is infrequently discontinued due to drug toxicity or intolerability (Table 3). One study administered itraconazole at 50–400 mg/day to 189 patients with a variety of systemic mycoses for a median of 5 months. Adverse reactions were observed in 39% of patients; however no fatalities and only rare toxicity requiring discontinuation of therapy were reported. The most frequent side effects included: nausea and vomiting (<10%), hypertriglyceridemia (9%), hypokalemia (6%), liver enzyme elevations (5%), skin rashes/pruritis (2%), headache and dizziness (<2%), and pedal oedema (1%) [32]. A higher frequency of side effects might however occur for patients receiving itraconazole for longer periods as in the treatment of chronic cavitary pulmonary aspergillosis.

Concerns for the potential disruption of mammalian steroidogenesis were evaluated in several studies and no effects were observed at doses up to 400 mg per day. However hypertension and hypokalemia (4 of 8 patients), similar to that seen in Conn's syndrome, and adrenal insufficiency (1 of 8 patients) were encountered when patients received 600 mg per day during treatment of refractory invasive mycoses suggesting this dose as the upper limit of tolerability [33].

Gastrointestinal intolerance (46%) is exceedingly common with the oral HPCD solution at doses >400 mg per day [34]. This formulation was evaluated in a paediatric cohort of patients at a dose of 5 mg/kg/day with vomiting the most frequent complaint (12%), followed by liver enzyme elevation (5%), and abdominal pain (3%). Therapy was discontinued in 18% of patients due to adverse events [35].

Hepatic injury and hepatitis are of concern given the hepatic metabolism of itraconazole, but appear infrequent with few cases reported [36]. The myocardial depressant effects of itraconazole are also well known and cases of congestive heart failure have been reported [37]. Cardiac negative inotropic effects may be a particular problem for patients on concomitant treatment with calcium channel blockers.

4.1.6 Drug Interactions

The majority of drug–drug interactions are mediated by itraconazole's effects on the cytochrome P450 system (Table 2). Through non-competitive inhibition of the heme moiety within CYP3A4 itraconazole effectively decreases the oxidative metabolism of many CYP3A4 substrates leading to accumulation of medications that require this pathway for metabolism (Table 4). The HMG-CoA reductase inhibitors, simvastatin, atorvastatin, and lovastatin, all have markedly increased serum levels when co-administered with itraconazole and accompanying rhabdomyolysis has been reported [38–41]. The statins not metabolised through CYP3A4, fluvastatin and pravastatin appear safe, however caution even with these agents is indicated. The benzodiazepines, alprazolam, midazolam, triazolam, also may reach toxic levels when co-administered with itraconazole, however a single IV bolus, such as given for short-term procedures, seems of low risk [42, 43]. Lorazepam, oxazepam and temazepam are not likely to be affected due to little or no CYP3A4 metabolism [44]. Other agents known to accumulate due to CYP3A4 inhibition when given concurrently with itraconazole include: phenytoin, carbamezepine, cyclosporine, tacrolimus, sirolimus, methylprednisolone, buspirone, alfentanil, the dihydropyridine calcium channel blockers verapamil and diltiazem, the sulfonylureas, rifampin, rifabutin, vincristine, busulphan, docetaxel, trimetraxate and the protease inhibitors ritonavir, indinavir, saquinavir [45–51].

Itraconazole has also been associated with QTc prolongation [31] and co-administration with other agents known to have similar effects (cisapride, terfenadine, astemizole, mizoloastine, dofetilide, quinidine, and pimozide amongst others) should be avoided [52–54].

Table 2 Summary of azole-mediated cytochrome P450 drug–drug interactions [1]

	Drug		
Drug mechanism	Itraconazole	Voriconazole	Posaconazole
Inhibitor			
2C19		+++	
2C9	+	++	
3A4	+++	++	+++
Substrate			
2C19		+++	
2C9		+	
3A4		+	

Note: interactions from minimal (+) to extensive (+++). Reproduced with permission from [1].

Several of these agents have effects on CYP3A4 themselves, which may in turn either increase or decrease itraconazole levels. Rifampin, rifabutin, isoniazid, carbamezipine, phenobarbital, and phenytoin are each CYP3A4 inducers and thus decrease plasma levels of itraconazole [47, 55]. Yet itraconazole levels may also be increased when administered with the CYP3A4 inhibitors ritonavir, indinavir, clarithromycin and erythromycin [56].

Careful consideration should be given to the addition of medications with potential interaction with itraconazole and therapeutic drug monitoring is strongly recommended.

4.1.7 Clinical Evidence

Itraconazole was the first triazole agent to demonstrate clinical efficacy in the treatment of aspergillosis. Oral itraconazole was shown in early studies to be as effective as amphotericin B (AMB)-treated historical controls. Although this was not a head-to-head comparison, effective oral therapy for IA was demonstrated for the first time [57]. Subsequently, another non-comparative study demonstrated similar findings. In this study IV itraconazole for 2 weeks followed by oral therapy for 12 weeks revealed similar response rates to historical controls [58]. The use of itraconazole capsules and poor absorption of this formulation likely reduced the efficacy of itraconazole in both of these studies. The current availability of itraconazole solution and availability of therapeutic drug monitoring may allow for more frequent attainment of target serum concentrations, however clinical trials using this formulation are limited and the success of other orally available triazoles (voriconazole) has relegated itraconazole to second-line therapy during the treatment of IA. Itraconazole is thus licensed in the United States of America (USA) only for salvage therapy of IA [2].

Itraconazole has also been evaluated for use in neutropenic fever when fungal infection is suspected. A direct comparative trial with AMB revealed a greater response rate in patients receiving itraconazole (defined as defervescence) and fewer drug-related adverse events [59]. This prompted the Food and Drug Administration (FDA, USA) to approve the use of itraconazole for empiric treatment of invasive fungal infections in patients with neutropenic fever. Since this time additional agents have shown greater efficacy and current guidelines recommend caspofungin or AMB in the care of these patients [60].

The utility of itraconazole for antifungal prophylaxis in patients at risk for invasive mycoses, such as allogeneic haematopoietic stem cell transplant (HSCT) or leukemic patients has also been investigated. A large meta-analysis of these trials concluded itraconazole reduced the rate of IA by 48% in a dose-dependent fashion. In this review, itraconazole oral solution was most effective at doses of 400 mg per day emphasizing the importance of the cyclodextrin vehicle for adequate absorption [61].

The decision to use itraconazole should thus be based on its potential side-effect drug interaction profile, the availability of only oral formulations, and the availability of newer and more effective medications as other potentially therapeutic options.

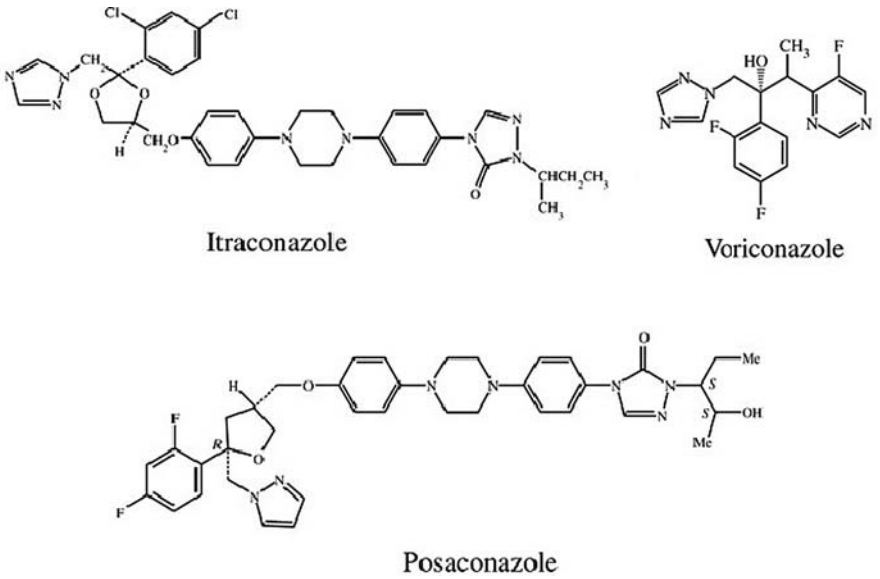

Fig. 2 Structural formulas of itraconazole, voriconazole, posaconazole

4.2 Posaconazole

Posaconazole is a lipophilic second-generation antifungal triazole with a similar molecular structure to that of itraconazole (Fig. 2). However posaconazole's spectrum of activity includes agents of the Zygomycetes, and improved activity against *Aspergillus* spp. compared to itraconazole [62].

4.2.1 Pharmacodynamics

Posaconazole is considered fungicidal against *Aspergillus* spp. Similar to itraconazole, its activity is both time and concentration dependent [63, 64]. The AUC/MIC ratio has been demonstrated in a neutropenic murine model to be the critical pharmacokinetic-pharmacodynamic parameter associated with treatment efficacy [65].

4.2.2 Pharmacokinetics

Posaconazole is insoluble in water and thus administered as a cherry-flavoured suspension using polysorbate 80 as the emulsifying agent [64]. No IV formulation has been developed. Optimal dosing of posaconazole is obtained when given as 2–4 divided doses administered with food or a liquid nutritional supplement [66, 67]. Unlike itraconazole, initial studies suggested that changes in gastric acidity do not affect posaconazole absorption [68]. However, subsequent work has shown

that concomitant use of posaconazole and cimetidine should be avoided due to a significant reduction in the AUC of posaconazole. In addition, the effects of other H_2-receptor antagonists and proton pump inhibitors on plasma levels of posaconazole have not been studied, but a reduction in bioavailability may occur and if possible co-administration should be avoided [64, 69–71].

Posaconazole has demonstrated dose-dependent pharmacokinetics with saturable absorption >800 mg per day; thus oral loading is not possible and steady state is typically achieved after 7–10 days of therapy [72]. This prolonged time required to reach steady-state levels may impact the use of posaconazole as primary therapy for invasive fungal infections. This agent also has a large volume of distribution despite its high protein binding and a half-life of approximately 24 h (Table 1).

Peak serum concentrations have shown considerable inter-patient variability for reasons that remain unclear. Krishna *et al*. reported maximum concentrations of 817 ± 689 ng/ml in adults given 200 mg four times daily or 400 mg twice daily [73]. Differences in concentration were not correlated with patient sex, age, body mass, renal or hepatic function [67, 73]. Others have proposed genetic polymorphisms within P-glycoprotein to play a role as posaconazole is both a substrate and inhibitor, but this remains unproven [74]. Glucuronidation plays a minor role in posaconazole metabolism and single-nucleotide polymorphisms within *UGT* (uridine diphosphoate-glucuronotransferase) have also been proposed to account for these differences but confirmatory studies are lacking [75]. This unpredictable variation in serum posaconazole levels has heightened interest and the necessity of therapeutic drug monitoring (which is discussed in more detail elsewhere in this book).

Posaconazole is hepatically metabolised and as discussed above undergoes minimal glucuronidation. Renal clearance plays a minor role in the clearance of posaconazole which is predominantly eliminated faecally.

4.2.3 Dosing

Currently, 200 mg three times daily is recommended for prophylaxis, and 800 mg divided in 2 or 4 doses is recommended in the salvage setting. For patients not tolerating food, a liquid nutritional supplement has been recommended to increase absorption [74]. Paediatric dosing schedules have yet to be established [2]. Dose adjustment by age, sex, race, and hepatic or renal insufficiency is not necessary given the minimal glucuronidation and renal clearance of posaconazole [76].

Posaconazole may share the embryotoxic or teratogenic effects of other azoles and as it is secreted into breast milk administration should be avoided during pregnancy or whilst lactating [70].

4.2.4 Therapeutic Drug Monitoring

This topic is discussed in more detail by Drs. Howard and Hope elsewhere in this book.

4.2.5 Adverse Effects Profile

Posaconazole is usually well tolerated and infrequently requires discontinuation due to adverse events (Table 3). In a large randomised, double-blind trial comparing posaconazole to fluconazole for prophylaxis in severe graft versus host disease (GVHD) similar adverse event rates were reported between the posaconazole (36%) and fluconazole (38%) groups with no fatalities reported [77]. The most frequent side effects were gastrointestinal (14%), whilst transaminase elevation and hyperbilirubinemia occurred with equal frequency (3%).

During a safety and tolerability study using rising single and multiple oral posaconazole doses no clinically significant changes in laboratory or electrocardiographic values were observed [72]. A large multicentre randomised trial comparing posaconazole prophylaxis to fluconazole or itraconazole in patients with neutropenia reported serious adverse events in 6% of patients treated with posaconazole versus 2% treated with fluconazole ($p = 0.01$). Three cardiac events were reported among those possibly related to posaconazole treatment including decreased ejection fraction, QTc prolongation, and *torsades de pointes* [78]. Nevertheless for most patients posaconazole is well tolerated and even long-term therapy (>6 months) is frequently devoid of toxicity [79].

4.2.6 Drug Interactions

Posaconazole is not significantly metabolised through the cytochrome P450 system and serum levels are unlikely to be increased by concomitant administration of P450 inhibitors.

Posaconazole, similar to other azoles, is a CYP3A4 inhibitor but has no effects on CYP2C9 or 2C19 (Table 2). Similar to itraconazole, non-competitive inhibition of CYP3A4 decreases the metabolism of multiple other medications dependent upon this process. These medications were reviewed in detail as above (Table 4).

The co-administration of CYP3A4 inducers (rifampin, rifabutin, phenytoin) does result in decreased posaconazole serum levels [70, 80]. The considerable variability of posaconazole serum levels and saturable absorption suggest that CYP3A4 inducers should be avoided during therapy as escalating posaconazole doses have not been proven to increase serum levels in this circumstance.

Posaconazole has also been associated with QTc prolongation and other agents known to prolong the QTc should be avoided [70, 78].

Careful consideration of additional medications and a review of potential synergistic toxicities should thus be considered when posaconazole is prescribed and therapeutic drug monitoring is strongly recommended.

4.2.7 Clinical Evidence

Oral posaconazole was proven effective in the prevention and treatment of pulmonary and disseminated aspergillosis in animal models [81, 82]. The success of this early animal work led to large clinical trials that have proven prophylactic

Table 3 Profile of common or important adverse effects of itraconazole, posaconazole, and voriconazole

Organ system	Itraconazole	Posaconazole	Voriconazole
Gastrointestinal disorders	Nausea, vomiting (5%), diarrhoea (3%); abdominal pain (<2%)	Nausea (8%), vomiting (6%), abdominal pain (4%), diarrhoea (4%)	Nausea, vomiting (<5%) Abdominal pain (<10%)
Skin and appendages	Pruritis, rash (<5%) Potentially exfoliative	Rash (2%)	Pruritis, rash (<10%) Potentially exfoliative
Liver and biliary system	Elevation of hepatic transaminases (<5%) Hepatitis (rare)	Abnormal liver function tests (3%)	Elevation of hepatic transaminases (<15%), hepatitis (rare)
Kidney	–	–	–
Bone marrow	–	–	–
Immunologic	Anaphylaxis reported	Anaphylaxis reported	Anaphylaxis reported
Endocrine System	Syndrome of mineralocorticoid excess: synthesis (all rare) Pedal edema; decreased testosterone	–	Adrenal insufficiency (rare)
Cardiovascular system	Potential to prolong QTc interval Congestive heart failure (rare)	Potential to prolong QTc interval	Potential to prolong QTc interval
Special senses	–	–	Altered/enhanced perception of light; Photophobia, blurred vision (<30%)
Nervous system	Headache, dizziness	Headache (8%), dizziness (<2%)	Hallucinations, confusion (10%), Headache
Maximum tolerated dosage in clinical trials and limiting events	600–800 mg/day – Endocrinologic effects	Not reported – Gastrointestinal intolerance	800 mg/day (10 mg/kg/day) have been tolerated without dose-limiting events for a period of 14 days

Modified with permission from [140]

Table 4 Drug interactions with itraconazole, voriconazole, and posaconazole

Drug	Itraconazole	Voriconazole	Posaconazole
Alfentanil	+	+	
Alprazolam[a]	+	+	
Astemizole	+	X	+
Buspirone	+	+	
Busulphan	+	+	
Carbamazepine	+		
Cisapride	X	X	X
Cyclophosphamide	+		
Cyclosporine	+	+	+
Digoxin	+		
Docetaxel	+		
Dofetilide	+		
Efavirenz	+	X	
Eletriptan	+		
Eplerenone	+		
Ergot alkaloids	X	X	X
Erlotinib	+		
Eszopiclone	+		
Felodipine[b]	+	+	+
Fexofinadine	+		
Gefitinib	+		
Glipizide [c]	+	+	
Haloperidol	+		
Ibuprofen		+	
Imatinib	+		
Irinotecan	+		
Lovastatin[d]	X	+	+
Methadone	+	+	
Methylprednisolone[e]	+	+	
Midazolam[a]	X	+	+
Omeprazole		+	
Paroxetine	+		
Phenytoin		+	+
Pimozide	X	X	X
Quinidine	X	X	X
Ramelteon	+		
Ranolazine	+		
Rifabutin	+	+	X
Risperidone[f]	+		
Saquinavir[g]	+		
Sirolimus	+	+	X
Solifenacin	+		
Sunitinib	+		
Tacrolimus	+	+	+
Terfenadine	+	X	+
Triazolam[a]	X	+	+

Table 4 (continued)

Drug	Itraconazole	Voriconazole	Posaconazole
Vardenafil[h]	+	+	
Vincristine[i]	X	+	+
Vinorelbine	+	+	
Warfarin[j]	+	+	
Zolpidem	+	+	

Note: Interactions demonstrated or predicted (+); co-administration contraindication (X).
Data adapted from, and reproduced with permission [141].
[a]Includes most benzodiazepines except: bromazepam, diazepam, temazepam, and estazolam.
[b]Includes most calcium channel blockers.
[c]Includes glyburide and tolbutamide.
[d]Includes simvastain and atorvastatin, but not pravastatin, or fluvastatin.
[e]Includes betamethasone, dexamethasone, hydrocortisone, fludrocortisone, budesonide, and fluticasone, but not prednisolone.
[f]Includes ziprasidone.
[g]Possibly includes all protease inhibitors.
[h]Includes tadalafil, sildenafil.
[i]Includes vinblastine.
[j]Includes acenocoumarol.

posaconazole effective in neutropenic patients with acute myelogenous leukaemia (AML) and in HSCT recipients with GVHD [77, 78].

In the first study, an international, randomised, double-blind trial, 600 patients with severe GVHD were randomised to posaconazole 200 mg three times daily or fluconazole 400 mg daily at the time immunosuppressive therapy was initiated. At the end of the fixed 112-day treatment period, posaconazole was found as effective as fluconazole in the prevention of all fungal infections (5.3% vs. 9.0%; $p = 0.07$), and was superior to fluconazole in the prevention of proven or probable IA (2.3% vs. 7.0%; $p = 0.004$). Additionally, fewer breakthrough infections were observed in the posaconazole arm (2.4% vs. 7.6%; $p = 0.046$) [77].

The second study, also a blinded, randomised multicentre trial, compared posaconazole prophylaxis to fluconazole or itraconazole prophylaxis in patients with prolonged neutropenia. Patients received prophylaxis during each cycle of chemotherapy and until either recovery from neutropenia, complete remission, or 12 weeks of therapy. Posaconazole was found superior to fluconazole or itraconazole prophylaxis for proven or probable invasive fungal infections (2% vs. 8%; $p = <0.001$). Significantly fewer patients receiving posaconazole developed aspergillosis (1% vs. 7%; $p = <0.001$) and survival was significantly longer in patients receiving posaconazole ($p = 0.04$). However as discussed above, more adverse events occurred in those receiving posaconazole (6% vs. 2%) [78]. Therefore the benefits of posaconazole prophylaxis should be measured against the potential risks of therapy and prophylaxis prescribed only to patients at sufficient risk of acquiring invasive fungal disease. These landmark trials emphasize the proven efficacy of posaconazole in the prevention of IA and posaconazole is now FDA approved for the prevention of IA in these patient groups.

The efficacy and safety of posaconazole monotherapy (800 mg daily in divided doses) for IA or other mycoses was evaluated in an open-label multicentre trial of patients refractory to or intolerant of conventional antifungal therapy [83]. The overall success rate was 42% for posaconazole and 26% for controls ($p = 0.006$). These differences in outcomes were preserved in subsequent subset analyses including: infection site, haematologic malignancy, HSCT, baseline neutropenia, and reason for enrollment. Although this study predated both echinocandins and voriconazole, the authors concluded posaconazole is an alternate to salvage therapy in patients refractory to or intolerant of other antifungal agents.

4.3 Voriconazole

Voriconazole is a low-molecular weight water soluble second-generation triazole with a chemical structure similar to fluconazole (Fig. 2). Voriconazole exhibits a broad spectrum of activity against moulds with the exception of the Zygomycetes [84].

4.3.1 Pharmacodynamics

Species and strain specific fungicidal activity have been found for voriconazole [85, 86]. As seen with the other triazoles the AUC/MIC ratio correlates best with antifungal efficacy, however the non-linear kinetics and inter-patient metabolic variability have made the evaluation of concentration-effect relationships difficult [14].

4.3.2 Pharmacokinetics

Voriconazole is available in both oral and IV formulations. Similar to itraconazole, the IV form is dependent upon sulfobutyl ether β–cyclodextrin (SBECD) for solubility [84]. When 3–6 mg/kg of daily voriconazole is administered, steady state levels are reached in 5–6 days. However, if IV loading is given (see Section 4.3.3 below) steady-state can be reached within 1 day [87].

The oral formulation also obtains steady-state levels within 24 h if a loading dose is administered, with peak plasma levels obtained 1–2 h after intake. Bioavailability when administered 1 h before or after a meal is >90%, and is independent of gastric acidity. Fatty foods, however, have been found to reduce bioavailability by 80% [88].

Although voriconazole in children has demonstrated linear pharmacokinetics, in adults non-linear metabolism has been observed, likely secondary to saturable metabolic enzymes required for drug clearance [87]. The isoenzymes CYP2C9, CYP2C19, and CYP3A4 are responsible for the clearance of voriconazole and the majority of drug-drug interactions seen with this agent (see Section 4.3.6 below). Inter-patient serum concentration differences have been largely attributed to polymorphisms within CYP2C19, the major metabolic pathway for voriconazole [84]. Up to 20% of non-Indian Asians have low CYP2C19 activity and voriconazole

serum levels are thus up to 4 times higher than those found in white or black populations in which the "poor metabolizer" status is uncommon [89]. The unpredictability of patient enzymatic activity has generated an increased interest in the routine use of voriconazole serum level determination [90].

Plasma protein binding is 58% and the mean volume of distribution 4 l/kg. Tissue levels may exceed those found in serum, and cerebrospinal fluid levels, although lower than brain levels, are typically 50% of concurrent serum values (Table 1).

4.3.3 Dosing

Loading doses with the use of both oral and IV formulations have been recommended based on the rationale outlined above. For IV administration in patients 12 years and older, 6 mg/kg twice daily, followed by 4 mg/kg IV twice daily for the duration of therapy is recommended. The oral dosages in adults are weight based. For those weighing >40 kg, 400 mg twice daily, followed by 200 mg twice daily until completion of therapy is suggested, whilst those weighing <40 kg should receive 200 mg twice daily followed by 100 mg twice daily [84].

Paediatric patients are known to hypermetabolize voriconazole and for this reason an IV dose of 7 mg/kg twice daily and oral dosing of 200 mg twice daily without loading is recommended [91].

Dose adjustments are necessary for patients with liver dysfunction. In these patients standard loading doses should be given, but the maintenance dose reduced by 50%. The safety of voriconazole use in severe liver disease remains uncertain. No dosage adjustment is required if oral drug is given to patient's with renal insufficiency. However similar to itraconazole, the presence of a cyclodextrin vehicle within the IV formulation of voriconazole has caused concerns about vehicle accumulation in renal insufficiency or dialysis dependence. Intravenous administration is avoided in patients with a creatinine clearance <50 ml/min unless potential benefit outweighs risks [84].

Similar to the other azoles previously discussed, voriconazole is teratogenic in animals and is best avoided during pregnancy or whilst the mother is breast feeding [56].

4.3.4 Therapeutic Drug Monitoring

The importance of monitoring voriconazole levels is discussed elsewhere in this book.

4.3.5 Adverse Effects Profile

Voriconazole is typically well tolerated, and the side effect profile is similar to other triazoles with few exceptions (Table 3). In a comparative trial of voriconazole versus fluconazole in the treatment of oesophageal candidosis, more treatment-related adverse events were reported in voriconazole treated patients (30%) than those receiving fluconazole (14%) [92]. Gastrointestinal side-effects were common (9%)

and equivalent between voriconazole and fluconazole treated patients. However, the majority of those experiencing a reported adverse reaction to voriconazole described abnormal vision (23%) that was transient, infusion related, and without sequalae. This unique side-effect of voriconazole is poorly understood, and no pathologic retinal changes or long-term sequalae have been found [84]. This effect typically occurs 30 min after infusion and abates 30 min after onset.

In a randomised, international, multicentre trial comparing IV voriconazole to liposomal amphotericin B for empiric antifungal therapy in febrile neutropenia, infusion-related side effects were also the most common adverse reaction attributed to therapy, with 21.9% of patients describing flashing lights (photopsia) or similar visual disturbance [93]. Discontinuation of voriconazole due to adverse events was infrequent in these trials.

Other well known effects of voriconazole therapy include skin rash and transaminase elevation [94]. Photosensitivity is particularly of concern in patients on long-term treatment with voriconazole (such as patients with chronic cavitary pulmonary aspergillosis) [95]. Although rare, cases of skin malignancy have been reported in association with prolonged voriconazole therapy [96, 97]. Baseline evaluation of hepatic function has been recommended before and during treatment, and rare cases of hepatic failure during voriconazole use have been reported [98]. Similar to other triazoles QTc prolongation has been attributed to voriconazole [99].

Elevated voriconazole serum levels have been attributed to the majority of side effects encountered in clinical practice, and higher levels (>5.5 mg/l) although associated with favourable outcomes, have also been suggested responsible for the uncommon potential side effects of encephalopathy or hallucinations [100–102].

4.3.6 Drug Interactions

Voriconazole is both a substrate and inhibitor of CYP2C19, CYP3A4, and CYP2C9, and this shared metabolic pathway is responsible for the majority of drug-drug interactions encountered (Table 2). Non-competitive inhibition of CYP3A4, similar to that described for itraconazole, may significantly increase serum levels of cyclosporine, tacrolimus, methadone, benzodiazepines, diltiazem, vincristine, statins, omeprazole, warfarin, sulfonylureas, phenytoin, protease inhibitors and non-nucleoside reverse transcriptase inhibitors (Table 4). These interactions require dose adjustment when medications are given concurrently [80, 91, 103–109].

Conversely voriconazole serum levels are reduced by CYP3A4 inducers such as rifampin, long-acting barbiturates, and carbamazepine [110, 111].

As voriconazole is both a substrate and inhibitor of the isoenzymes CYP2C19, CYP3A4, and CYP2C9, 2-way drug interactions are also observed such as when concomitant efavirenz, phenytoin, or rifabutin are used. Rifabutin decreases in voriconazole serum levels are discussed above, but the inhibition of the cytochrome system by voriconazole is also known to increase rifabutin levels. For instance, omeprazole increases voriconazole AUC_T concentrations of voriconazole by an average 40%.

The aforementioned potential for QTc prolongation during voriconazole therapy should give caution if an additional agent known to prolong the QTc interval is to be co-administered. Careful consideration should be given to the addition of medications with potential interactions and therapeutic drug monitoring should be considered if concern for subtherapeutic or toxic levels exists.

4.3.7 Clinical Evidence

Voriconazole has become the drug of choice for most cases of IA [2]. This recommendation is based on a large multinational, randomised phase III clinical trial comparing voriconazole to conventional amphotericin B, followed by other antifungal therapy in patients with IA [112]. At week 12, 52.8% of voriconazole patients demonstrated a response to therapy versus only 31.6% in the amphotericin treated arm (95% confidence interval, CI, 10.4–32.9). Survival was also increased in the voriconazole treated group (70.8%) compared to amphotericin B (57.9%) (95%, CI 21.1–42.6). Side effects during voriconazole therapy were also significantly less frequent than those treated with AMB (13.4% vs. 24.3%; $p = 0.008$).

The empiric use of voriconazole during persistent neutropenic fever was evaluated in an open-label, randomised international trial comparing liposomal amphotericin B to voriconazole. Although voriconazole did not meet predetermined non-inferiority criteria, there were significantly fewer breakthrough infections (including those caused by *Aspergillus* spp.) in patients receiving voriconazole [93].

This agent has also proven effective in non-pulmonary cases of IA, such as the central nervous system, and bone infection [113, 114].

Combination therapy with an echinocandin may prove even more effective than voriconazole alone, and prospective trials are ongoing [115, 116].

4.4 New and Emerging Azoles

Despite the utility and proven efficacy of itraconazole, voriconazole, and posaconazole, therapeutic problems remain. Poor drug bioavailability, toxicity concerns, and/or lack of IV formulations have prompted the search for additional antifungal agents. Ravuconazole, isavuconazole, and albaconazole are new extended spectrum triazoles in pre-clinical development that have shown promise in the treatment of fungal infections. Clinical trials of these agents are limited and the following discussion focuses primarily on in vitro and animal data.

4.4.1 Ravuconazole

Ravuconazole (Eisai Co. Ltd) (formerly BMS-207147, ER-30346) is a promising new triazole with activity against both yeast and moulds (Fig. 3).

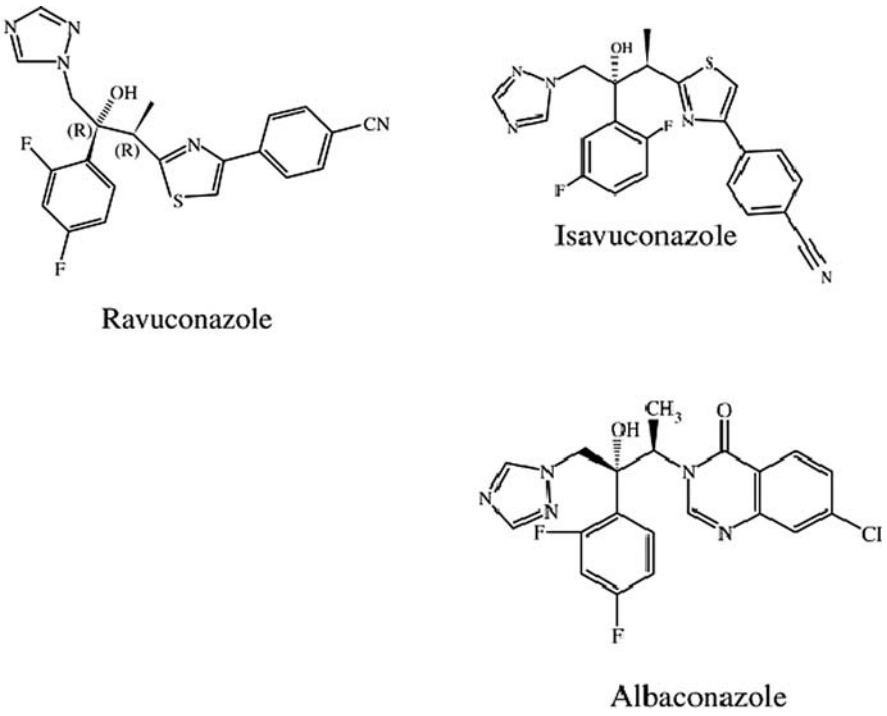

Fig. 3 Structural formulas of the investigational azoles: ravuconazole, isavuconazole, and albaconazole

Pharmacodynamics

Multiple reports have documented the in vitro activity of ravuconazole against *Aspergillus* spp. [117–119]. MIC geometric means ranged from 0.3 to 1 μg/ml across all *Aspergillus* isolates tested. Additionally, ravuconazole resistance appears uncommon with less than 2% of *A. fumigatus*, and 3% of other species, possessing MICs of ≥4.

Evaluation of the IV ravuconazole formulation has suggested both concentration and time-dependent effects on *Aspergillus* hyphae [120].

Pharmacokinetics

The IV pro-drug of ravuconazole is BMS-379224 (ravuconazole dilysine phosphoester). Contrary to the cyclodextrins, the lysine and phosphate residues present in IV ravuconazole are easily cleared and do not accumulate in renal insufficiency. A dose-proportional increase suggesting linear pharmacokinetics has been demonstrated [3].

Ravuconazole has an extended half-life (76–202 h), and is highly protein bound (98%). This agent is not known to induce CYP3A isoenzymes, although nelfinavir

levels were decreased during concurrent administration [3, 121]. This decreased ability to inhibit CYP3A4 has prompted speculation that ravuconazole may carry fewer drug–drug interactions than its triazole counterparts; however, its effects on CYP2C19 and CYP2C9 have yet to be determined.

Dosing

Optimal human dosing of ravuconazole has yet to be determined.

Therapeutic Drug Monitoring

The linear pharmacokinetics seen with ravuconazole and potential for fewer drug-drug interactions may obviate therapeutic drug monitoring for this agent, yet further data is needed.

Adverse Effects Profile

Toxicity has yet to be reported in animal studies of ravuconazole and in preclinical dose finding studies headache and abdominal pain have been the most frequent adverse events with rates similar to those observed with placebo [122, 123]. No patients in either of these trials required drug discontinuation due to laboratory abnormalities.

Drug Interactions

Minimal data has been reported concerning drug–drug interactions with ravuconazole, however inducers of CYP3A4 are likely to lower serum ravuconazole levels [124].

Clinical Evidence

Ravuconazole prophylaxis has been proven effective in a murine neutropenic model of IA. In this study oral ravuconazole significantly delayed mortality compared to prophylactic itraconazole when both were used at 10 mg/kg, however when both drugs were administered at 40 mg/kg these differences were negated [125]. Ravuconazole was subsequently evaluated in an immunosuppressed temporarily neutropenic guinea pig model of IA [126]. This study directly compared oral ravuconazole (5, 10, and 25 mg/kg daily), oral itraconazole (2.5 and 5 mg/kg daily) and intraperitoneal (IP) amphotericin B (1.25 mg/kg). Both mortality and tissue burdens were reduced with all three tested antifungals, however 100% survival was observed only with the use of ravuconazole 5 and 10 mg/kg, and itraconazole at 10 mg/kg. This dose-dependent effect was also observed in a neutropenic rabbit model of pulmonary aspergillosis. This study showed a significant reduction in mean pulmonary infarct score with 5 and 10 mg/kg of ravuconazole, but not with 2.5 mg/kg compared to untreated controls. Galactomannan levels and computed tomography (CT)-measured pulmonary injury were also reduced in treated animals [120].

The clinical efficacy of ravuconazole during the treatment of IA remains to be determined. Phase III studies are not yet available with this drug.

4.4.2 Isavuconazole

Isavuconazole, is a new water-soluble extended spectrum triazole with activity against both yeast and moulds currently manufactured by Basilea Pharmaceutica (Basel, Switzerland). This compound has shown promise against a variety of invasive fungal infections and phase III clinical trials are ongoing.

Pharmacodynamics

In vitro findings have illustrated potent activity against 118 clinical *Aspergillus* isolates, including *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger* [127]. Isavuconazole was also fungicidal against all isolates with minimum fungicidal concentrations (MFC) within 2 dilutions of the isolates' MIC. It has been suggested in a mouse model of candidaemia that the AUC/MIC ratio best predicts isavuconazole efficacy [3].

Pharmacokinetics

Both oral and IV preparations of isavuconazole have been developed. The pro-drug, isavuconazonium (BAL-8557) is cleaved by plasma esterases to the active form of isavuconazole (formerly BAL-4815) and the inactive metabolite BAL-8728 [128]. After oral administration peak concentrations are observed 1.5–3 h after dosing and the mean half-life ranged from 56 to 104 h after administration. Preliminary data show that food has no relevant effect on the pharmacokinetics of isavuconazole [129], with a slight delay in drug absorption occurring in presence of food. In animal models, isavuconazole enjoys a large volume of distribution and is highly protein-bound [130]. These findings have been confirmed in human trials. The reported volume of distribution with oral isavuconazole ranges from 155 to 292 l and with the IV formulation 304–494 l.

The water-solubility of isavuconazole avoids the necessity of cyclodextrin to obtain solubility. The lack of the cyclodextrin vehicle in IV preparations thus obviates concerns for accumulation in patients with a reduction in renal function – a potential advantage over IV voriconazole and the now discontinued IV itraconazole formulations.

Isavuconazole is primarily metabolised by CYP3A4 raising concerns for potential drug interactions similar to those seen with other triazoles.

Dosing

Optimal human dosing has not yet been determined. Preliminary data presented in abstract form shows that isavuconazole dosage might need adjustment in individuals with mild or moderate hepatic impairment [131].

Therapeutic Drug Monitoring

Isavuconazole has shown low to moderate inter-subject variability in serum levels (<40%), compared to voriconazole (up to 100-fold) [128]. Although this early data is encouraging, the utility and necessity of therapeutic drug monitoring remains to be determined.

Adverse Effects Profile

Animal data has shown no evidence of mutagenesis, allergenicity, or phototoxicity, and initial dose escalation trials showed no adverse effects. Initial pharmacokinetic data reported side effects of headache, rhinitis and nasopharyngitis and mild hepatitis in one patient. QTc interval changes have not been reported for isavuconazole. Results of phase II studies in the treatment of oesophageal candidosis have shown comparable toxicities to those of fluconazole [132].

Drug Interactions

Similar to other medications metabolised through the hepatic CYP3A4 system, multiple drug interactions are predicted. A 35-fold increase in isavuconazole clearance, a 4-fold reduction in C_{max}, and a 40-fold reduction in the AUC have been observed when the CYP3A4 inducer rifampicin was co-administered [133]. However warfarin and cyclosporine levels were unaffected when co-adminstered with isavuconazole [134, 135].

Clinical Evidence

Phase III clinical trials investigating isavuconazole for the prophylaxis or treatment of invasive pulmonary aspergillosis are currently ongoing. A non-randomised trial examining the safety and efficacy of escalating isavuconazole doses in the prophylaxis of acute myeloid leukaemia patients is also currently underway. This study will provide not only the efficacy for antifungal prevention, but additional pharmacokinetic data. An additional head-to-head trial comparing isavuconazole to voriconazole in the treatment of IA will also be of great interest to clinicians. The results of these trials will provide insight into the proper use of isavuconazole in the treatment and prevention of invasive mycoses [3].

4.4.3 Albaconazole

Albaconazole (Stiefel Laboratories, Inc., Coral Gables, FL) (formerly UR-9825, Laboratories Uriach & Cía S.A., Barcelona, Spain) is another potent and broad spectrum triazole in pre-clinical development.

Pharmacodynamics

Albaconazole also has activity against both yeast and moulds, and potent in vitro activity against *Aspergillus* spp. has been demonstrated (MIC of 0.06–0.5 μg/ml) [136]. Animal models have not evaluated the use of albaconazole in the treatment of IA to our knowledge, although effective prophylaxis was observed in a steroid-immunosuppressed rat model [3]. A dose-response relationship was reported, with 100% survival at the 50 mg/kg dose – similar results to those seen in the AMB arm (2 mg/kg IV daily) [137].

Pharmacokinetics

Oral ravuconazole is rapidly absorbed with the C_{max} reached within 2–4 h of ingestion. Both the C_{max} and the AUC are dose-proportional at doses ranging from 5 to 80 mg, however at escalating doses non-linear pharmacokinetics are observed [3].

Dosing

Optimal human dosing has not yet been determined

Therapeutic Drug Monitoring

The utility and necessity of therapeutic drug monitoring for albaconazole remains to be determined.

Adverse Effects Profile

Minimal toxicity has been reported in animal models evaluating the efficacy of albaconazole. During long term treatment (>150 consecutive days) gastrointestinal disturbance has been seen in dogs [138] whilst in mouse models using albaconazole no toxicity has been reported [139].

Drug Interactions

Drug-drug interactions similar to the other azoles are likely to be observed, but the metabolism and potential for therapeutic interactions has yet to be evaluated.

Clinical Evidence

No human trials evaluating albaconazole in the treatment or prophylaxis of IA have been performed. In a steroid-immunosuppressed rat model of disseminated aspergillosis comparing albaconazole to amphotericin B, albaconazole was protective in a dose-related manner [137].

5 Conclusion

The incidence of infection with *Aspergillus* spp. continues to rise with the increasing immunosuppressed patient population. The recently expanded antifungal armamentarium offers the potential for more effective and less toxic therapy for this often lethal infection, and these agents offer distinct pharmacologic profiles and indications for use. The triazoles in development may offer additional benefits such as an extended half-life compared to their currently available counterparts, however their role in the treatment of IA has not been fully determined.

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Echinocandins

David S. Perlin and William W. Hope

Abstract The echinocandin drugs are potent inhibitors of glucan synthase and are the first class of antifungal agents to target the fungal cell wall. The three principal drugs, caspofungin, micafungin and anidulafungin are highly serum protein bound, and display favourable pharmacokinetic and pharmacodynamic properties, as well as an excellent safety profile. Although initially approved for salvage therapy for invasive aspergillosis, treatment regimens are still evolving. The echinocandin drugs are moderately fungistatic with *Aspergillus* spp. but their in vivo effectiveness appears to be derived from drug-induced enhancement of the local immune response. Drug resistance is a rare event that has been linked to changes in glucan synthase subunit Fks1p.

Keywords Anidulafungin · Antifungal · Caspofungin · Echinocandins · FKS1 · Micafungin · Pharmacodynamics · Pharmacokinetics

Contents

D.S. Perlin (✉)
UMDNJ-New Jersey Medical School, Public Health Research Institute, Newark, NJ, USA
e-mail: perlinds@umdnj.edu

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1 Introduction

In late January 2001, the Food and Drug Administration (FDA, USA) ushered in a new era of antifungal therapy by approving Cancidas® (caspofungin acetate) as a new antifungal medication for patients who were unresponsive to or could not tolerate standard therapies for invasive pulmonary aspergillosis (IPA). This was soon followed by an expanded label indication in empirical therapy for presumed fungal infections in febrile neutropenic patients. Mycamine® (micafungin) was approved in 2005 for the treatment of patients with oesophageal candidosis and prophylaxis of *Candida* infections in patients undergoing haematopoietic stem cell transplantation (HSCT). In 2008, the label was extended for the treatment of patients with candidaemia, acute disseminated candidosis, *Candida* peritonitis and abscesses. Eraxis® (anidulafungin) was approved in 2006 for the treatment of candidaemia, oesophageal candidosis, *Candida* peritonitis and intra-abdominal abscesses. Caspofungin is the only drug with an indication for invasive *aspergillosis*, although all three drugs are mould active in vitro and show efficacy in animal models [1–6].

The echinocandins are the first new class of antifungal agents in more than a decade and the only class that directly targets the fungal cell wall [7–9]. They are semisynthetic lipopeptide inhibitors of β-1,3-D-glucan synthase, which catalyzes the biosynthesis of the principal glucan component of fungal cell walls [10]. The drugs are cyclic hexapeptides N-linked to a fatty acyl side chain, which constitutes a major feature distinguishing these chemically related drugs (Fig. 1). The echinocandins are broadly active against *Candida* and *Aspergillus* spp., and are fully active on triazole-resistant yeasts and moulds [11], and biofilms [12] because they act on a target apart from azole drugs and are not substrates for common drug efflux transporters [13].

The echinocandins are fungicidal against yeasts but only moderately fungistatic against moulds, where they cause the tips of some growing hyphae to lyse but do not completely block cell growth [14, 15]. Hyphae exposed to echinocandin drugs grow much slower, show thickened cell types and display altered colony morphology resembling a multi-budded rosette structure (Fig. 2). This altered morphological state for *Aspergillus* species has implication for pathogenicity and host response. The drug-induced morphological change also serves as an endpoint for susceptibility testing assays yielding a minimum effective concentration (MEC) [16].

Echinocandin drugs are now widely used for antifungal therapy against yeasts and moulds [17–23], although they are generally inactive against invasive infections

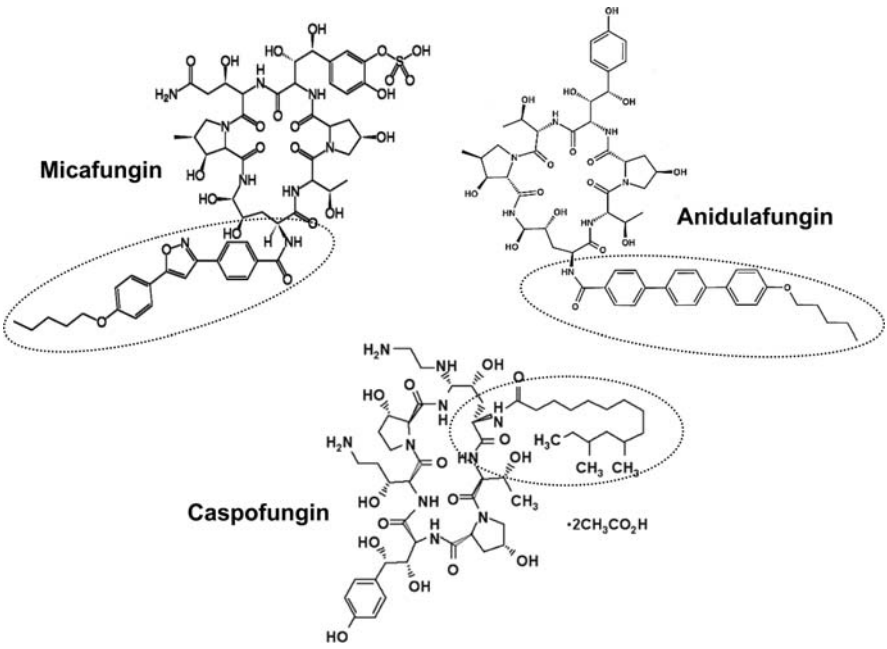

Fig. 1 Chemical structures of the licensed echinocandins

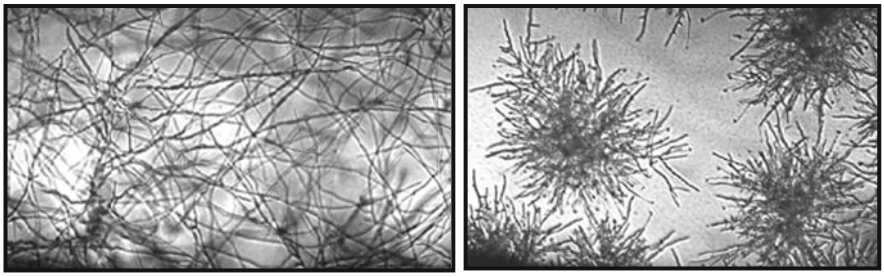

Fig. 2 Typical changes observed when *Aspergillus* hyphae are exposed to echinocandin drugs

caused by species of *Zygomycetes, Cryptococcus*, *Fusarium*. Overall, these drugs have excellent safety and tolerability profiles with few drug-related adverse events [24–27].

2 Pharmacokinetics

The pharmacokinetics (PK) of the major echinocandin drugs following intravenous (IV) administration have been extensively reviewed [21, 28–31]. The human PK of micafungin [32–34] and anidulafungin [35, 36] are well-described using linear

population PK models. A population PK of caspofungin was developed by Merck, but has not been published. For caspofungin, IV delivery results in a multiphasic decline with a dominant phase that exhibits log-linear behaviour up to 48 h in which plasma levels decrease 10-fold. Approximately 92% of the drug is distributed to tissues by 48 h after a single dose. There is little excretion or biotransformation of caspofungin during the first 30 h. Caspofungin is metabolized via hydrolysis and N-acetylation. It also undergoes spontaneous chemical degradation, resulting in a microbiologically inactive open ring degradation product. Anidulafungin shows a rapid distribution phase followed by protracted elimination. It is widely distributed in tissues (liver, spleen, kidneys, and lungs), approximately 10-fold greater compared to plasma. The half-life of anidulafungin is 1–2 days following a once daily dose. It is eliminated by slow chemical degradation with only a small amount of drug in faeces and no measurable drug in urine. Anidulafungin does not rely on enzymatic degradation or hepatic or renal excretion and, it is therefore safe to use in patients with hepatic or renal insufficiencies. Micafungin plasma concentrations decline bi-exponentially with a terminal half-life of approximately 4–6 h. Drug distribution to tissues is rapid (<5 min) post-dose with the highest drug levels found in the lungs and kidneys. Micafungin is metabolized to M-1 (catechol) by arylsulfatase, with further metabolism to M-2 (methoxy) by catechol-O-methyltransferase. A new echinocandin that has not yet entered phase III studies (aminocandin) shows a longer half-life which should allow for less frequent dosing (e.g., once or twice weekly) [31].

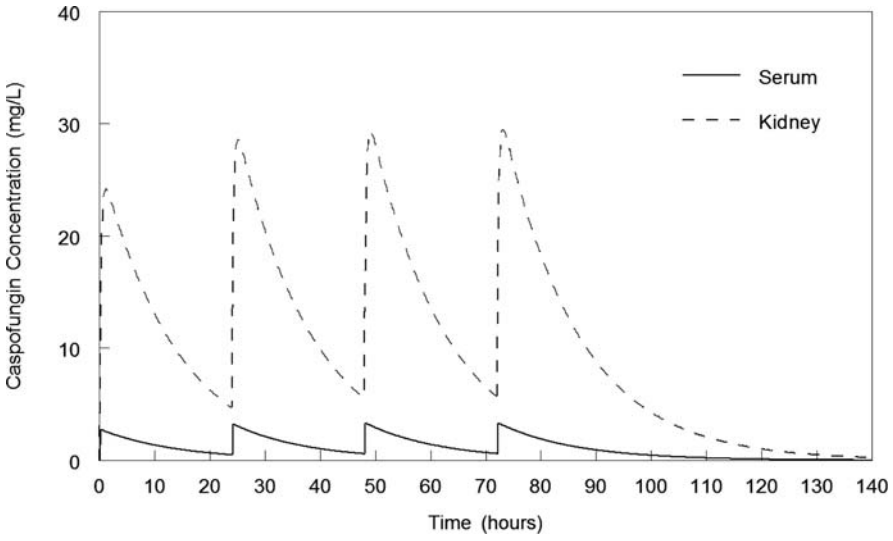

Fig. 3 Caspofungin pharmacokinetics as reflected by serum and kidney drug concentrations, after once daily dosing. The drug traffics rapidly into tissues and shows a relatively long elimination half-life

The echinocandins achieve therapeutic levels in the lungs, liver, spleen and kidneys [37–39], and detectable levels can also be found in the brain of laboratory animals, albeit to a lesser extent [40]. Echinocandins are not reliably found in the cerebrospinal fluid (CSF), although high levels are found in the surrounding meninges [40]. The successful treatment of CNS infections with echinocandins may require dosage escalation to achieve therapeutic concentrations [40].

The concentration-time profiles of the echinocandins are predominantly determined by the rate of trafficking of drug into and out of tissue, rather than by biotransformation or excretion. The echinocandins traffic rapidly into tissues, but flux back relatively slowly, and consequently exhibit relatively long mean tissue residence times [41, 42] (Fig. 3). This phenomenon accounts for the relatively long elimination half life of the echinocandins, as well as the persistent antifungal effect at the site of infection despite serum levels failing beneath the limit of detection. While these principles have been derived in murine models of disseminated candidosis using the kidneys as the effect sites, the same almost certainly applies to the lung for IPA where antifungal effect is better understood using pulmonary drug concentrations rather than serum.

3 Pharmacodynamics

The pharmacodynamic (PD) properties of the echinocandins against *Aspergillus* species remain poorly defined due to the absence of suitable biomarkers, which enable the antifungal effect to be readily quantified. While dose-dependant morphological changes are observed, there is not a decrement in conventional measures of residual fungal burden, such as quantitative organ cultures and circulating galactomannan concentrations. The reason for this remains unclear, but is probably related to the unique mechanism of drug action. Drug-affected hyphae may fragment more easily during the homogenization of tissue, and therefore appear more numerous, or they may liberate excessive amounts of galactomannan as a component of drug-induced stress response. In contrast, however, the echinocandins induce modest prolongation in survival and a decrease in surrogate measures of organism-mediated pulmonary injury (lung weight and pulmonary infarct score) and these findings provide the experimental basis for the clinical use of these agents for invasive aspergillosis [43, 44]. The mechanism of survival prolongation and decrease in pulmonary injury despite an apparent increase in fungal burden has been explained in terms of a decreased propensity for angioinvasion. Alternatively, the echinocandins may lead to exposure of immunoreactive cell wall epitopes which are normally embedded deep within the fungal cell wall and normally shielded from immunological surveillance and effector mechanisms – in this sense, the echinocandins may function as immunomodulatory agents (see below). The echinocandins appear to exhibit concentration – (as opposed to time-) dependent killing and this has been demonstrated for both *Candida* and *Aspergillus* species [41, 45]. Whether the ratio between peak concentration and minimal inhibitory concentration (MIC) or the ratio between area under the curve (AUC) and the MIC is the parameter which best links

drug exposure with the antifungal effect is debatable (a detailed discussion of which is beyond the scope of this chapter). Nevertheless, the concentration-dependant pattern of killing suggests that less fractionated regimens are appropriate modes of echinocandin administration, and this may be especially valuable for the use of these agents for prophylaxis or use in ambulatory settings.

A relevant clinical question arising from experimental models is whether viable, but echinocandin-affected organisms can resume growth following the removal of drug. Relatively limited experimental data suggest that this does not occur; withdrawal of therapy in the setting of persistent immunosuppression does not appear to result in an increase in late mortality in murine models of disseminated aspergillosis [3, 14].

Laboratory animal models of combination antifungal therapy for invasive aspergillosis are notoriously difficult from both a technical and analytical perspective. Perhaps the most comprehensive study investigated the combination of ravuconazole and micafungin in persistently neutropenic rabbits with IPA. The combination resulted in an improvement in survival, lung weight, pulmonary infarct score, fungal burden and galactomannan antigenaemia [46]. The potential benefits of voriconazole and caspofungin have also been investigated in a guinea pig model of disseminated aspergillosis. Combination therapies with both drugs reduced colony counts in tissues 1,000-fold over those for the controls [47]. A more critical assessment of combination therapy awaits on-going clinical trials.

4 Serum Factor

A variety of host factors can influence the behaviour of echinocandin drugs, which contribute to their ultimate in vivo efficacy. There is compelling evidence that serum influences the in vitro antifungal efficacy of echinocandin drugs [48–50], which has implications for therapy in patients [51]. The echinocandin drugs are heavily serum protein bound resulting primarily from interactions with albumin and, to a lesser extent, α-1-acid glycoprotein. A variety of animal and human studies indicate that caspofungin is up to 96% bound [37, 52], anidulafungin is greater than 84% [53], and micafungin more than 99% protein bound [54]. The in vitro antifungal potency of echinocandin drugs is reduced in the presence of 50% human serum, which is observed as a diminished efficacy in assays of whole cell growth [48–50] and direct enzyme inhibition [49]. In contrast, low levels of serum appear to enhance the relative antifungal properties of caspofungin in growth assays of *A. fumigatus* [55]. In the absence of serum, in vitro differences between drugs in MEC assays may vary by 5- to 20-fold. However, these differences between the echinocandin drugs were eliminated in the presence of 50% serum yielding nearly equivalent MEC values for *Aspergillus* spp. [49]. Given the behaviour of echinocandin drugs in serum, monitoring of serum in patients may be advisable [56]. For many drugs, only the free or unbound drug is biologically active. Yet, protein-bound drug can be recovered from target tissues. It is not immediately clear whether echinocandin drugs are partially active in the presence of bound serum protein. Overall, the data indicates that the

antifungal properties of echinocandin drugs are influenced by serum protein binding, and MEC values obtained in the absence of serum may not accurately predict in vivo efficacy.

5 Paradoxical Effect

The "paradoxical effect" refers to the growth of echinocandin-susceptible fungi at highly elevated drug concentrations, far in excess of the MIC. The phenomenon was first documented by Stevens and colleagues as turbid growth observed with a subset of *C. albicans* isolates at high concentrations of caspofungin [57]. These strains demonstrated a normal susceptibility pattern with a typical low MIC, but then paradoxically at high levels of drug (>16 μg/ml) showed break-through growth. This drug-insensitive growth at high drug levels behaviour was conditional, as strains undergoing paradoxical growth showed normal susceptibility properties when cultured and re-challenged with drug. The mechanism responsible for paradoxical growth remains elusive. It is not related to modification of the glucan synthase (FKS1 mutation) complex or to its up-regulation in the presence of drug [58]. The growth behaviour observed is consistent with adaptive stress responses observed in *S. cerevisiae* and *C. albicans,* which can lead to reduced susceptibility [59, 60]. In one example, chemical analysis of the cell wall of a *C. albicans* strain undergoing paradoxical growth revealed a 900% increase in chitin content. It was suggested that the elevated chitin level compensated for a decrease in β˜-1,3 glucan and β˜-1,6-glucan following caspofungin action [61]. Apparent paradoxical behaviour was reported for caspofungin in a murine model of pulmonary aspergillosis [45], although it was not reproduced in a systemic candidosis mouse model with micafungin utilizing strains displaying paradoxical behaviour [62]. More recently, a paradoxical increase in circulating *Aspergillus* antigen during treatment with caspofungin in a patient with pulmonary aspergillosis [63] and with micafungin and caspofungin in animal models [29]. The clinical significance of the paradoxical effect remains unclear, since the drug levels required would appear to exceed normal dosing levels, and the impact of drug bound to serum protein is ill-defined.

6 Drug Resistance

Echinocandin resistance resulting in patient failure due to high MIC infecting strains is uncommon amongst susceptible species, such as the *Candida* spp. Increasingly, there are reports of resistance in *C. albicans*, *C. glabrata* and *C. albicans* due to mutations in Fks1p, the major subunit of glucan synthase [64–70]. In most cases, the patients experienced repeated and prolonged exposure to the drug prior to the emergence of resistant break-through strains. Resistance to echinocandin drugs in a wide range of fungi is associated with amino acid substitutions in two "hot-spot" regions of Fks1p [71]. The mutations result in elevated MIC values and confer cross-resistance amongst the class of echinocandin drugs. Most significantly,

FKS mutations can diminish the drug sensitivity of glucan synthase by more than a thousand-fold, which is also observed as a comparable reduction in efficacy in animal models [70]. The Fks1 resistance mechanism accounts for intrinsic reduced susceptibility of *C. parapsilosis* [72]. Fks1 mutations can confer resistance in *A. fumigatus* [73], and Fks1 is partially linked to resistance in *Fusarium solani* indicating that this mechanism is conserved amongst most fungi [73]. Echinocandin resistance has been observed rarely for *A. fumigatus* infections. Recently, *A. fumigatus* isolates with significant reduced susceptibility were characterized from a Danish cystic fibrosis patient on prolonged and multi-antifungal regimens, and an Austrian patient treated with caspofungin monotherapy for invasive aspergillosis. The isolates did not contain Fks1 mutations, but did show up-regulation of the gene [74]. Finally, *Aspergillus* infections have been noted with strains showing inherent reduced susceptibility to echinocandins such as *A. lentulus* [75] and *A. flavus* [76, 77]. A concern with *Aspergillus* infections is that as the echinocandin drugs are used more prominently in combination with other drugs, prolonged exposure may lead to the emergence of strains with reduced susceptibility. Such an occurrence would not be uncommon for a drug that is only weakly fungistatic against most moulds.

7 Immunogenic Properties

The known efficacy of echinocandin drugs in animal models [43, 45, 78] and in patients for primary therapy [79] and/or prophylaxis [80] is non-intuitive given the moderate fungistatic antifungal properties of these drugs in vitro on *Aspergillus* species. As mentioned previously, the echinocandin drugs do not prevent growth of *Aspergillus* hyphae but rather alter cell and colony morphology [16]. It was initially assumed that in vivo efficacy reflected altered virulence of the treated infecting strains, as they assumed a new morphological form in the presence of drug. However, this explanation can only partially account for in vivo success because lung infections that respond to drug fail to recrudesce when the drug is removed. Yet, *Aspergillus* will grow happily when removed from the local tissue environment. It is apparent that like other antifungal agents, there is a pronounced drug-mediated host response that helps limit the infection [81]. Early evidence for this immune priming activity came from studies of *C. albicans* in which it was shown that low levels of caspofungin altered the cell wall architecture resulting in the unmasking of β-1,3 glucans [82]. This glucan unmasking could then activate immune responses via the dectin-1 receptor [83]. Dectin-1 is a pattern recognition receptor that synergizes with Toll-like receptors to initiate specific responses to infectious agents [84]. In *A. fumigatus*, caspofungin- or micafungin-treated hyphae stimulate macrophages responses, which correlate with increased β-glucan exposure consistent with Dectin-1-mediated inflammatory responses [85]. Echinocandin mediated exposure of β-glucans was examined in a wide range of fungi including *A. fumigatus, A. terreus, Rhizopus oryzae, F. solani, F. oxysporum, Scedosporium prolificans*, and *S. apiospermum* hyphae. It was determined that pre-exposure to caspofungin induced expression of Dectin-1 by polymorphonuclear leukocytes (PMN) resulting

in enhanced hyphal damage [86]. These studies suggest that echinocandins induce β-glucan unmasking by echinocandins and enhancement of PMN activity against mould hyphae, and it further supports an immunopharmacologic mode of action of echinocandins [86].

8 Treatment Guidelines

The currently licensed dosages for all of the echinocandins have been determined using studies of disseminated candidosis. The dosing regimen of caspofungin of 50 mg/day following a loading dose of 70 mg was designed to ensure serum levels are above the MIC_{90} of *Candida* spp. The dosages of micafungin (100–150 mg/day) and anidulafungin (50–100 mg/day) are also based upon clinical trials of disseminated candidosis. The optimal human dosage for all the echinocandins against *Aspergillus* species is not known, and remains an extremely difficult question to resolve.

The PK of the echinocandins in neonates and children has been studied to help guide appropriate paediatric dosing. With the possible exception of anidulafungin, body size is an important determinant of clearance [40, 87]. Since there is a non-linear relationship between weight and clearance, a larger than expected weight-based dosage is required in smaller patients [88–90]. For example, the adult dosage for micafungin is 1–2 mg/kg, but 8–9 mg/kg is required to achieve equivalent drug exposure in premature neonates. A number of dosing strategies have been described to ensure comparable levels of drug exposure in paediatric patients with a wide range of weights to those observed in adults for caspofungin and micafungin. Caspofungin should be dosed on the basis of surface area and micafungin [90, 91] can be dosed on the basis of a nomogram [33]. The appropriate dosing of anidulafungin remains less clear, since the relationship between weight and clearance is less clear. Weight may also be an important covariate in adults; patients >66.3 kg receiving micafungin have higher clearances and therefore a smaller drug exposure (AUCs) compared with patients <66.3 kg receiving the same absolute dosage. Consequently, a higher dose may be appropriate in larger patients. Whether this is important for caspofungin and anidulafungin remains unclear.

8.1 Empirical Antifungal Therapy

The efficacy and safety of caspofungin for the treatment of neutropenic patients with prolonged fever unresponsive to broad-spectrum antimicrobial agents has been established in a randomised clinical trial which compared caspofungin with liposomal amphotericin B [92]. There was no difference in the overall incidence of breakthrough fungal infection in patients treated with these agents and the incidence of breakthrough invasive aspergillosis in patients receiving caspofungin and liposomal amphotericin B arm was 10/556 and 9/539, respectively [92]. Caspofungin was associated with significantly less infusional events (35.1% versus 51.6%)

and nephrotoxicity (2.6% versus 11.5%), both of which are important causes of morbidity and mortality in their own right [93, 94]. Caspofungin also provides a survival advantage when compared with liposomal amphotericin B after 7 days of therapy [92]. A more recent study reported a slightly higher 6% breakthrough rate of invasive aspergillosis patients treated empirically with caspofungin [95]. Micafungin and anidulafungin are not licensed for the empirical therapy given the absence of a suitable clinical data base.

8.2 Primary Therapy of Invasive aspergillosis

There are relatively few data to direct the use of the echinocandins for the primary therapy for invasive aspergillosis. The lack of fungicidal activity observed in experimental models poses some concern, especially in profoundly neutropenic hosts. The inability to suppress biomarkers and the relatively modest reductions in other measures of pulmonary lung injury in experimental models have lead some to question whether the echinocandins have any clinically useful activity against *Aspergillus* spp. [96]. The rate of response to first-line therapy was recently estimated to be 42% in a small number of patients [97]. At the time of writing, an EORTC-sponsored trial is underway to examine the efficacy of caspofungin as first-line therapy in patients with a variety of haematological malignancies. Data presented in abstract form suggests that the response rate is approximately 30–40%, which is considerably lower than has been reported for other agents, although the patient population is comprised of high-risk patients with low performance scores, which is likely to have a detrimental impact on overall success rates [98]. There is only a limited data set available for micafungin [99] and no data for anidulafungin [100].

8.3 Salvage Therapy of Invasive aspergillosis

The efficacy of caspofungin in patients who are refractory or intolerant of conventional antifungal therapy was initially established in 83 patients and a portion of this dataset formed the basis of FDA approval of this agent. A favourable response to caspofungin when used in a salvage setting ranges from 38 to 64% of patients [97]; the range of estimates probably reflects the patient case mix as well as methodological issues such as when the assessment of response is made.

8.4 Combination Antifungal Regimens in Clinical Settings

The advantage of combining an echinocandin and a triazole has been investigated in a number of clinical settings [101–103], although data from definitive randomised trials are still pending. Results from a retrospective study showed that patients receiving a combination of voriconazole and caspofungin had superior responses to patients receiving voriconazole alone following initial treatment with various

lipid preparations of amphotericin B [101]. The utility of this combination has been confirmed in a prospective multicentre trial for patients who are refractory or intolerant of standard antifungal therapy [103]. Solid organ transplant recipients receiving a combination of voriconazole and caspofungin had similar overall mortality to historical controls receiving a lipid preparation of amphotericin B. Subgroup analysis, however, revealed that patients with renal failure and *A. fumigatus* infection receiving combination therapy had superior 90-day mortality outcomes ($p = 0.02$ and 0.019, respectively). There have been ongoing concerns regarding the increased cost and toxicity of combination therapy. The safety and efficacy of the combination of voriconazole and anidulafungin compared with voriconazole alone is now being defined in a randomised clinical trial (ClinicalTrials.gov identifier NCT00531479).

9 Conclusion

The echinocandin drugs are an important addition to the relatively limited armentarium against invasive fungal diseases, including those due to *Aspergillus* species. These agents have demonstrated potency, an excellent safety profile and a low propensity to generate drug resistance. The echinocandins appear to have an especially important role for the treatment of invasive aspergillosis, as a component of antifungal therapy.

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Polyene Antifungal Agents

Russell E. Lewis

Abstract Polyene antifungals have been the cornerstone treatment for invasive aspergillosis for over 40 years. Whilst new treatment options have somewhat changed their role, lipid-associated amphotericin B regimens remain important therapeutic options for aspergillosis due to their broad-spectrum of activity and limited cross-resistance with triazole antifungals. Recent studies have demonstrated the importance of accurate speciation of *Aspergillus* species during amphotericin B therapy, as some non-*fumigatus* species, particularly *A. terreus* and *A. flavus* are relatively resistant to polyenes. Furthermore, insights into the pharmacokinetic/pharmacodynamic behaviour of the polyenes suggest a limited role for dosage escalation in the treatment of invasive pulmonary aspergillosis. The continued development of new formulation approaches and interest in aerosolised delivery strategies may open new opportunities for the use of polyenes in the prevention of fungal pneumonia caused by a wide range of moulds, including *Aspergillus* species.

Keywords Amphotericin B deoxycholate · Amphotericin B colloidal dispersion · Amphotericin B lipid complex · Liposomal amphotericin B · Polyenes

Contents

R.E. Lewis (✉)
University of Houston College of Pharmacy and, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA
e-mail: rlewis@uh.edu

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1 Introduction

The antifungal agents nystatin (1954), amphotericin B (1958), natamycin (1958), and mepartrican (1975) represent a fraction of the over 200 polyene macrolide antibiotics produced by the soil actinomycete *Streptomyces*. Polyene antibiotics are characterised by a rigid non-polar cyclic ring with multiple conjugated bonds that are linked to a polar side chain comprising a large number of hydroxy groups and an amino sugar mycosamine (Fig. 1). These structural characteristics are responsible for the amphipathic nature of polyene compounds and their chemical instability. Amphotericin B is the only polyene macrolide antifungal that has been successfully developed into a systemic antifungal agent for the treatment of invasive aspergillosis (IA).

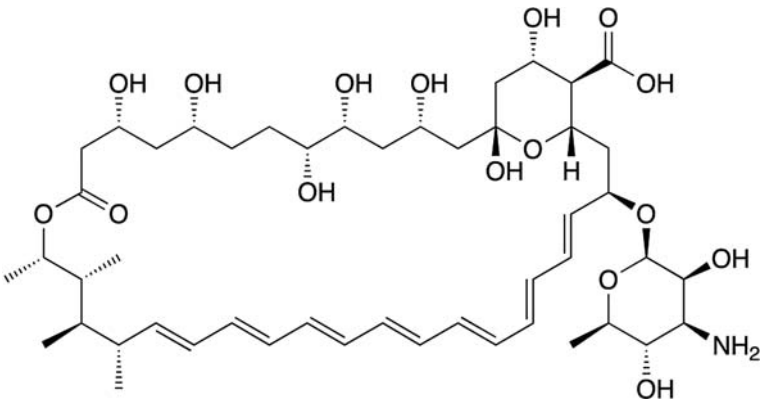

Fig. 1 Chemical structure of amphotericin B

2 Amphotericin B

Amphotericin B (AMB) has been the cornerstone of antifungal therapy for *Aspergillus* infections for over 40 years. Following the isolation of nystatin by Hazen and Brown in 1950 and its development as topical agent by Squibb

Laboratories, screening of *Streptomycete* cultures was continued to identify analogues of nystatin that could prove effective against systemic mycoses. In January of 1953, a fermentation culture of *Streptomyces nodosus* isolated from the Orinico basin in Venezuela was found to possess antifungal activity superior to nystatin. Subsequent purification of the fermentation extracts revealed two polyene compounds designated amphotericin A and B. Chemical fractionation of the mixed product soon revealed the broader spectrum and activity of the B component, which prolonged survival after oral administration in mice and rats with experimental fungal infections [1].

Unfortunately, canine and human experiments eventually confirmed that AMB could not be absorbed from the gastrointestinal tract of higher species [1]. Exhaustive attempts to develop AMB into a bioavailable formulation, however, did eventually lead to a procedure that made it possible to administer the antifungal intravenously. A mixture of AMB with sodium deoxycholate formed a complex that could be lyophilised and, when reconstituted in glucose solution, formed a micellular suspension that could be administered intravenously. Bloodstream concentrations of AMB achieved with this formulation were sufficiently high to inhibit a number of pathogenic fungi including cryptococcosis, histoplasmosis, coccidioidomycosis, candidosis, and aspergillosis [1]. In 1964, the amphotericin B deoxycholate (d-AMB) formulation was introduced onto the market by Squibb Laboratories under the trade name Fungizone®, 14 years after the discovery of nystatin [1].

Early formulations of d-AMB were plagued with impurities that elicited allergic reactions and endotoxin-like responses [2]. Although improvements in manufacturing process over the last 30 years has improved the tolerability of the drug, infusion-related reactions and renal dysfunction are still commonplace and often compromise the effectiveness of d-AMB in critically-ill patients with systemic mycosis [2]. The reformulation of AMB into lipid carriers in the late 1980s and early 1990s, however, improved the tolerability of the polyene allowing clinicians to deliver higher daily doses with less nephrotoxicity [3]. As a result, AMB continues to play an important role in the treatment of IA in severely immunosuppressed patients due to the drug's broad spectrum of activity and limited cross-resistance with triazole and echinocandin antifungals.

2.1 Chemical Properties

AMB is a polyene macrolide containing seven double bonds (heptane), an internal ester, a free carboxyl group, and a glycoside side chain with a primary amino group (Fig. 1). AMB is relatively insoluble in water and derives its name from its amphoteric properties that lead to the formation of methanol soluble salts in both acidic and basic conditions [2, 4]. The current clinical formulation is provided as sterile lyophilised powder providing 50 mg AMB and 41 mg sodium deoxycholate with 20.2 mg sodium phosphate as a buffer.

Several attempts have been made to modify the structure of AMB molecule with the aim of improving its aqueous solubility and therapeutic index. Methylesters

of AMB were developed in the 1980s and found to have significantly less nephrotoxicity than the conventional formulation [5]. Unfortunately, leukoencephalopathy was noted in several patients during early clinical trials leading to the abandonment of these water-soluble analogues [5]. Newer synthetic techniques, however, may provide opportunities for the development of a newer generation of safer, water-soluble analogues of AMB.

2.2 Mechanism of Action

Although the exact mechanism of action of polyenes remains somewhat controversial, there is a consensus that the initial steps of AMB activity involve the irreversible binding of the drug to ergosterol in the fungal cell plasma membrane. Binding to ergosterol results in the formation of a transmembrane pore comprised of 8–10 AMB molecules that allow leakage of monovalent cations, membrane depolarisation and disorganisation, intracellular acidification, and precipitation of cytoplasmic components leading to cell death [6]. The selectivity of AMB for the fungal cell membrane versus mammalian membrane can be explained, in part, by the greater affinity of ergosterol versus cholesterol for forming Van der Waals-type bonds with the rigid cyclic heptane backbone of polyenes [6]. However, AMB does retain some affinity for cholesterol – a property that plays an important role in the cellular toxicity and nephrotoxicity if the molecule in mammalian hosts [6].

AMB also inhibits exogenous and endogenous respiration in *Aspergillus fumigatus* by mechanisms independent of fungal cell membrane sterol interactions [7]. There is also evidence to suggest that AMB-mediated killing may be due in part to its oxidative properties that result in the generation of reactive oxygen intermediates and peroxidation of lipids in fungal cell membrane [8, 9].

AMB has long been recognised to have potent immunostimulatory effects that may influence the overall antifungal activity of the drug. AMB can activate microbial pattern recognition receptors, including toll-like receptor 2 and CD14, which are expressed in immune effector cells and in the respiratory epithelium [10, 11]. Exposure of mononuclear and polymorphonuclear leukocytes to AMB induces the production of IL-1β, tumour necrosis factor alpha (TNF-α), IL1-Rα and nitric oxide synthesis in human macrophages [12–15]. Collectively, these immunostimulatory mechanisms account for the acute infusion-related toxicities of AMB (fever, chills, rigors, myalgias) during intravenous infusion of the drug. Whilst these immunostimulatory effects have been shown in laboratory studies to enhance both human and murine neutrophil conicidal activity and hyphal damage of *Aspergillus* species [10, 12, 16, 17], their contribution to the overall efficacy of AMB therapy remains unproven, particularly to patients with severe immunosuppression who are predisposed for developing IA.

2.3 In Vitro Spectrum Against Aspergillus Species

AMB has broad antifungal spectrum that includes most of the common yeast and moulds that cause deep mycoses in humans, making it a useful agent for empiric

Table 1 Comparative in vitro susceptibility of selected *Aspergillus* species to amphotericin B

	Amphotericin B minimal inhibitory concentration (μg/ml)		
Organism (*n*)	MIC_{50}	MIC_{90}	Range
A. fumigatus (292)	1.0	2.0	0.25–4.0
A. terreus (43)	2.0	2.0	0.5–4.0
A. flavus (35)	1.0	2.0	0.5–4.0
A. niger (21)	0.5	1.0	0.25–1.0
A. nidulans (13)	1.0	2.0	0.25–2.0

Legend: MIC_{50}, minimum inhibitory concentration 50%; MIC_{90} minimum inhibitory concentrations 90%.
Source: Data compiled from references [35, 69].

therapy when the fungal pathogen is unknown [18]. Whilst many *Aspergillus* species are inhibited at AMB concentrations of <1 μg/ml, relative resistance to the fungicidal effects of the polyenes may be more common than previously thought for both *A. fumigatus* and other *Aspergillus* species (Table 1) [19–21]. For example, the SENTRY microbial resistance surveillance program recently reported a marked increase in the prevalence of polyene resistance with only 11.5% of isolates inhibited at AMB concentrations of ≤1 μg/ml [20]. However, longitudinal measurements of antifungal resistance amongst *Aspergillus* species are not available making it difficult to determine whether polyene resistance is truly increasing [22]. Moreover, susceptibility breakpoints for AMB have not been established; although many clinicians consider minimum inhibitory concentrations (MIC) of >1 μg/ml to be resistant [22]. Rare *Aspergillus* species, including *A. nidulans, A. ustus* and *A. lentulus*, and *Neosartorya pseudofischeri,* which often have diminished susceptibility to other antifungal agents, may also be relatively resistant to AMB killing in vitro [22–26]. Therefore, accurate speciation of clinical *Aspergillus* isolates is critical in patients receiving AMB regimens, as newer triazoles or echinocandins may provide more reliable activity for some of these relatively resistant moulds. The importance of classical and molecular methods for speciation of *Aspergillus* species are discussed elsewhere along this book.

A. terreus is inherently more resistant to AMB killing both in vitro (Table 1) and in animal models [27, 28]. In a review of 29 patients with IA treated with d-AMB monotherapy at a dosage of 1.5 mg/kg/day, all patients with isolates exhibiting AMB MIC of <2 μg/ml survived, but 22 of the 24 patients who were infected with isolates with MIC of >2 μg/ml died, including all of the 9 patients infected with *A. terreus* [29]. The poor clinical outcome of AMB-treated *A. terreus* infections was further demonstrated in a multicentre, retrospective analysis of 83 cases of proven or probable aspergillosis due to *A. terreus* that showed improved overall survival in patients who received voriconazole compared to AMB-based regimens [28]. Prior AMB therapy has also been described as a risk factor for subsequent breakthrough infections with *A. terreus* [23, 28]. Collectively, these data demonstrate that AMB monotherapy is a poor therapeutic choice for IA caused by *A. terreus.*

2.4 Mechanisms of Polyene Resistance

Most of what is understood about mechanisms of polyene resistance amongst *Aspergillus* species has been elucidated from studies of intrinsically (primary) resistant isolates [23]. Considerably less is known about adaptive (secondary) resistance in species such as *A. fumigatus*, which is considered to be rare phenomena [22, 23]. Studies in *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans* suggest that mutations in the ergosterol biosynthesis pathway (primarily *ERG3* encoding α8,7-isomerase or α5,6-desaturase) leads to the accumulation of alternative sterols in the fungal cell membrane and reduced AMB binding [23, 30]. However, recent studies examining the basis of intrinsic AMB resistance in *A. terreus* did not reveal differences in fungal cell membrane ergosterol content, cell wall composition or extent of lipid peroxidation when resistant isolates were compared to susceptible *A. fumigatus* [31].

Because AMB fungicidal activity has been partially attributed to its action as an oxidising agent, resistance might be induced though increased production of neutralizing (reducing) enzymes (e.g., catalases, hyperoxide dismutase) that confer resistance to oxidising stress [23]. Catalase production is significantly higher in *A. terreus* when compared to *A. fumigatus*, a factor that may account, in part, for the reduced susceptibility of this species to AMB [31].

2.5 Polyene Antagonism

Given that azole antifungals inhibit ergosterol synthesis in susceptible fungi, it has long been argued that the combined use of mould-active triazoles with AMB would be antagonistic in the treatment of aspergillosis [32]. The pharmacokinetic and pharmacodynamic interactions between polyenes and triazoles, however, are poorly understood [32]. Retrospective analyses of clinical experience with these combinations have not been helpful, as combination therapy is more heavily utilised in patients with a poorer underlying prognosis of IA. For example, a large retrospective analysis of 179 patients with proven or probable IA who received primary antifungal therapy with liposomal AMB (L-AMB) alone or L-AMB in combination with itraconazole showed equally poor response in both study arms (10% vs. 0%, respectively) [33]. Unfortunately, the controversy surrounding the use of azole and polyene antifungals for aspergillosis is unlikely to be resolved through a randomised clinical trial.

In vitro, most combination studies have shown antagonism or indifference against *A. fumigatus* when an azole is combined with AMB, with occasional reports of synergy when tested with low concentrations of AMB [32]. In vivo studies evaluating combinations of AMB and an azole for the treatment of aspergillosis have shown antagonism or indifference [32] with antagonism more likely if the azole is administered before AMB [34, 35]. Using multiple endpoints for drug activity (fungal burden, pulmonary infarct lesions, lung weight and galactomannan index) in a well-established rabbit model of IPA, Meletiadis and colleagues found a 20–69%

reduction in the in vivo efficacy of L-AMB when the drug was administered simultaneously with ravuconazole, despite no evidence for a pharmacokinetic interaction [36]. Therefore, it can be concluded that the bulk of pre-clinical evidence suggests that combinations of triazoles and polyene antifungals may be antagonistic in the treatment of IPA [32].

2.6 Pharmacokinetics

Absorption of AMB from the gastrointestinal tract is negligible. When administered intravenously, AMB is released from the deoxycholate complex and becomes highly protein bound (>95%) to plasma lipoproteins, including albumin and α1-acid glycoprotein, erythrocytes and cholesterol [2, 18]. Peak serum concentrations are related to the dose, rate, and frequency of intravenous infusions [37, 38]. In adults, an intravenous infusion of 0.6–1.5 mg/kg daily infused over 4–6 h produces peak serum concentrations ranging from 1 to 4 μg/ml that rapidly decline to achieve a plateau phase of 0.2–0.5 μg/ml 24 h later (Table 2) [18, 38]. Administration of twice the daily dose on alternate days results in modestly higher peak concentrations but no difference in the minimum plasma concentrations. Similarly, increasing the rate of infusion from 4–6 h to 45 min yields higher peak plasma concentrations but similar trough concentrations 18–42 h post-infusion [18].

AMB is widely distributed to many tissues with the highest concentrations found in the liver and spleen, with lesser amounts of drug in the kidneys and lungs [39]. AMB does not extensively distribute into adipose tissue suggesting that dosing should be based on lean body mass or ideal body weight [40]. Concentrations in the pleura, peritoneum, synovium, aqueous humour and bronchial secretions are usually less than half the concurrent serum concentrations [2, 41]. Very little drug (<5%) penetrates the cerebral spinal fluid (CSF), vitreous humour, or normal amniotic fluid. However, AMB is the drug of choice for life-threatening fungal infections in pregnant patients [40]. Urine concentrations of unchanged AMB average between 2 and 5% of total dose with daily administration.

Until recently, the metabolic fate of AMB was poorly understood as no metabolites of the polyene had been identified. Following the administration of ^{14}C-labeled AMB in healthy volunteers, Bekersky and colleagues demonstrated that nearly two-thirds of the total dose was excreted in the urine (20.6%) and faeces (42.5%) with over 90% of the dose accounted for by 1 week, suggesting that metabolism plays at most a minor role in AMB elimination [42]. The terminal elimination half-life of AMB was 5–7 days after a single dose [42], but has been reported to persist more than 15 days with multiple dosing [2, 38]. In fact, AMB can be detected in tissues such as the liver, spleen and kidney for as long as 12 months after therapy has been terminated [2, 41]. Hence, the prolonged terminal elimination phase of AMB most likely represents the slow release of the drug from tissues (Table 2).

Because less than 20% of the drug is eliminated through renal or hepatic mechanisms, no dosing adjustments are required in patients with renal or hepatic failure to minimize the risk of drug accumulation. However, doses may be reduced or with-

Table 2 Comparative adult pharmacokinetics of amphotericin B formulations

Characteristic	Amphotericin B deoxycholate (Fungizone®)	Amphotericin B lipid complex (Abelcet®)	Amphotericin B colloidal dispersion (Amphotec®)	Liposomal amphotericin B (AmBisome®)
Formulation	Micelle	Ribbons/sheets (DMPC & DMPG; 7:3)	Disks cholesterol sulphate	Unilamellar vesicles (cholesterol sulphate; 5 EPC and DSPG; 10:4)
Size	<10 nm	1,600–11,000 nm	122 nm	0.25–4.0 nm
Pharmacokinetic dose	0.6 mg/kg	5 mg/kg × 7 days	5 mg/kg × 7 days	5 mg/kg × 7 days
C_{max}	1.2 μg/ml	1.7 μg/ml	3.1 μg/ml	83 μg/ml
C_{min}	0.5 μg/ml	0.7 μg/ml	ND	4 μg/ml
Volume of distribution	3–5 l/kg	131 l/kg	4.3 l/kg	0.10 l/kg
Half-life (β)	91 h	173 h	28.5 h	6.8 h
AUC	14 μg/ml·h	17 μg/ml·h	43 μg/ml·h	555 μg/ml·h
Clearance	38 ml/kg/h	436 ml/kg/h	0.117 ml/kg/h	11 ml/kg/h

Legend: AUC, area under the curve; C_{max}, maximum serum concentration; C_{min}, minimum serum concentration; EPC, egg phosphatidylcholine; DMPC, Dimyristoyl phosphatidylcholine; DMPG, Dimristoylphosphatidyl glycerol; DSPG, Disteroylphasphatidylglycerol.
Source: Data compiled from [3, 18, 40].

held in some patients to minimize additive toxicity [18, 43]. Hemodialysis does not appreciably affect AMB serum concentrations, except in hyperlipidemic patients who have decreased bloodstream concentrations, apparently due to binding of the AMB-lipoprotein complex in the dialysis membrane [43].

Several pharmacokinetic parameters of AMB are different in children compared to adults. Children have a smaller volume of distribution and faster drug clearance than adults resulting in lower peak concentrations after equivalent doses [44–46]. Indeed, children from 3 months to 9 years of age often tolerate higher doses of AMB more readily than adults [18, 46]. In neonates, extreme intra-individual variability in AMB volume of distribution, clearance, and serum half life is observed but CSF penetration is improved with concentrations reaching 40–90% of simultaneously obtained serum concentrations [47].

2.7 Pharmacodynamics

In vitro, AMB displays characteristics consistent with a concentration-dependent, fungicidal antibiotic [48, 49]. AMB exhibits a steep concentration-effect curve in vitro against susceptible *A. fumigatus* species when tested without plasma proteins, with inhibition of hyphal growth at concentrations of $\leq$1 μg/ml and fungicidal activity at concentrations $\leq$4x MIC [49, 50]. A shallower dose-response curve is generally observed against *A. terreus* and some isolates of *A. flavus* with growth inhibition at concentrations >1x MIC and a lack of fungicidal activity even as concentrations surpass 4–16x MIC [50]. The post-antifungal effect (PAE), a measure of persistent cellular damage or growth inhibition after an antifungal has been removed or concentrations fall below the MIC, has been reported to persist for 12–14 hour in *Aspergillus* species following AMB exposure [51]. Characterisation of AMB pharmacodynamic patterns can vary, however, from laboratory to laboratory depending on the *Aspergillus* species and morphotype (i.e. conidia, germlings, mature hyphae) tested. The fungicidal activity of AMB is often diminished, for example, when drug is tested against mature hyphae versus conidia or germlings [49, 52, 53].

Multiple studies evaluating AMB activity in animal models of IPA have confirmed dose-dependent improvements in animal survival and fungal clearance, but a limited capacity for AMB to "sterilise" tissues infected with *Aspergillus* [54]. Systematic characterisation of polyene pharmacodynamic behaviour in animal models, however, has been limited somewhat by the narrow therapeutic index of the compound and limited dosing intervals tested [55]. Due to the interrelationship between pharmacokinetic/pharmacodynamic components (PK/PD) (i.e. the C_{max}/MIC ratio, AUC/MIC ratio, and the percentage of time above the MIC), these studies have not been able to discern the critical parameter for effective dosing [56]. Dose fractionation studies, by varying the dosing interval and dose, allow for further discrimination between these PK/PD parameters to identify which component of the dosing strategy is critical for treatment success.

Only one published study has utilised a dose-fractionation design to identify the PK/PD parameter most closely linked to AMB activity in experimental IPA [57].

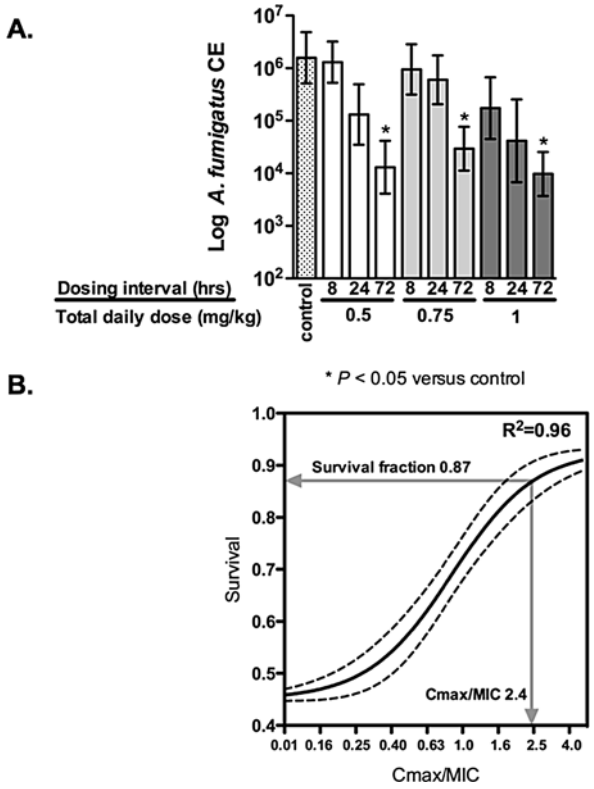

Fig. 2 In vivo pharmacodynamics of amphotericin B deoxycholate in experimental aspergillosis characterised by dosage fractionation experiments [57]. Mean (± standard error) *log Aspergillus* qPCR CE are plotted on the *y* axis. Each bar represents a group of mice receiving different doses of amphotericin deoxycholate at different dosing intervals. Each group (0.5, 0.75, and 1.0 mg/kg) was fractionated into three different dosing intervals (q8h, q24h, or q72h)

Using a neutropenic murine model of IPA, Wiederhold and colleagues found that reductions in pulmonary fungal burden and improvements in survival were maximised at the regimens that achieved the highest peak plasma exposures at each dosing range, with maximal activity in the model occurring when the plasma C_{max}/MIC approached 2.4 (Fig. 2) [57]. No dosing regimen completely cleared *A. fumigatus* from the lung as determined by quantitative polymerase chain reaction (PCR) analysis of *Aspergillus*-specific DNA.

This surprisingly-low "ceiling" for AMB concentration-dependent activity may represent the limit of soluble bioactivity of the drug in the lungs [58]. A similar phenomenon has been noted following amphotericin B treatment and in human autopsy studies, where viable *Aspergillus* could be recovered from the lungs even through AMB tissue concentrations that exceeded the MIC by 50- to 100-fold [39, 41]. Collette and colleagues reported wide variations in AMB bioactivity depending on tissue sites (i.e. liver, lung brain); therefore PK/PD relationship may vary from one tissue site to the next depending on drug distribution and patterns of drug solubility/protein binding [39]. These pre-clinical data suggest that dosage-escalation may not be an effective strategy for optimising therapeutic outcomes with AMB in the treatment of IPA [58].

Further support for the concept of a "PK/PD ceiling" for AMB in the lung was recently provided by a double-blinded, randomised clinical trial comparing two dosing strategies in patients with proven or probable aspergillosis [59]. Cornely and colleagues randomised patients to an initial dosing strategy or 3 mg/kg or 10 mg/kg of L-AMB for the first two weeks of therapy, followed by de-escalation to a dose of 3 mg/kg daily in the 10 mg/kg dosing group [59]. End of treatment efficacy for the 3 mg/kg dose was equivalent to that of 10 mg/kg L-AMB, although the higher dose regimen was associated with increased toxicity [59]. Hence, there appears to be no clinical benefit for increasing daily dosages of lipid AMB formulations beyond the currently recommended dosing of 3–5 mg/kg/day for IPA [60]. In addition, a recent trial [61] comparing two d-AMB doses (1.0 mg/kg/day vs. 0.7 mg/kg, in association with 5-flucytocine) for the treatment of cryptococcal meningitis showed that higher AMB dosage is more rapidly fungicidal, at the cost of more toxicity [62].

2.8 Adverse Effects

Infusion-related reactions characterised by fever, chills, rigor, anorexia, nausea, vomiting, myalgias, arthralgias and headache, are the most common acute toxicity of all AMB formulations. Frequently, these reactions are more severe in patients with adrenal insufficiency [40]. Acute infusion reactions result from pro-inflammatory activity of AMB and correlate with increased blood levels of inflammatory cytokines [13, 40]. Hypotension, flushing and dizziness are less common, but bronchospasm and true-anaphylactic reactions have been reported with both the conventional and lipid formulations of AMB [63]. Severe hypokalemia and cardiac arrhythmias have also been described in patients with central venous catheters that received rapid infusions or excessive doses of conventional AMB. Therefore, slower infusion rates ($\geq$4–6 h) and electrocardiogram monitoring should be considered in patients with underlying cardiac conduction abnormalities. Thrombophlebitis is a common local side effect with infusion, which often necessitates the placement of a central venous line for therapies lasting >1 week. Slower infusion rates, rotation of infusion sites, application of hot packs, low-dose heparin, and avoidance of concentrations >1 g/l can minimise thrombophlebitis.

Acute reactions generally subside over time and with subsequent AMB doses. Lipid formulations reduce to varying degrees, the incidence of infusion-related reactions. It has been suggested, that the concentration of each formulation (% weight per volume) plays a role in the incidence of infusion reactions, and has been shown to correlate with degrees of pro-inflammatory activity in leukocytes ex vivo [13, 64, 65]. In the past, a test-dose of d-AMB (i.e. 1–5 mg) was recommended prior to initiating full-dose therapy, but is no longer considered useful for screening patients for hypersensitivity reactions. Pre-medications such as low dose hydrocortisone (1 mg/kg), diphenhydramine, meperidine (0.5 mg/kg) and non-steroidal anti-inflammatory agents may be administered to blunt symptoms of acute reactions, although there is limited data from organised clinical trails supporting their routine use [2, 18]. Pre-medication should also be considered on an "as-needed" basis

for lipid AMB formulations even though infusion reactions are somewhat reduced (formulation-dependent).

Nephrotoxicity is the most significant and often dose-limiting delayed toxicity of AMB therapy in patients with aspergillosis that can be classified by glomerular and tubular mechanisms [2, 18]. AMB directly constricts the afferent arteriole resulting in a decrease of renal blood flow and a drop in glomerular filtration (increased serum creatinine), eventually leading to azotemia. AMB-induced azotemia can be delayed in some patients by ensuring adequate hydration prior to starting therapy and by *sodium loading* (administering intravenous normal saline 0.5–1 hour before and after AMB infusion in patients who can tolerate extra fluid) to maintain renal blood flow and adequate glomerular filtration pressure [66]. Although the exact amount of sodium needed to reduce nephrotoxicity is unknown, studies in human adults receiving AMB deoxycholate have examined a range of 85–600 mEq/day of sodium before AMB administration [18, 66]. Two small non-randomised studies have also suggested that the administration of AMB by continuous infusion can delay glomerular toxicity [67, 68]; however, this dosing approach has not been widely adopted and has unproven efficacy in patients with documented aspergillosis [58]. Azotemia and renal tubular acidosis with AMB is generally reversible, although 5–10% of patients may have persistent renal impairment after discontinuation of therapy. Renal toxicity progressing to dialysis is more common in recipients of haematopoietic stem cell transplantation, patients with diabetes mellitus, patients with pre-existing renal dysfunction, or patients receiving concomitant nephrotoxic agents [69]. Wingard et al. [70] showed that duration of d-AMB use was an independent predictor of death in immunocompromised patients treated with d-AMB for suspected or proven aspergillosis (Hazard Ratio – HR, 1.03/day $p = 0.015$). Other variables associated with death in this study were the use of other nephrotoxic agents (HR, 1.96; $p = 0.017$) and haemodialysis requirement (HR, 3.09; $p < 0.001$).

Damage to distal tubular membranes by AMB leads to impaired urinary acidification, impaired urinary concentrating ability, and wasting of potassium and magnesium. Hypokalemia, an indication of AMB tubular damage, is common in patients receiving either conventional or lipid formulations of AMB and may require the administration of up to 15 mmol of supplemental potassium per hour [18]. Hypokalemia and hypomagnesaemia frequently precede decreases in glomerular filtration (increased serum creatinine), especially in patients who are adequately hydrated or receiving lipid formulations of AMB. Continued tubular damage, however, eventually results in decreases in renal blood flow and glomerular filtration through tubuloglomerular feedback mechanisms that constrict the afferent arteriole [66]. Therefore, it is the opinion of the author that renal protective measures such as *sodium loading* should still be considered for patients receiving lipid AMB formulations (provided additional fluid can be tolerated) even though the benefits of this practice are not well-proven.

Although abnormalities in liver function tests are common in patients receiving AMB formulations, other drugs, intercurrent viral infections, or underlying diseases are often more prominent causes of hepatic dysfunction during the treatment of aspergillosis [71]. AMB has been associated with hepatocellular (elevated alanine

aminotransferase), cholestatic (elevated alkaline phosphatase and total bilirubin), and mixed patterns of liver injury that is reversible with discontinuation of therapy [71], although rare cases of irreversible hepatic failure have been reported with AMB therapy [71].

Patients who receive prolonged courses of AMB may develop normochromic, normocytic anemia due to the inhibitory effects of AMB on renal erythropoietin synthesis. Non-cancer patients may experience decreases in haemoglobin of 15–35% below baseline that will return to normal within several months of discontinuation of the drug. Administration of recombinant erythropoietin (Epoietin) has been recommended in select patients with symptomatic anaemia during AMB therapy [18].

Drug interactions with AMB can be classified as pharmaceutical, pharmacokinetic, or as toxicodynamic interactions [72, 73]. All AMB formulations are prone to pharmaceutical interactions due to the limited solubility of polyene molecule and its inherent instability when complexed with either deoxycholate or the phospholipid carriers. Consequently, AMB formulations generally should not be mixed with other drugs and diluted only in 5% dextrose water, since any precipitation increases the risk of toxic reactions [73].

Pharmacokinetic interactions may arise due to the nephrotoxic effects of AMB, which is often additive to that caused by other agents that cause damage to the distal tubules of the kidney (i.e. cyclosporine, tacrolimus, aminoglycosides, cisplatin, vancomycin) [72]. Toxicodynamic interactions are frequently manifestations of electrolyte disturbances that develop during therapy with AMB (hypokalemia, hypomagnesemia) and are often additive to other medications (i.e. loop and thiazide diuretics, steroids), or augment the pharmacological effect of drugs such as antiarrythmics, non-depolarizing skeletal muscle relaxants, and digoxin [74]. AMB-induced nephrotoxicity can also lead to the accumulation of drugs such as 5-flucytosine, which may result in additional toxicities [75]. Steroids enhance AMB-induced hypokalemia and have contributed to reversible cardiomyopathy in due to the augmentation of steroid hypokalemic and salt/water retention effects [76]. Therefore, this combination should be avoided whenever possible in patients with a history of congestive heart failure.

Drug interactions that arise from AMB-associated nephrotoxicity are difficult to avoid in the medically-complex patient who is predisposed to developing IA [74]. Management of these interactions focuses on preventative measures to limit the severity of the reaction, and careful monitoring and supplementation of electrolyte deficiencies. Lipid formulations of AMB should be substituted for d-AMB whenever possible in patients with underlying renal dysfunction or concomitant nephrotoxic agents. Careful monitoring of renal function – serum creatinine, blood urea nitrogen and electrolytes (i.e., K^+, Mg^{++} and PO_4) is essential in aspergillosis patients receiving AMB-based therapy [74]. Blood or serum concentration monitoring of renally-eliminated drugs with a narrow therapeutic index require intensive monitoring even in patients without evidence of declining glomerular filtration rate, as shifts in serum concentration of these drugs generally precede changes in the serum creatinine [18, 74, 75].

3 Lipid Formulations of Amphotericin B

The amphipathic characteristics of AMB made it possible to formulate the drug into lipid carriers for intravenous infusion without the bile salt deoxycholate. Three such formulations are currently marketed: amphotericin B lipid complex (Abelcet®, ABLC); amphotericin B colloidal dispersion (Amphotec®, Amphocil®, ABCD), and a liposomal formulation (Ambisome®; L-AMB). All three formulations were offer two important advantages over conventional d-AMB formulation: (i) the ability to administer higher daily dosages of drug; and (ii) reduced or delayed nephrotoxicity [3]. Consequently, the lipid formulations of AMB are the preferred formulations for the treatment of IA [77].

3.1 Chemistry and Pharmacokinetics

When AMB is incorporated into a lipid carrier, the pharmacokinetics of the drug change depending in the particle size, phospholipid content, shape, bilayer rigidity, and electrostatic charge of the formulation (Fig. 3) [3]. ABLC, the formulation with the largest particle size, is rapidly removed from the bloodstream by macrophages and other cells of the monophagocytic system and deposited into deep tissue sites such as the liver, spleen and lungs [78]. Consequently, ABLC displays a substantially larger volume of distribution and faster clearance of AMB from the bloodstream compared to the conventional d-AMB formulation [78] (Table 2). L-AMB, which has the smallest particle size of the three formulations, circulates in the bloodstream for longer periods before being sequestered into deep tissue sites [3]. Therefore, clearance of this formulation and the volume of distribution are significantly lower than d-AMB. The clinical significance of these pharmacokinetic differences between different AMB formulations remains somewhat controversial. However, all of the three lipid formulations accumulate to a lesser degree in the kidneys, which accounts for their reduced rates of nephrotoxicity [3]. For the same reason, lipid formulations should not be used for the treatment of renal infections caused by fungi.

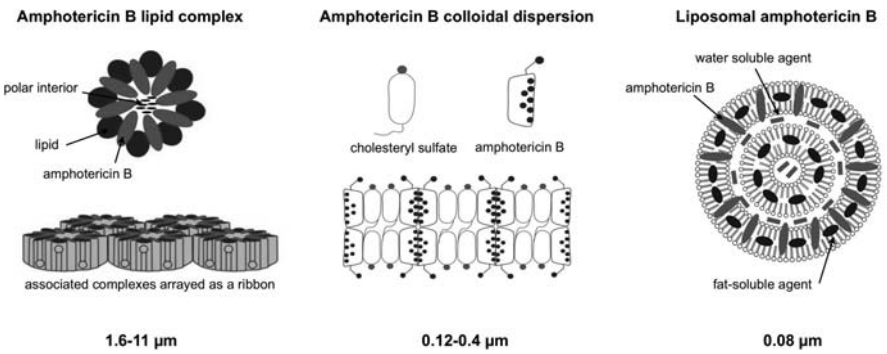

Fig. 3 Comparative structural features of the lipid-associated amphotericin B formulations. Figures were adapted from references [98, 99]

Whilst most experts consider all three of the AMB lipid formulations to have similar efficacy in the treatment of aspergillosis, pharmacokinetic differences between the formulations may be an important consideration based on the site of infection. For example, ABLC rapidly distributes to the lung after intravenous administration achieving high tissue concentrations with the first dose, suggesting a possible benefit as initial therapy in acutely-ill patients with IPA [79, 80]. L-AMB has been reported to achieve higher drug concentrations in brain tissue compared to the conventional d-AMB or the other lipid formulations [3, 81], which could be important in patients with cerebral aspergillosis. The importance of these pharmacokinetic differences in the treatment of IA, however, is not well established in clinical studies.

3.2 Pharmacology

All three lipid formulations have an improved therapeutic index compared to d-AMB and as such, are administered at doses 3–5 times higher (mg/kg basis) than conventional formulation. Whilst the reasons for the renal sparing effects of lipid AMB is not completely understood, several mechanisms have been proposed [78]:

1. Selective tissue targeting via uptake my macrophages, thus limiting free drug in the bloodstream.
2. Selective transfer of the AMB from the lipid carrier to the fungal cell membrane, thus limiting opportunities for the polyene molecule to interaction with cholesterol. Extracellular phospholipases in some fungal species may enhance the release of the AMB at the site of infection, producing higher drug concentrations at the site of infection.
3. Sequestering AMB from interactions with low density lipoproteins, which appear to be more nephrotoxic than AMB bound to lipid carriers or high-density lipoproteins.
4. Immunomodulatory effects of the lipid carriers, which elicit reduced pro-inflammatory cytokine response (i.e. TNF-α, IL-1, IL-8, etc.) from host cells compared to d-AMB. These pro-inflammatory cytokines could be mediators of not only infusion-related reactions, but also renal injury. Indeed, liposomes have been shown to possess potent anti-inflammatory effects and attenuate AMB signalling through TLR-2, resulting in decreased production of reactive oxygen species by host immune cells.

The unifying concept of each of these mechanisms is the ability of lipid formulations to limit drug delivery to the kidneys and opportunities for free drug to non-specifically interact with cholesterol in mammalian cell membranes.

Pharmacodynamic characteristics of the lipid amphotericin B formulations are complicated by their pharmacokinetic differences and cannot be broadly assumed to mimic d-AMB. However, pre-clinical models have generally supported the concept that, similar to d-AMB, these formulations exhibit a steep dose-response curve that plateaus against susceptible *Aspergillus* species when daily dosages approach 5–10 mg/kg/day [79, 82]. In comparative studies of ABLC and L-AMB activity in

neutropenic murine models of IPA, dosing regimens that achieved lung tissue concentrations in greater than 3–4 μg/g were associated with clearance of *Aspergillus* species compared to dosing regimens that failed to achieve this target concentration [79, 83]. Dosing regimens that achieved 10-fold higher tissue concentrations did not substantially improve clearance of *Aspergillus* from the lung [79].

3.3 Adverse Effects

All three lipid-based formulations of AMB are less nephrotoxic than d-AMB. However, only L-AMB has reduced frequency of infusion related toxicities compared to d-AMB [84]. In addition to the reactions mentioned previously with d-AMB, a unique triad of dyspnoea associated-symptoms, severe abdomen, flank, or leg pain; and flushing and urticaria has been reported with each of the lipid formulations [85]. Most of these reactions can be effectively managed by diphenhydramine administration and interruption of the lipid amphotericin B infusion [85]. Moreover, idiosyncratic reactions with one lipid formulation do not preclude use of another lipid AMB formulation [60]. Other adverse effects reported with the lipid formulations include headache, hypotension, hypertension, nausea, vomiting and rashes. Laboratory abnormalities include hypokalemia, hypomagnesaemia, hypocalcaemia, abnormal liver function, and thrombocytopenia [3].

4 Clinical Evidence Supporting the Use of Amphotericin B for Aspergillosis

Current clinical guidelines recommend the use of lipid AMB formulations in the treatment of aspergillosis as an alternative to voriconazole, or as salvage therapy in patients who have failed or are intolerant to other antifungal therapies [60]. d-AMB is not recommended as initial primary or salvage therapy due to the high doses (1–1.5 mg/kg/day) and prolonged treatment courses required for aspergillosis, which are poorly tolerated in many patients with IA [60]. ABLC and ABCD are approved at dosages of 5 mg/kg/day and 3–4 mg/kg day, respectively, and L-AMB is approved at a dosage of 3–5 mg/kg/day for salvage therapy of IA [60]. Although L-AMB has been safely administered at dosages as high as 15 mg/kg/day, a clinical benefit was not observed with these higher doses [86]. Furthermore, a prospective randomised trial of L-AMB, which compared a dosage of 3–10 mg/kg/day for primary treatment of proven or probable aspergillosis in 201 patients, found no improvements in response rates with greater toxicity in the high-dose group [59]. Routine administration of AMB in combination with a second antifungal as primary therapy for aspergillosis is not recommended, except in the possible context of salvage therapy [60]. Because of antagonism concerns with azoles, echinocandins may be the preferred agents for combination therapy with AMB therapy. A recent small prospective trial suggested that a combination of caspofungin plus L-AMB dosed at 3 mg/kg was more effective than 10 mg/kg L-AMB in leukemic patients with Proven or probabe IA [87]. A summary of major clinical trials supporting the use of AMB in the treatment of aspergillosis is provided in Table 3.

Table 3 Major controlled clinical trials assessing the safety and efficacy of amphotericin B formulations for invasive aspergillosis

Study	Year	Study design	No. of subjects	Drugs (doses) compared	Success rate (%)	*P*	Adverse events
Ellis et al. [100]	1998	Randomised, double-blind, prospective	87	L-AMB 1 mg/kg/day vs. L-AMB 4 mg/kg/day	1 mg/kg/day (49%); 4 mg/kg/day (46%)	NS	SAE including renal toxicity: 36.5% (1 mg/kg/day) vs. 54.3% (4 mg/kg/day)
Herbrecht et al. [101]	2002	Randomised, open, prospective	277	d-AMB (1–1.5 mg/kg/day) vs. VOR (6 mg/kg ×2 doses followed by 4 mg/kg every 12 hours)	d-AMB (31.6 %) vs. VOR (52.8%)	<0.05	SAE including renal toxicity: 24.3% (d-AMB) vs. 13.4% (VOR)
Bowden et al [102].	2002	Randomised, double-blind, prospective	174	ABCD (6 mg/kg) vs. d-AMB (1–1.5 mg/kg/day)	ABCD (35%) vs. d-AMB (35%)	NS	Renal toxicity (100% increase in SCr): 12% (ABCD) vs. 38% (d-AMB)
Cornely et al. [59]	2007	Randomised, double-blind, prospective	201	L-AMB (3 mg/kg/day) vs. L-AMB (10 mg/kg/day for 2 weeks, then 3 mg/kg/day)	3 mg/kg/day (50%) vs. 10 mg/kg/day (46%)	NS	Renal toxicity (doubling in SCr): 14% (3 mg/kg group) vs. 31% (10 mg/kg group)
Caillot et al. [87]	2007	Randomised, open, prospective	30	L-AMB 3 mg/kg/day plus CAS 70 mg day#1, then 50 mg/day vs. L-AMB 10 mg/kg/day	L-AMB 3 mg/kg/day plus CAS (67%) vs. L-AMB 10 mg/kg/day (27%)	0.028	Renal toxicity (doubling in SCr): 6.7% (combination group) vs. 23.5% (10 mg/kg/day L-AMB group)

Legend: CAS, caspofungin; d-AMB, amphotericin B-deoxycholate; L-AMB, liposomal amphotericin B; VOR, voriconazole; SAE, serious adverse events; SCr, serum creatinina; NS, non-significant.

5 Non-Intravenous Approaches for Amphotericin B Administration

Alternative approaches to intravenous dosing are occasionally utilised with AMB formulations to improve drug delivery at the site of infection whilst minimizing systemic exposures and toxicity [88]. As sinopulmonary inhalation is the most common route for exposure to *Aspergillus*, several clinical trials have explored the use of inhaled AMB administered through a nebulizer or as a nasal spray as an adjunctive or preventative treatment strategy for IA. Concentrations of d-AMB range between 1 and 2 mg/l, with either sterile water or 5% dextrose as the solvent and dosed 5–20 mg once to three times daily [88]. Overall, the incidence of invasive fungal infections whilst receiving aerosolised d-AMB range from 0 to 22.7% versus 5.1–72% in the control groups [88]. Only two of these trials, however, demonstrated significant reductions in the absolute number of fungal infections [89, 90]. Adverse effects associated with aerosolised administration of d-AMB included nausea, taste disturbances, coughing, increased sputum production, dyspnoea, decreased lung peak flow (bronchial constriction) bronchospasm, and epistaxis [88]. Toxicity is particularly attributed to the presence of deoxycholate, which is toxic to lung surfactant [91].

Lipid-based formulations of AMB have also been examined in the prevention IA in patients with persistent neutropenia or recipients of lung transplantation. A randomised, double-blind controlled trial compared tolerability of aerosolised d-AMB with ABLC for the prevention of invasive fungal infections [92]. Patients received d-AMB 25 mg or ABLC 50 mg via nebulisation once daily for 4 days followed by weekly administration for $\leq$7 weeks. Inhalation doses were doubled in patients on mechanical ventilation. Intolerance of the inhalation therapy resulted in discontinuation of prophylaxis in 6/49 (12.2%) of patients receiving d-AMB and 3/51 (5.9%) of those on ABLC. Invasive fungal infections developed in 7/49 (14.3%) of patients on d-AMB versus 6/51 (11.8%) of patients on ABLC [92]. A randomised, placebo-controlled trial evaluated the prophylactic efficacy of twice-weekly aerosolised L-AMB (2.5 ml of a 5 mg/ml solution) administered by an adaptive aerosol delivery system administered over 30 min in patients with haematological malignancies with expected neutropenia $\geq$10 days [93]. Amongst the 271 patients studies during 407 neutropenic episodes, 2/91(2.2%) of patients in the L-AMB arm developed IA versus 18/132 patients (13.6%) in the placebo arm, ($P = 0.007$) (on-treatment analysis). Non-severe adverse effects such as coughing were reported more frequently in the L-AMB group ($p = 0.002$) resulting in higher discontinuation rates for at least 1-week (45% vs. 30%, respectively, $P = 0.01$). However, no differences were detected in overall survival [93].

Whilst these results are encouraging, aerosolised delivery has important limitations. First, it is still unclear whether aerosolised delivery of AMB provides adequate drug distribution to the periphery of the lung, particularly for pleural-based lesions, which are common in IA in neutropenic patients. Second, aerosolised AMB is not effective against extra-pulmonary aspergillosis so many high-risk patients require addition of a second (systemic) antifungal [88]. Third, long-term administration is

not always feasible in patients outside the hospital where drug stability may be problematic and foaming of the preparation in the nebulizer can occur if doses are not appropriately prepared by patients [88].

More recently, novel dry-powder formulations of AMB have been developed that can be administered with small-hand-held nebulisers. NKTR-024 is a small-particle powder formulation of AMB that has demonstrated significant activity against *A. fumigatus* in experimental models of IA [94] and was well tolerated in adults at doses up to 25 mg [95]. Further dose-ranging studies and clinical trials will help determined the prophylactic potential of this novel formulation.

Percutaneous injections or direct endobronchial instillations of AMB into fungal balls has been attempted using various extemporaneously prepared formulations, including solutions, gels, and pastes [88]. Percutaneous therapy is often a dosing strategy of last resort, when surgical resection is not feasible or refused by the patient. A majority of published cases have described daily percutaneous instillations of AMB solution (50 mg in 20 ml of 5% dextrose solution) into fungal balls over 3–5 min with capping of the catheter for 8–12 h [88]. In some cases, AMB instillation is followed by injection of 10% *N-acetylcystine* or bromohexine (8 mg/10 ml), and continuous suctioning of the catheter overnight [96, 97]. Overall success rates reported in the literature are quite favourable (60–80%), but likely reflect a degree of publication bias [88]. Reported toxicities of the instillations include coughing, fever, headache, and vomiting [88].

6 Conclusions

AMB remains an important treatment option for IA due the drug's broad spectrum, minimal cross-resistance with other antifungals, and predictable pharmacokinetics. Continued development of novel formulations and administration strategies, that take advantage of the drug unique pharmacokinetic and pharmacodynamic profile, will open new opportunities for safer and more effective uses of this polyene antifungal.

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5-Flucytosine

Johan W. Mouton and Paul E. Verweij

Abstract Flucytosine (5-fluorocytosine; 5-FC) is the only antifungal agent that belongs to the class of nucleoside analogs and is primarily used to treat yeast infections. Flucytosine is seldomly used in the treatment of invasive aspergillosis, primarily because isolates of *Aspergillus* species are usually not susceptible to 5-FC in vitro. Most minimum inhibitory concentrations are >64 mg/l under standard test conditions. Some in vitro studies show synergism with other antifungals agents, but the results are difficult to interpret. However, recent studies have shown that the activity of 5-FC is pH-dependent and the drug has been shown to be effective in an *Aspergillus* infection model in a dose-dependent manner against some strains. Good clinical studies showing a benefit of 5-FC are still unavailable. Most current guidelines do not recommend the use of 5-FC for the treatment of invasive aspergillosis.

Keywords Animal model · Combination therapy · Flucytosine · 5FC · PK/PD · Synergism

Contents

J.W. Mouton (✉)
Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Ziekenhuis Nijmegen, Nijmegen, The Netherlands
e-mail: Mouton@cwz.nl

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1 Introduction

Flucytosine (5-fluorocytosine; 5-FC) is the only antifungal agent that belongs to the class of nucleoside analogs and has first been used for the treatment of invasive mycoses in 1968 [1]. It has always been viewed as having a narrow spectrum of antifungal activity that includes *Candida* species and *Cryptococcus neoformans* [2, 3]. Flucytosine is seldomly used in the treatment of invasive aspergillosis, primarily because isolates of *Aspergillus* species are usually not susceptible to 5-FC in vitro and most minimum inhibitory concentrations (MIC) are >64 mg/l [4] although in some centres it has been used in combination with another antifungal agent such as amphotericin B or other antifungal [3, 5], despite the lack of convincing clinical evidence. More recently the drug has been used less frequently due to the availability of other antifungal agents with efficacy against aspergillosis.

However, recent studies have shown that the activity of 5-FC is pH-dependent [6, 7]. Moreover, 5-FC was shown to be effective in an *Aspergillus* infection model in a dose- dependent manner. In the present chapter we will provide an overview of 5-FC pharmacology, susceptibility testing against *Aspergillus* spp., pharmacodynamic relationships and possible clinical use.

2 Pharmacology

2.1 Mode of Action

Flucytosine is an agent of the fluoropyrimidin class and was discovered in 1957 as an analogue to the cytostatic chemotherapeutic agent fluorouracil (5-FU) [8]. Figure 1 shows the structural formula of 5-FC. After penetration through the cell wall, controlled by the enzyme cytosine permease, 5-FC is converted to 5-FU by the enzyme cytosine deaminase [9] and then further to 5-fluorouridine. This is a crucial step, and fungi that lack this enzyme are not susceptible to 5-FC [10]. After three phosporylation steps, incorporation into RNA instead of uracil results in a blocking of protein-synthesis. Alternatively, 5-FU may be converted to 5-fluorodeoxyuridine monophosphate that acts as an inhibitor of thymidylate synthetase and thereby blocks DNA synthesis. Which of the two pathways plays the keyrole in 5-FC activity is not clear [11].

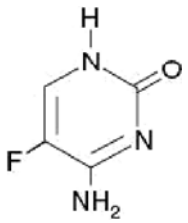

Fig. 1 Structural formula of 5-flucytosine

2.2 Pharmacokinetics

Flucytosine can be administered both orally as well as intravenously. After oral administration, it is readily and almost completely absorbed [12]. Peak concentrations are reached between 0.5 and 2 h after administration. The half-life is around 3–4 h but is much longer in patients with renal insufficiency [13–15]. Elimination is primarily by glomerular filtration and correlates well with creatinin clearance [12, 13, 16]. Since the drug is usually administered 3–5 times daily accumulation does occur during the first days. Protein binding is virtually absent. The volume of distribution approaches that of total body water. The drug distributes well into almost all body fluids, including the eyes, urine, and cerebrospinal fluid (CSF). This latter feature – penetration into CSF – is important in the treatment of infections in the central nervous system, for instance cryptococcal infections.

2.3 Toxicity

Flucytosine is generally well tolerated. The relatively weak toxicity of flucytosine in humans can largely be explained by the absence of cytosine deaminase in human cells. However, at high concentrations – above 100 mg/l – it may induce liver and bone marrow toxicity, including leukopenia and enterocolitis [17]. Liver toxicity can be possibly avoided with careful maintenance of levels below that target level, so therapeutic drug monitoring has been recommended [18], in particular in the presence of renal impairment. This is discussed in more detail in the chapter by Drs Howard and Hope. The mechanism of toxicity is not fully clear, but it is speculated that 5-FC is converted to 5-FU by the intestinal microbiota [19]. Reabsorption of 5-FU may then cause both local as well as systemic toxicity.

3 Susceptibility

In general, 5-FC is not very active against *Aspergillus* spp. under standard test conditions [20], and *Aspergillus* spp. are therefore not susceptible to 5-FC in vitro [6, 21, 22] However, the in vitro activity of a drug is affected by a variety of test conditions, such as incubation time, test medium, medium pH, and endpoint determination criteria. TeDorsthorst and colleagues showed that the activity of 5-FC against a variety of filamentous fungi is pH-dependent [7]. For *A. fumigatus, A. flavus and A. terreus* there was a significant difference in MIC between values measured at pH 7 and pH 5 [6], with MIC coming down to ranges that are clinically relevant when determined at pH 5 (Table 1). This was shown in RPMI as well as in YNB and compares well for similar effects found in yeasts [21, 23]. Figure 2 shows the pH effect in a more detailed manner for one *Aspergillus fumigatus* isolate. The MIC decreases dramatically immediately below values of pH = 7. In a collection of 50 clinical *A. fumigatus* isolates the median MIC decreased from 128 mg/l at pH 7 to 0.125 mg/l

Table 1 MIC_{50} (range, in mg/l) of 5-flucytosine against 21 *Aspergillus* isolates determined in two different media at two different pH values

	RPMI 1640		YNB	
Species (*n*)	pH 5	pH 7	pH 5	pH 7
A. fumigatus (10)				
ITZ susceptible (5)	0.063 (0.031– 0.25)	16* (2 – 128)	0.063 (0.016 – 0.25)	16* (1 – 64)
ITZ resistant (5)	0.188 (0.063 – 0.5)	24* (2 ≥ 256)	1 (0.125 – 1)	48* (8 – 256)
A. flavus (5)	0.25 (0.125 – 0.5)	1.5* (0.25 – 2)	0.25 (0.125 – 0.5)	3* (2 – 4)
A. terreus (6)	0.063 (0.063 – 0.125)	2* (0.5 ≥ 256)	0.25 (0.063 – 1)	4* (1 – 64)
All isolates (21)	0.125 (0.031 – 0.5)	4 (0.25 ≥ 256)	0.25 (0.016 – 1)	8 (1 – 256)

Note: MIC of 5-flucytosine were read at the lowest concentration that showed no more than 50% growth in comparison with that of the growth control (MIC-2). Asterisks indicate a statistically significant difference between MIC found at pH 5.0 and 7.0 ($p \leq 0.01$). Modified from [6].

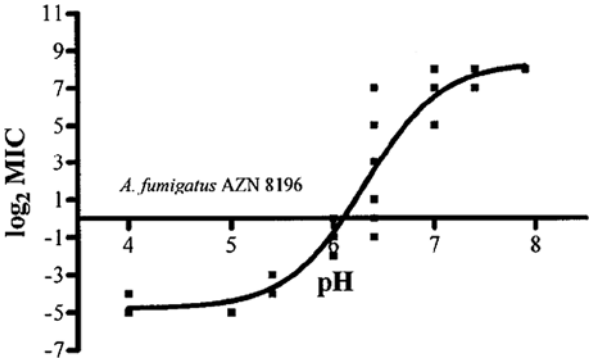

Fig. 2 Effect of pH on the minimum inhibitory concentration (MIC) of 5-flucytosine in an *Aspergillus fumigatus* isolate. Reproduced from TeDorsthorst et al., with permission [31]

at pH 5 (Verweij et al., submitted). Although a significant decrease was observed for 48 of 50 (96%) isolates, two isolates retained a high MIC at pH 5 [24]. This observation is in sharp contrast with amphotericin B. This drug shows an increase in MIC at lower pH values.

4 In Vivo Activity

Although many animal studies have been performed looking at the effect of 5-FC in yeast infections, few studies have been performed to determine the effect of 5-FC in *Aspergillus* infections. This has been due to the relatively weak activity of the drug in vitro, but also because *Aspergillus* models of infection are relatively difficult to set up and standardise. The studies that have been performed have shown relatively little effect of 5-FC [25–28]. However, since the pH at the site of infection may be lower than the pH at which susceptibility testing is performed due to necrosis [29], the production of organic acids by fungi [30], or lysosome-activity of granulocytes

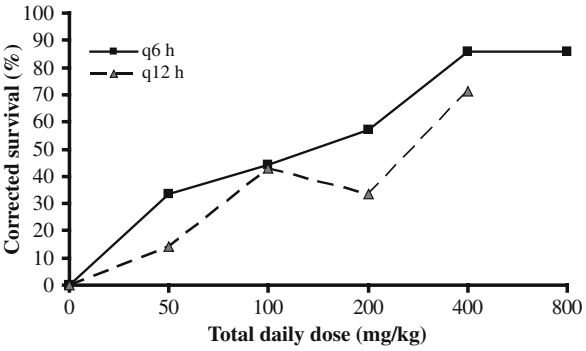

Fig. 3 Efficacy of 5-flucytosine in a non-neutropenic mouse model of *Aspergillus* infection. Increasing doses of 5-flucytosine were administered either q6h or q12h and resulted in increased survival. Reproduced from TeDorsthorst et al., with permission [7]

and macrophages [31], 5-FC might have retained some efficacy in vivo. In a non-neutropenic animal model of infection with an *A. fumigatus* isolate using survival as an endpoint, TeDorsthorst and colleagues showed a dose dependent activity of 5-FC (Fig. 3) [32] during and after 7 days of treatment. Flucytosine was administered following three different dosing regimens with intervals of 6, 12 and 24 h. Although there seemed to be some effect of dosing interval, this was not significant. In addition, the pharmacodynamic index that best correlated with efficacy appeared to be the AUC/MIC and not T>MIC (Fig. 4), indicating that it is the total daily dose that best correlates with efficacy. Since the MIC was pH-dependent, 128 mg/l at pH 7 and 0.5 mg/l at pH 6, this may explain the efficacy found in this model. However, 5-FC was not effective against another isolate that retained a high MIC at pH 5, indicating that in this model efficacy of 5-FC could be explained by a pH-dependent MIC. Furthermore, the MIC value, measured at pH 5, showed a good correlation with efficacy in this model of invasive aspergillosis (Verweij et al., submitted).

The slightly better correlation of efficacy with AUC/MIC rather than T>MIC is in contrast with results found for yeasts, where frequent dosing was more efficacious than once daily dosing [33, 34]. However, in these models animals were treated for a much shorter period of time, and this may have had an effect on the pharmacodynamic characterization – as has also been shown for antimicrobials [35].

5 Activity in Combination

Flucytosine has been studied in in vitro combination studies, mainly using a checkerboard approach. However, the results derived from checkerboards are ambiguous, both with respect to the method itself, the reading of the results as well as interpretation of the results [36], in particular when the fractional inhibitory concentration (FIC) is used as an endpoint. In their study of the effect of 5-FC in combination with amphotericin B or itraconazole, TeDorsthorst and colleagues showed that, depending on the endpoint used to determine the FIC (MIC-0, MIC-1 or MIC-2), the combination was either synergistic, indifferent, or antagonistic (Fig. 5). New approaches such as a surface response do show promise

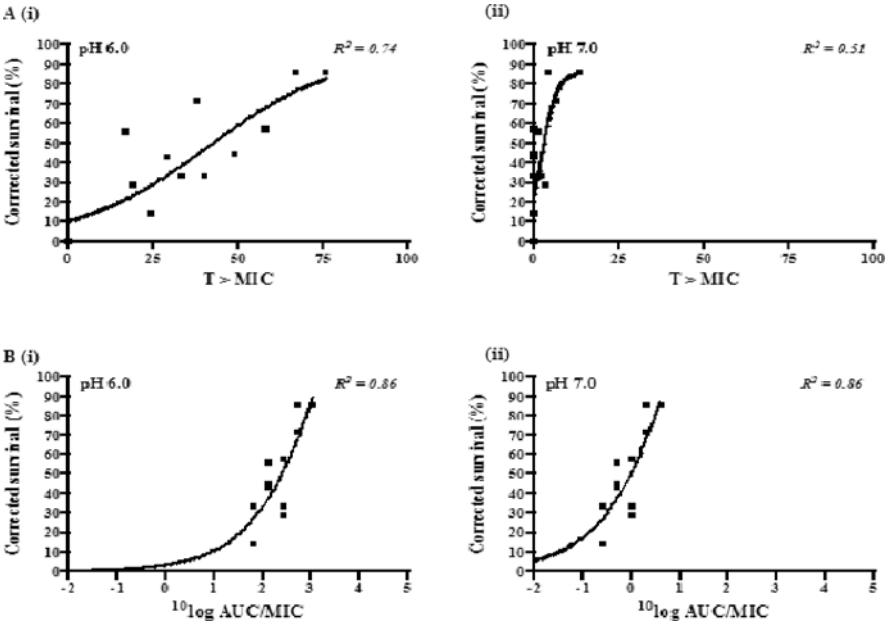

Fig. 4 Pharmacodynamic relationships of 5-flucytosine in a non-neutropenic mouse model of *Aspergillus* infection, indicating that AUC/MIC (area under the curve divided by the minimum inhibitory concentration) is best correlated with efficacy. Since the MIC is lower at a lower pH, the AUC/MIC curve is shifted to the right using MIC values obtained at a pH of 6. Reproduced from TeDorsthorst et al., with permission [7]

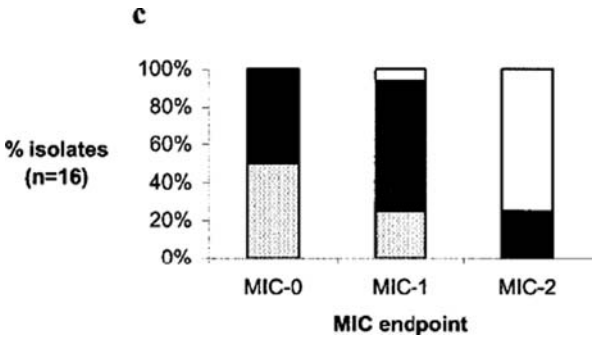

Fig. 5 Interactions found by using different minimum inhibitory concentration (MIC) endpoints for the determination of the fractional inhibitory concentration (FIC) index for the combination of itraconazole and 5-flucytosine. *Shaded*, antagonism; *black*, indifference; *white*, synergism. Reproduced from TeDorsthorst et al., with permission [6]

and are probably more relevant than the FIC approach. In the TeDorsthorst study the response surface approach as described by Greco [37], indeed gave more consistent results. However, the correlation between in vitro interaction studies and in vivo efficacy remains to be determined. Thus, even when in vitro studies do show

synergy or antagonism, it is not clear what the impact is for clinical use. In conclusion, there are no unambiguous data showing that there is a synergistic treatment effect of 5-FC with other drugs. However, because of the pharmacokinetic properties of 5-FC it might have some use to treat *Aspergillus* infections in body compartments where concentrations of other antifungals are relatively low.

6 Conclusions

Most current guidelines do not recommend the use of 5-FC for the treatment of invasive aspergillosis [38], although some guidelines recommend its use in difficult to treat infections, such as *Aspergillus* osteomyelitis [39]. The main reason is that there are no unequivocal clinical data that do show an additional value of 5-FC in *Aspergillus* infections. This applies both to single drug therapy as well as combination therapy. However, based on preclinical data, it could be hypothesised that 5-FC could be effective if the *Aspergillus* isolate shows a low MIC when determined at a pH of the medium <7. In the absence of convincing clinical evidence, the clinical use of 5-FC to treat *Aspergillus* infections will remain limited.

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Terbinafine

Manuel Cuenca-Estrella and Juan Luis Rodriguez-Tudela

Abstract Terbinafine belongs to the allylamine group of antifungal agents. It exhibits a broad spectrum of activity in vitro which includes *Aspergillus* spp. although a significant rate of *Aspergillus fumigatus* clinical isolates have decreased susceptibility in vitro to that compound. This chapter summarises the mechanism of action, spectrum of activity and clinical uses of this agent and details the susceptibility profile in vitro of *Aspergillus* spp. to terbinafine. The doubtful clinical effectiveness of terbinafine in the treatment of aspergillosis is also discussed. Finally, the usefulness of combination therapy with terbinafine is analyzed.

Keywords Allylamines · Combination therapy with terbinafine · Resistance to terbinafine · Treatment of aspergillosis with terbinafine

Contents

1 Mechanism of Action, Spectrum of Activity and Clinical Uses of Terbinafine

Terbinafine is a member of allylamine group of antifungal agents which includes naftifine as well. Allylamines inhibit ergosterol biosynthesis via inhibition of squalene epoxidase. This allows for a highly selective mode of action on fungal cells,

M. Cuenca-Estrella (✉)
Servicio de Micología, Instituto de Salud Carlos III, Centro Nacional de Microbiología, Madrid, Spain
e-mail: mcuenca-estrella@isciii.es

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and little cross-reactivity with mammalian targets. Terbinafine is a fungicide agent since its effect causes the death of the fungal cell due to the increased membrane permeability mediated by the accumulation of high concentrations of squalene [1].

Terbinafine is a lipophilic compound very active in vitro against a variety of yeasts and filamentous fungi such as *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., and the dermatophytes [2–5].

This antifungal agent is available in topical and oral formulations. It is rapidly absorbed and widely distributed in body tissues following oral administration. It achieves high concentrations in skin, nail, and fatty tissues and has low adverse side effects because of its high selectivity for fungal enzymes [1, 6, 7].

Terbinafine is considered the drug of choice today to treat various dermatomycosis. It exhibits high rate of efficacy after a relatively short course of therapy compared with other agents used in the treatment of superficial fungal infections. This allylamine is very efficient for the management of tinea capitis, tinea corporis, tinea cruris, tinea pedis, and tinea manuum at oral doses of 250 mg/day for 2–6 weeks [8, 9].

The properties of terbinafine are particularly significant to treat dermatophyte infection of the fingernails or toenails [6]. Onychomycosis is an infection difficult to treat which requires longer therapeutic course with agents as fluconazole and itraconazole. However terbinafine has shown to be at least as effective as its alternatives when used at doses of 250 mg/day for 6–12 weeks. Many experts maintain that oral terbinafine in combination with topical antifungal agents is the therapeutic regimen of choice in the treatment of onychomycosis caused by dermatophytes and by other fungal species [10–12]. Onychomycosis due to *Aspergillus* species is discussed by Dr. Negroni elsewhere in this book.

With regard to the use of terbinafine to treat deep mycoses, small clinical case series have revealed that the monotherapy with terbinafine could be also efficacious in the management of refractory pulmonary aspergillosis of non-immunosuppressed patients, and other studies indicated that it could be effective in treating subcutaneous and some systemic mycoses as well such as sporotrichosis and chromoblastomycosis [1, 9, 13–15].

2 Profile of Activity In Vitro Against *Aspergillus* Species

As described above, terbinafine exhibits a good activity in vitro against most of *Aspergillus* species [3, 16, 17]. However, according to data from several reports and from the Spanish Medical Mycology Reference Laboratory, the allylamine compound has a weak activity in vitro against *Aspergillus fumigatus*, the most common species of *Aspergillus* [12]. Table 1 displays the data of profile susceptibility in vitro for the seven most common organisms belonging to *Aspergillus* genus. *A. fumigatus* is the most resistant species to terbinafine in vitro, with geometric mean (GM) of minimal inhibitory concentration (MIC) values around 3 mg/l. Several reports have indicated that average plasmatic levels of oral terbinafine at doses of

Table 1 In vitro susceptibility data of *Aspergillus* species to terbinafine. Data from Spanish Medical Mycology Reference Laboratory

Species	Number of isolates	Geometric means	MIC_{90}	Range	Rate of strains with MIC ≥ 4 mg/l (%)
A. fumigatus	608	2.9	8.0	0.03–32.0	63
A. flavus	155	0.16	1.0	0.15–4.0	2.5
A. terreus	221	0.25	1.0	0.03–32.0	1.5
A. niger	115	0.32	1.0	0.03–4.0	0.5
A. nidulans	45	0.42	1.0	0.03–32.0	2
A. sydowii	44	0.17	1.0	0.03–4.0	2
A. versicolor	40	0.45	1.0	0.03–4.0	2
Total	1,228	0.85	4.0	0.015–32.0	32

Note: MIC values in mg/l. MIC_{90}: MIC value including 90% of isolates.

250 mg/day stand at 2–4 mg/l [6, 7, 18]. Clinical breakpoints of terbinafine have not been establish yet, but from an epidemiological point of view it should be noted that more than 60% of *A. fumigatus* clinical strains exhibit a MIC value of terbinafine ≥4 mg/l what means that many isolates may be resistant in vitro to the allylamine.

Mechanisms of resistance in vitro of *Aspergillus* to terbinafine have been analyzed at molecular level. Extra copies of the *A. fumigatus* gene encoding squalene epoxidase have been observed in strains which had resistance to terbinafine [19]. Replacement of phenylalanine with leucine at position 391 in squalene epoxidase gene has been identified as being responsible for terbinafine resistance in mutants of *Aspergillus* [20]. In addition, it has been reported recently that terbinafine resistance could be mediated by overexpression of salicylate 1-monooxygenase, a well-characterized enzyme in bacteria with capability to degrade the naphthalene ring. It suggests that the increase of the monooxygenase could confer terbinafine resistance due to degradation of the naphthalene ring contained in the allylamine [21], mechanism of resistance which has not been described to other families of antifungal compounds.

With regard to other *Aspergillus* species, *A. flavus*, *A. terreus, A. niger*, *A. nidulans*, *A. sydowii* and *A. versicolor* are susceptible in vitro to terbinafine with average GM of MIC values <0.5 mg/l. Percentages of clinical strains belonging to those species with MIC values ≥4 mg/l are less than 3%.

Table 2 shows susceptibility results for infrequent species of this genus. Most of isolates of uncommon species of *Aspergillus* are susceptible in vitro to terbinafine [22]. The genus *Aspergillus* is undergoing extensive taxonomic re-evaluation and a rearrangement of most species has been proposed. Classification of those species requires sequencing of different targets of DNA such as ribosomal DNA (internal transcriber spacers and intergenic spacers) and β-tubulin gene. Isolates in Table 2 were classified by DNA sequencing. It should be notes that some uncommon species seem to be resistant in vitro to amphotericin B and azole agents [23].

Table 2 In vitro susceptibility data of uncommon *Aspergillus* species to terbinafine. Data from Spanish Medical Mycology Reference Laboratory

Species	Number of isolates	Geometric means	Range	Rate of strains with MIC ≥ 4 mg/l (%)
Aspergillus lentulus	9	1.85	0.50–2.0	0
Aspergillus ustus	9	0.41	0.12–2.0	0
Aspergillus alliaceus	9	0.21	0.03–32.0	10
Neosartorya hiratsukae	5	0.31	0.06–0.25	0
Neosartorya pseudofischeri	5	0.70	0.50–2.0	0
Aspergillus niveus	4	0.25	0.25–0.50	0
Aspergillus ochraceus	4	0.19	0.03–0.12	0
Aspergillus wentii	3	0.50	0.25–2.0	0
Aspergillus fumigatiaffinis	3	1.5	1.0–2.0	0
Aspergillus sclerotium	2	0.17	0.12–0.25	0

Note: MIC values in mg/l.

3 Clinical Use of Terbinafine in *Aspergillosis*

Terbinafine has been only occasionally used in the treatment of aspergillosis due to various reasons. It was commercialized to treat dermatophytosis and that is still its main clinical indication. There are no clinical trials comparing the efficacy of terbinafine with those of antifungal agents with clinical indications against aspergillosis and animal model studies showed that the allylamine is ineffective to control experimental brochopulmonary aspergillosis in rats [24]. In addition, the lack of an intravenous formulation of terbinafine does not allow for its use as monotherapy in patients suffering from severe cases of invasive aspergillosis.

Some studies have indicated that terbinafine is less effective in rodent models because of a rapid hepatic first-pass effect and that is metabolized differently in humans than in rats [14]. Taking into account those results, terbinafine has been evaluated in the management of some patients with pulmonary aspergillosis. Reports depict about 30 cases with lower respiratory tract infections due to *Aspergillus* in non-immunocompromised patients [14, 15, 25] and seldom in immunosuppressant individuals [26, 27]. Terbinafine was used as salvage therapy in chronic and subacute aspergillosis cases which were unresponsive to the usual antifungal treatment. Terbinafine was administered at doses ranging from 5 to 15 mg/kg/day depending on the clinical severity of the disease, and was given for 90–270 days depending on clinical progress and compliance. Partial response with marked clinical and radiological improvement was observed in half of the cases. These results suggest that there is not sufficient evidence to recommend terbinafine as therapy of pulmonary aspergillosis.

However, terbinafine has shown having effectiveness to treat superficial aspergillosis such as cutaneous infections and external otitis [9, 27], and more commonly onychomycosis. Non-dermatophytic onychomycoses represent 1.5–17.5%

of all fungal nail infections [12]. Epidemiological studies have indicated that *Aspergillus* spp. are an emerging pathogen of toenail onychomycosis [28, 29]. Indeed, after *Scopulariopsis* spp. the genus *Aspergillus* seems to be the second most common cause of non-dermatophytic onychomycosis. A clinical trial has evaluated terbinafine in the treatment of *Aspergillus* onychomycosis [12]. The allylamine was administered intermittently, 500 mg/day for 1 week each month for 3 months. The study included 34 cases of onychomycosis caused by *Aspergillus* spp. what represented 2.6% of all onychomycoses. Their clinical features were chalky deep white nail, rapid involvement of lamina and painful perionyxis without pus. At the follow-up, 12 months after the start of therapy with pulsed terbinafine, clinical and mycological recovery was confirmed in 30/34 patients (88%), indicating that terbinafine is particularly effective in the treatment of *Aspergillus* spp. nail infections.

4 Combination Therapy with Terbinafine

Another point to consider is the combination therapy with terbinafine. Rationale to use antifungal compounds in combination is to improve the clinical results of monotherapy. Theoretical benefits of combination can be to achieve a greater effect for the usual dosage of each drug, equally efficacy for a lower dose of each drug, and pharmacological advantages in which one drug clears infection from one body system whilst the other drug clears it from another site. In addition, combination therapy could be utilized in an attempt to prevent or delay the emergence in vivo of resistant populations of the pathogenic fungus [30].

From the mechanistic point of view, combinations of azoles and terbinafine are predicted to exhibit positive interaction since they are acting at different steps of the same pathway, the ergosterol biosynthesis. This theoretical concept has been corroborated in several studies in vitro. Combinations of terbinafine with fluconazole, itraconazole, voriconazole or posaconazole have shown synergy in vitro against species of *Candida*, *Aspergillus* and *Zygomycota*, even against *Candida* isolates with resistance to fluconazole and itraconazole-resistant *Aspergillus* strains [31–37]. A study based on the response surface modelling revealed that the most potent combination against *Aspergillus* spp. was itraconazole plus terbinafine [38]. Other works described the effects in vitro of several combinations of antifungal agents against *Scedosporium prolificans*, a multi-resistant species [39]. Combinations of terbinafine with miconazole, voriconazole e itraconazole showed a potent positive interaction in vitro [40, 41].

The interaction of terbinafine and amphotericin B or 5-flucytosine has been assessed as well. Checkerboard and time-kill curve studies have indicated that these combinations were generally indifferent or antagonist against *Aspergillus*. Regarding other fungal species, one study showed that amphotericin B plus terbinafine had synergistic effects against a 20% of strains of *Zygomycota* tested. In addition, other work signalled an additive effect of this combination against the majority of isolates of *Zygomycota* analyzed apart from *Rhizopus oryzae* [31, 32, 34, 40].

Clinical data on the question of efficacy of combinations with terbinafine to treat aspergillosis are discouraging. A small, randomized study compared amphotericin B plus placebo with amphotericin B plus terbinafine (750 mg daily) in the treatment of invasive aspergillosis [42]. The study showed a significant higher mortality in the combination group. Antagonism between terbinafine and amphotericin B had been previously reported in a rabbit model of experimental invasive aspergillosis [43]. The efficacy of this combination has been understandably questioned.

But on the other hand, some reports have documented successful treatments of individual cases of mycoses caused by *Aspergillus* species [27, 44] particularly by species other than *A. fumigatus* such us *Aspergillus ustus* [45–48].

Finally, some reports have communicated successful treatments of fungal infections by other species with terbinafine plus azole agents. To begin with, a patient with oropharyngeal candidosis due to a fluconazole-resistant strain of *C. albicans* was recovered with a combination of fluconazole plus terbinafine [49]. An invasive facial infection due to *Pythium insidiosum* and one case of refractory chromoblastomycosis by *Fonsecaea pedrosoi* were successfully treated with itraconazole plus terbinafine [50, 51]. Notably, combinations of voriconazole plus terbinafine have resulted in the cure or control of deep infections due to *S. prolificans* [52–55]. Theses species are resistant to all systemic antifungal agents available today [56], and disseminated infections are almost uniformly fatal. Occasionally, combination therapy was given in addition to aggressive surgical debridement [30].

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PART II
INVASIVE PULMONARY ASPERGILLOSIS

Epidemiology of Invasive Pulmonary Aspergillosis

Carol A. Kauffman and Nelson P. Nicolasora

Abstract The epidemiology of IA, the major invasive mould infection in immunocompromised patients, has evolved over the last several decades. During the 1990s, increasing morbidity and mortality from these infections, particularly amongst the increasing numbers of patients being treated for haematological malignancies and those undergoing allogeneic haematopoietic stem cell transplantation, became a universal experience in many tertiary care medical centres. Changes in transplantation practices, including prophylaxis directed against *Candida* spp. and viral infections, helped to redefine the population at risk and the timing of invasive aspergillosis during the post-transplantation period. Molecular epidemiological studies of these ubiquitous organisms have improved our understanding of the epidemiology of these infections in the hospital and have documented emerging *Aspergillus* species causing infection. Recent years ushered in an era of newer mould-active agents, the echinocandins and the extended-spectrum triazoles, voriconazole and posaconazole, to treat and prevent invasive fungal diseases. The potential impact of these antifungal agents in reshaping the epidemiology of invasive mould infections is yet to be established, but early reports from some medical centres have noted a decline in the incidence of invasive aspergillosis and the emergence of non-*Aspergillus* mould infections.

Keywords Invasive aspergillosis · Epidemiology · Haematological malignancy · Haematopoietic stem cell transplant

C.A. Kauffman (✉)
Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Medical School, Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, MI, USA
e-mail: ckauff@umich.edu

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Contents

1 Introduction

The epidemiology of invasive aspergillosis (IA) is dependent on the population of susceptible hosts and the environmental exposure to the organism, and is influenced by the prophylactic measures taken to prevent this infection (Fig. 1).

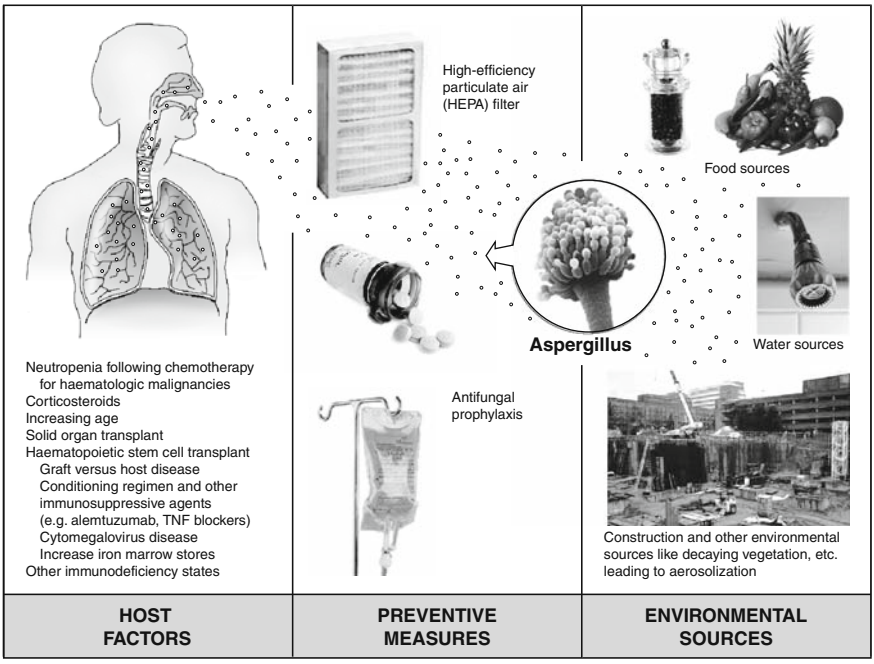

Fig. 1 Schematic diagram showing primary sources of *Aspergillus* spp., key factors that predispose patients to IA, and measures employed in hospitals to prevent infection with these moulds

During the 1990s, increasing numbers of cases of IA were reported from many tertiary care medical centres, some of which noted an incidence of ≥10% in certain populations [1–8]. The reason for this increase was likely due to the expanding population of immunocompromised patients, who were surviving longer because of improved treatment of their underlying condition, changes in transplantation practices, and institution of effective prophylaxis for common infections, such as those caused by *Candida* species and cytomegalovirus (CMV) [9, 10]. These patients were often profoundly immunosuppressed, increasing the risk for invasive mould infections. Mortality rates exceeded 50% and reached 90% in allogeneic haematopoietic stem cell transplant (HSCT) recipients [3, 11–13]. In some centres, the increase in cases of aspergillosis was accompanied by shifts in the species of *Aspergillus* causing infection [14, 15]. In the last several years, the introduction of improved mould-active antifungal agents, including the echinocandins and the extended-spectrum triazoles voriconazole and posaconazole, has improved treatment options for IA but has also been associated with an apparent decline in aspergillosis and an increase in non-*Aspergillus* mould infections in some medical centres [16].

This chapter will describe the epidemiology of IA, initially from the perspective of the organism and then from the perspective of the host, with emphasis on epidemiological features in patients who have haematological malignancies or have received a HSCT. Specifies about IA in other populations are discussed in detail in separate chapters among this book.

2 Organism Factors

The epidemiology of aspergillosis in immunocompromised patients involves exposure both inside and outside the health care setting. *Aspergillus* species are ubiquitous in the environment; they can be found in decaying vegetation, water, soil, building materials, dust, and various foodstuffs. Because of this, it is difficult if not impossible to avoid the organism in the course of everyday living [17]. The extent of exposure is one factor in the risk for development of infection, but few data are available that quantify this for individual patients. Many studies have been performed to document the concentrations of *Aspergillus* conidia in the air, both inside and outside the hospital setting. Wide fluctuations are common, intermittent bursts of large numbers of conidia have been noted periodically, and the link between numbers of conidia in the ambient air and development of infection in individual patients is difficult to prove [18].

Most of what we know is derived from outbreaks in which exposure is likely to have been to large numbers of *Aspergillus* conidia from a discrete point source. Even in outbreaks, the source of the organism has often not been defined. Simply finding the same species in the environment and as a cause of a patient's infection is not adequate because there are numerous different strains that exist in the environment [17, 19]. Molecular typing methods are now required to establish a link between an environmental source and an infecting strain [20].

2.1 Aspergillus *Species*

Very few of the over 900 *Aspergillus* species that have been identified have been reported to cause human disease. In a study of *Aspergillus* isolates recovered from clinical specimens in 1995 in 24 North American tertiary care medical centres that were part of the Mycoses Study Group, the leading causes of IA in all patients were *A. fumigatus*, causing 67% of infections, *A. flavus*, 16%, and *A. terreus*, *A. niger,* and *A. nidulans*, each less than 5% [21]. A subsequent report from 19 United States of America (USA) transplant centres, noted that the *Aspergillus* species isolated from HSCT recipients who had IA were *A. fumigatus* 56%; *A. flavus* 19%; *A. terreus* 16%; *A. niger* 8%; and *A. versicolor* 1% [15]. These data in HSCT recipients contrast with earlier single centre data in HSCT recipients in which approximately 90% of cases were due to *A. fumigatus* [6]. Overall, *A. fumigatus* remains the most important cause of IA in all types of immune compromised states and in all forms of aspergillosis [17]. Most cases are sporadic, but outbreaks, many of which are hospital-associated, have been reported [19].

A. flavus, although isolated less often, is almost always associated with invasive infection and rarely with chronic pulmonary forms of aspergillosis [21, 22]. Outbreaks of *A. flavus* infection related to environmental contamination have been noted [20, 23, 24], but many cases are sporadic. *A. flavus* is the predominant cause of IA in HSCT recipients in Hong Kong, in contrast to data from the USA [25]. *A. flavus* is also particularly frequent as an agent of both invasive and allergic aspergillosis in India and in the Middle East [26]. Climate factors particularly temperature and humidity are important determinants of the presence of *Aspergillus* species in the air, with *A. flavus* being particularly frequent in certain countries and hospitals. *A. niger* is rarely a cause of invasive infection in immune compromised patients; this species is more likely to cause chronic pulmonary infection and colonise abnormal airways [21, 27].

In recent years, there have been an increasing number of reports of the isolation of *A. terreus*, a species that is considered resistant to amphotericin B, from immune compromised patients [14, 28, 29]. At the University of Alabama (USA), *A. terreus* isolates accounted for only 1.5% of *Aspergillus* isolates in 1996, but rose to 15.4% of all isolates by 2001 [14]. A review of 13 cases from another medical centre established the propensity of *A. terreus* to cause invasive infection in immunosuppressed hosts [28]. Of 83 cases included in a multi-centre retrospective review of *A. terreus*, 65% of patients had a haematological malignancy and 45% had undergone HSCT [29].

A. terreus infections occur worldwide, but most cases have been reported from warmer climates. In hospitals, *A. terreus* has been isolated from showers and air samples [30], and a hospital-associated outbreak has been reported in which *A. terreus* strains found in soil from potted plants were shown to be the same as those causing infection in four of nine leukemic patients [31]. In one investigation, inhalation with amphotericin B was found to be a risk factor for the acquisition of *A. terreus* [32].

Almost all cases of *A. ustus* infection have occurred in recipients of HSCT [33, 34]. This species is resistant to many different antifungal agents, including voriconazole, for some isolates. The environmental niche for *A. ustus* has not been defined.

2.2 Sources of Aspergillus *Species*

2.2.1 Air and Ventilation Systems

Most patients appear to develop aspergillosis following inhalation of conidia into the alveoli or the upper airways. Sampling of outside air routinely reveals *Aspergillus* species, and in hospitals and other types of buildings, *Aspergillus* species can be found in varying quantities [35, 36]. In some instances, variations in the number of colony forming units in air samples inside hospitals have been linked to corresponding changes in infection rates in immune compromised patients [37], but in other studies, this correlation could not be verified [38].

Outbreaks have been ascribed to aerosolisation of conidia from a discrete point source. These sources have included insulation, fire-proofing materials, and carpeting that were heavily contaminated with *Aspergillus* spp. [20, 39, 40]. Faulty ventilation systems have allowed increased numbers of conidia to be dispersed. This has been associated with contaminated air filters and defects intrinsic to air handling systems [23, 24, 37, 41]. In most cases, nearby construction was associated with the initial contamination of the ventilation system, which then dispersed the conidia [24, 37, 41–43].

Current guidelines recommend that allogeneic HSCT recipients should be placed in rooms that have >12 air exchanges per hour and point-of-use high-efficiency (>99%) particulate air (HEPA) filters that are capable of removing particles $\geq$0.3 mm in diameter [5]. This approach has been used to reduce the incidence of nosocomial outbreaks of aspergillosis from nearby construction sites and during renovation projects [44–46], but has not always been successful [47, 48]. In addition, sealing off patient areas from construction activity with impermeable barriers is necessary, as is inspecting the ventilation system to be certain that there is no air flow from the construction area [20, 49].

These outbreaks notwithstanding, most cases of IA in immune compromised patients arise sporadically and cannot be traced back to a specific instance of failure of a ventilation system or to nearby construction. For many patients, it is likely that exposure to *Aspergillus* may well have occurred at home or elsewhere outside of the hospital. Attempts to differentiate hospital-acquired from community-acquired infection generally assign disease onset after a certain defined period of hospitalisation as being hospital-acquired. For example, defining hospital-acquired aspergillosis as that occurring >7 days after admission and <14 days after discharge, revealed that about 70% of cases of aspergillosis were acquired in the community, even though hospital construction had been ongoing during the period studied [50]. Acquisition outside the hospital setting is especially likely for those HSCT

recipients who develop aspergillosis late after their transplant, often when they are on therapy with steroids for graft versus host disease (GVHD), an increasingly common risk factor for IA. Very little is known about risks in the home or other environments to which patients are exposed after they leave the hospital.

2.2.2 Water

Samples taken from showerheads, faucets, and shower drains yield *Aspergillus* species, but whether water is a major source of infection is still debated [20, 42, 51]. Anaissie et al. have developed strong arguments for water being a major source of infection, and have documented one case in which molecular typing showed similarity between a strain found on the shower wall in the patient's hospital room and the strain causing the patient's infection [30, 51]. This group has found higher concentrations of *Aspergillus* conidia in bathrooms than in patient rooms and hallways, noting that aerosolisation from water-use areas can occur, and showed that assiduous cleaning of the shower area reduces colony counts of *Aspergillus* obtained by air sampling [51, 52]. These studies focused on the hospital environment. Others have shown that municipal water supplies in some cities are routinely contaminated with filamentous fungi, including *Aspergillus* spp. [53], but no studies have assessed the role of tap water or showering in transmission of *Aspergillus* in the home setting. The evidence for a possible water source for *Aspergillus* has led some transplant centres to restrict HSCT patients from taking showers while in hospital post-transplant.

2.2.3 Other Sources of *Aspergillus*

Several studies have surveyed foodstuffs to which patients might be exposed. Routinely, pepper is heavily contaminated with *Aspergillus* conidia, and other foodstuffs, including tea in bags, freeze-dried soups, and various fruits with "fuzzy" surfaces may carry *Aspergillus* spp. [54]. Although no outbreaks of aspergillosis have been linked directly to contaminated foods, many stem cell transplant units routinely restrict the use of those foods that are likely to be contaminated from their units [55].

An unusual source of infection with *Aspergillus* spp. is *Cannabis*. Marijuana cigarettes routinely yield *Aspergillus* species on culture, but infections linked to smoking marijuana have been rare and not surprisingly, only in immunosuppressed hosts [56] (this is discussed in more detail in a chapter about aspergillosis in drug addicts). Potted plants are generally excluded from units in which immune compromised patients are cared for because of the high yield of moulds, including *Aspergillus* species, from the soil [57]. At least one outbreak of aspergillosis has been traced by molecular methods back to potted plants on a haematology ward [31].

Amongst children, aspergillosis has been linked to contaminated adhesive tape and arm boards used to secure intravenous lines [58, 59] (for more on this, please refer to the chapters on aspergillosis in children and cutaneous aspergillosis). Aerosolisation that took place during dressing changes of a heavily colonised

abdominal wound in a patient who had undergone liver transplantation was shown by molecular typing studies to be responsible for subsequent IA in a second liver transplant recipient who was cared for in the same open unit [60]. Rarely, aspergillosis in solid organ transplant recipients has been traced back to transfer of the organism with donor organs [61].

3 Host Factors

Patients with haematological malignancies account for most cases of IA. In a multi-centre international study, patients who underwent HSCT and patients with leukaemia or lymphoma comprised 61% of all cases of IA [62]. Other underlying risk factors, including solid organ transplantation, acquired immunodeficiency syndrome, chronic granulomatous disease, and chronic pulmonary diseases, each contributed less than 10% of cases. This chapter will focus on risk factors for IA amongst patients with haematological malignancies and those who have received a HSCT; other chapters deal in depth with other risk groups.

3.1 Patients with Haematological Malignancies

IA occurs more often in patients with acute leukaemia than in patients with chronic leukaemia, lymphomas or multiple myeloma [63]. This has remained true, as noted in an autopsy series from M.D. Anderson Cancer Center (USA), from 1989 through 2003 [64]. Most patients who died with invasive fungal diseases in that series also had uncontrolled leukaemia. The use of steroids increases the risk for aspergillosis in patients with diseases, such as myeloma, that generally are at low risk for IA [65]. The risk of invasive infection by *Aspergillus* species and other filamentous fungi is strongly correlated with the degree and duration of neutropenia. In studies conducted several decades ago amongst patients who had acute myelogenous leukaemia and who underwent induction chemotherapy, the risk of IA increased from 1% per day for the first 3 weeks of neutropenia to 4–5% per day after 5 weeks [66] In another study, the incidence was shown to be as high as 70% amongst patients who had been granulocytopenic for ~1 month [67]. Repeated cycles of prolonged neutropenia and concomitant therapy with steroids further increase the risk for IA [63]. The case fatality rate for IA amongst patients who have haematological malignancies is approximately 50% [12]. The recovery of granulocytes is essential for survival in this population [63, 68].

3.2 Haematopoietic Stem Cell Transplantation

IA is the most common mould infection amongst HSCT recipients. Autologous transplantation is associated with a far lower risk of IA than allogeneic transplantation. The incidence of aspergillosis for autologous transplants has ranged from 0 to 10% in various centres [6, 8, 69, 70], but current data note a diminishing risk, with

most centres reporting no aspergillosis cases amongst their autologous transplant recipients who have appropriate engraftment [11, 15, 69]. The decreased incidence relates to the use of growth factors and improved stem cell harvesting, leading to shorter periods of neutropenia before engraftment [13]. After engraftment, there should be minimal risk for development of IA unless other factors, such as use of steroids for other conditions, intervene.

Amongst allogeneic HSCT recipients, the incidence of IA has ranged from 0 to 20% over the last several decades [6–8, 11, 15, 69]. In the 1980s and early 1990s, aspergillosis occurred primarily in the early post-transplant period prior to engraftment, and neutropenia during pre-engraftment was the predominant risk factor [8, 71, 72]. The report from Wald et al., noting a bimodal distribution, with cases occurring late after engraftment as well as in the early pre-engraftment period, signalled a change in the epidemiology of IA in this population [8]. This shift was associated with shortened periods of neutropenia pre-engraftment as a result of the use of non-myeloablative transplantation using reduced-intensity conditioning regimens and peripheral stem cells for transplantation [13, 73]. Associated with these regimens, however, was an increased risk of GVHD. Currently, most cases of IA are reported post-engraftment, and GVHD has emerged as a major risk factor for late-onset aspergillosis [1, 6, 11, 69, 74, 75]. Several authors have noted that the risk of IA increases with the severity of GVHD; thus, the greatest risk is for those patients who have either class III or IV acute GVHD or chronic extensive GVHD [25, 74, 76, 77].

It is likely that the management of GVHD with aggressive immunomodulating regimens is the primary reason the risk of invasive fungal infection is increased in patients who have this complication of transplantation. Use of alemtuzumab (Campath-1H) which depletes peripheral blood T and B cells for a prolonged period of time markedly increases the risk for invasive fungal infections [78]. Infliximab, a tumour necrosis factor (TNF)-alpha-antagonist, is also used in the treatment of GVHD, and has been noted to be associated with a high risk of development of IA [79]. However, it is likely that the most important risk factor is the use of high dosage of steroids for the treatment of GVHD [1, 6, 11, 69, 74, 80]. A recent study found that use of $\geq$1 mg/kg methylprednisolone daily for $\geq$21 days for treatment of GVHD was an independent risk factor for the development of IA; those requiring lesser amounts to control GVHD had a decreased risk of aspergillosis [69].

The source of transplanted stem cells is an important issue regarding risk for aspergillosis. The shift from bone marrow to peripheral stem cells has increased the incidence of late-onset aspergillosis but not early aspergillosis; this is probably because the incidence of chronic GVHD is higher in those receiving peripheral stem cells [73]. The incidence of IA is lower in recipients of HLA-matched sibling donor cells (2.3%) than in those who receive unrelated donor cells (3.9%) [15]. Use of umbilical cord blood stem cells has been identified as a significant risk factor for aspergillosis that usually occurs early after transplantation. Delayed neutrophil engraftment and the use of anti-thymocyte globulin as part of the conditioning regimen are potential explanations for this observation [6, 81]. T cell-depleted and

CD34-selected cells are other identified risk factors for early-onset aspergillosis in some series [6].

CMV, which has immunomodulating properties, has been recognized as a risk factor for invasive fungal infections in both solid organ transplant and HSCT recipients [6, 82]. The association between CMV infection and IA has been noted both pre-engraftment and post-engraftment [6]. Other risk factors for IA include older age and increased marrow iron stores [83, 84]. Interleukin-10 promoter gene single nucleotide polymorphisms may be associated with risk of IA regardless of HLA disparity and chronic GVHD; the clinical significance of this finding is still being defined [85]. A deeper discussion about the importance of host immune system in *Aspergillus* infections is provided by Dr. Romani in the initial chapters of this book.

Amongst HSCT recipients, case fatality rates have been noted to be 80–90% [12, 13, 25, 76]. Specific risk factors that were associated with poor outcome in the mid to late 1990s included GVHD and cumulative prednisolone dosage >7 mg/kg in one study from France [7] and daily prednisolone dose >2 mg/kg at the time of diagnosis in another study from Seattle (USA) [6]. A recent analysis from France of risk factors for death from aspergillosis showed the highest risk was for uncontrolled GVHD, 4.02 (confidence interval, CI 95% 1.54–10.49) and >2 mg/kg prednisolone daily at the time of diagnosis, 3.05 (CI 95% 1.43–6.49) [11]. However, the mortality rate in this latter study was only 62%, an improvement from earlier studies. Likewise, a recent study from Seattle notes mortality rates in patients treated between 2002 and 2004 that are half of those of at every measured time point than those noted in patients seen from 1990 to 2001 [86]. The mortality at 30 days was 33% versus 69% in the earlier years. Prednisolone dose >2 mg/kg daily at the time of diagnosis of IA remained a significant risk factor for death.

4 Impact of Prophylaxis on the Epidemiology of Aspergillosis

The introduction of fluconazole for prophylaxis in neutropenic patients and bone marrow transplant recipients was associated with a reduction in *Candida* infections, but not surprisingly, no change in mould infections. Itraconazole, an agent active against *Aspergillus* species, did not prove terribly useful because of intolerance, problems with absorption, and drug-drug interactions [87]. The recent use of broader spectrum triazole agents and echinocandins for prophylaxis has a greater potential to change the epidemiology of IA in the highest risk patients (please refer to the chapter on antifungal prophylaxis for IA for more detail). In a blinded, randomised, multi-centre trial comparing 400 mg fluconazole with 50 mg micafungin daily during the period of neutropenia (pre-engraftment) after HSCT, micafungin was shown to significantly reduce invasive fungal infections when compared with fluconazole. There was a trend toward a reduction in the number of *Aspergillus* infections, but the numbers were small and the differences were not significantly

different amongst the two arms [88]. However, this is not the major period of risk for IA in most HSCT recipients, and thus, it is unlikely that there will be major shifts in the epidemiology of IA in the HSCT population related to early prophylaxis with this echinocandin.

Two recent trials using posaconazole for prophylaxis showed the benefit of this agent in decreasing mould infections in high-risk populations [89, 90]. It is possible that shifts in the epidemiology of IA will occur as the use of this agent becomes more widespread. In the blinded, randomised, multi-centre trial reported by Ullman et al., HSCT recipients who had chronic extensive GVHD or Grade II-IV acute GVHD treated with intensive immunotherapy received either fluconazole, 400 mg daily, or posaconazole, 200 mg three times daily, as prophylaxis for up to 112 days post-transplantation [90]. Posaconazole was shown to be superior to fluconazole in reducing IA and in reducing the number of fungal-related deaths. In the open-label, randomised trial reported by Cornely et al., posaconazole was compared with either itraconazole or fluconazole, depending on which agent was preferred by the patient's physician, for prophylaxis during induction therapy for patients who had acute myelogenous leukaemia or myelodysplastic syndrome and were expected to have prolonged neutropenia related to induction chemotherapy [89]. Significantly fewer patients (only 2) receiving posaconazole developed IA when compared with those receiving fluconazole or itraconazole (20 patients), and the overall mortality was lower in the posaconazole arm. Thus, both of these studies show the potential for a more effective *Aspergillus*-active agent to decrease the risk of development of IA in these high-risk populations. Breakthrough infections with other moulds were noted uncommonly in both studies.

Preliminary results from a blinded, randomised trial of prophylaxis with either voriconazole, 200 mg twice daily, or fluconazole, 400 mg daily, have been reported [91]. In this trial, prophylaxis was given for the first 100 days after HSCT, but was extended to 180 days if patients were on high-dose steroids and thus at greater risk for invasive fungal diseases. There were only 7 cases of IA in the voriconazole arm, but 16 cases in the fluconazole arm, and breakthrough infections with other moulds were uncommon in both arms.

Of great interest is whether new mould infections will emerge in high-risk patients if aspergillosis declines with effective prophylaxis. This is especially problematic with voriconazole use and the emergence of the zygomycetes, against which this agent has no activity. Early single-centre case reports noted a rise in cases of zygomycosis in patients who were receiving prolonged voriconazole, mostly for prophylaxis in those who had severe GVHD [92]. Two subsequent reports that included larger numbers of patients similarly found an association between voriconazole use and an increase in zygomycete infections [16, 93]. This association however was not noted in the randomised prophylaxis trial with voriconazole noted above, perhaps reflecting differences in the "standard-risk" population studied in the trial and those reported in the individual case reports and small series. At this point, it is fair to say that the impact of these new prophylactic agents on the epidemiology of IA is unknown, but will likely be very interesting.

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Pathogenesis of Invasive Pulmonary Aspergillosis

Ronen Ben-Ami and Dimitrios P. Kontoyiannis

Abstract The study of the pathogenesis of invasive pulmonary aspergillosis has advanced significantly over recent years because of scientific achievements on two fronts: sequencing of the genomes of three *Aspergillus* species and elucidation of the innate immune mechanisms involved in defence against *Aspergillus* infection. The principal events that lead to invasive aspergillosis take place in the alveolus, where inhaled conidia germinate into invading hyphae that penetrate the respiratory epithelium. Pulmonary innate immunity against *Aspergillus* infection consists of resident and recruited phagocytes, soluble immune factors, and antimicrobial proteins. Various *Aspergillus* traits, including the production of pigment, antioxidants, proteases, adhesins, siderophores, and mycotoxins, are implicated as potential virulence factors. The complex interplay amongst these factors, usually in the setting of depressed immunity, forms the basis for this medically important opportunistic mycosis.

Keywords Angioinvasion · Innate immunity · Mycotoxin · Virulence factor

Contents

D.P. Kontoyiannis (✉)
Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA
e-mail: dkontoyi@mdanderson.org

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1 Introduction

Members of the genus *Aspergillus* are ubiquitous in the environment, where they inhabit soil and organic debris and play an important role in biodegradation and nutrient cycling. *Aspergillus* conidia (asexual spores) are continuously dispersed into the air, where they reach concentrations of up to 100 conidia/m^3 [1]. Despite the fact that hundreds of conidia are inhaled into the human lung every day, invasive pulmonary aspergillosis (IPA) remains a rare disease, a testament to the efficiency with which the innate immune system clears these infectious propagules. However, whereas inhaled *Aspergillus* conidia present no danger of an invasive infection to an immunocompetent host, the risk of life-threatening IPA is significant in individuals with severe, sustained immune suppression [2–4]. Another group of susceptible individuals, those who have hyperactive immune responses to inhaled *Aspergillus* conidia, are prone to hypersensitivity reactions. These reactions include asthma, allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitisation (SAFS), and allergic fungal sinusitis and are discussed in detail in another section of this book.

Over the past decade, we have witnessed the discovery of multiple genes and metabolic pathways that affect the fitness and pathogenic potential of *Aspergillus* species (Table 1). Sequencing of the genomes of *Aspergillus fumigatus* [5], *A. nidulans* [6], and *A. oryzae* has contributed to this effort [7]. In this chapter, we review the pathogenesis of IPA, as well as the different characteristics of IPA in immunologically diverse hosts.

Table 1 Genes implicated in *Aspergillus* virulence

Gene	Gene product	Function	Phenotype of deletion mutant	References
Adhesion to respiratory epithelium				
rodA, rod B	Rodlets	Conidial dispersal, resistance to macrophage killing	*ΔrodA*, normal virulence	[51]
Asp f2	Asp f2	Binding to laminin	NA	[49]
Melanin				
pksP	Polyketide synthase	DHN melanin synthesis, cAMP signal transduction	Hypovirulent	[92, 95]
cAMP signalling pathway				
acyA	Adenylate cyclase	cAMP signal transduction	Hypovirulent	[98, 99]
gpaB	G protein			
pkaC1	Protein kinase A			
Antioxidants				
cat1	Cat1	Mycelial catalase	Cat1(–)/Cat2(–) double deletion mutant	[102, 104]
cat2	Cat2	Mycelial catalase	hypovirulent	
catA	CatA	Conidial catalase	Normal virulence	
Thermotolerance				
cgrA	CgrA	Orthologue of *A. nidulans* gene involved in ribosome biosynthesis	Hypovirulent	[108]
thtA	NA	Function unknown; required for growth at 48°C but not 42°C	Normal virulence	[109]
Proteinase				
	Elastase	Elastase	Hypovirulent	[115]
Calcineurin pathway				
cnaA	Calcineurin A	Polarisation of hyphal growth,	Hypovirulent	[124]
calA	Calmodulin A	conidial morphology (rodlet layer)	NA	[125]
Siderophores				
sidA	SidA	Catalyses first common step in synthesis of intracellular and extracellular siderophores	Hypovirulent	[130]
sidC	SidC	Synthesis of the intracellular siderophores ferricrocin and hydroxyferricrocin	Hypovirulent	
sidF	SidF	Synthesis of the extracellular	Hypovirulent	
sidD	SidD	siderophores fusarinine C and triacetylfusarinine C	Hypovirulent	
sidG	SidG	Synthesis of triacetylfusarinine C	Hypovirulent	

Table 1 (continued)

Gene	Gene product	Function	Phenotype of deletion mutant	References
Mycotoxins				
res	Restrictocin	Ribonucleotoxin	Normal virulence	[136]
Asp f1	Asp f1			
	Mutagillin			
gliP	GliP	GT biosynthesis	Hypovirulent in steroid-treated but not in neutropenic mice	[140, 154]
gliZ	GliZ	Transcriptional regulator of GT production	Normal virulence	[157]
laeA	*LaeA*	Regulation of secondary metabolism	Hypovirulent	[155, 156]

2 The Study of *Aspergillus* Virulence

Defining virulence in *Aspergillus* species is difficult given the opportunistic nature of these pathogens. The net state of immunosuppression of the host is well recognised as the predominant factor influencing the risk of invasive aspergillosis (IA). To illustrate this point, genomic fingerprinting of environmental and clinical strains of *A. fumigatus* has generally failed to identify specific clades or morphological traits associated with IA as opposed to saprophytic growth [8–11]. These findings support the hypothesis that any strain of *A. fumigatus* has the potential of becoming invasive if inhaled at a sufficient quantity by a susceptible host.

The pathogenic species *A. fumigatus* has long been considered a stricktly asexual organism due to a cryptic sexual state that only becomes evident after prolonged (6 months) incubation [14]. Thus, traditional genetic methods that depend on sexual mating were not available to study genetic determinants of the virulence of this species. Researchers have used several approaches to overcome this obstacle. *A. nidulans*, although rarely implicated as causing aspergillosis in humans, has a sexual cycle and produces diploid forms under natural conditions; therefore, investigators have used this species extensively as a model for studying the virulence of *A. fumigatus* [12]. Because the genomes of both *A. fumigatus* and *A. nidulans* have been sequenced, identification of homologous genes has become straightforward. The discovery that a parasexual cycle may be induced in *Aspergilli*, including haploid organisms such as *A. fumigatus*, provides an additional tool for genetic studies [12]. In the parasexual cycle, two fungal strains can be induced to form stable heterokaryons when grown in close proximity to each other. The haploid nuclei of these two strains will fuse to form a single diploid nucleus [13]. The recent discovery of a sexual cycle in *A. fumigatus* (teleomorph *Neosartorya fumigata*) should help us to better understand the biology and evolution of this important species [14].

As with other human fungal pathogens, generation of mutants deficient in genes encoding putative virulence factors has been the mainstay for studying the

pathogenesis of *Aspergillus* species. The virulence of the loss-of-function mutant as compared with that of its isogenic wild-type counterpart is typically assessed using an animal model, most commonly an immunosuppressed rodent. The criteria for Koch's molecular postulates are met if the loss-of-function mutant is less virulent than the isogenic wild-type strain, and the virulence of the mutant is restored by the reintroduction of the wild-type gene [15]. The molecular techniques used for the generation and identification of *Aspergillus* mutants have been the subject of several comprehensive reviews [1, 12, 16].

Although investigators have successfully used these approaches to identify multiple genes of potential importance to *Aspergillus* pathogenicity (Table 1), no single gene or pathway solely accounts for the virulence of true opportunistic moulds such as *Aspergillus* species. Rather, *Aspergillus* virulence appears to be multifactorial, involving several genetic and possibly epigenetic traits. Hence, genes responsible for diverse fungal characteristics, such as thermotolerance, germination rate, hyphal growth rate, resistance to host killing mechanisms, ability to attach to and subsequently invade host epithelium, procurement of nutrients from the host environment, and production of secondary metabolites, all contribute to the pathogenicity of *A. fumigatus*. For the most part, however, the products of these genes cannot be viewed as virulence factors in the classic sense because they lack host-specific inhibitory function. Instead, these genes determine or regulate functions that are important for fungal growth both in the saprophytic state and in the host environment.

3 *Aspergillus* Species and Pathogenicity

Of the more than 185 known species of *Aspergillus*, fewer than 20 are known to infect humans, and four species (*A. fumigatus*, *A. flavus, A. terreus*, and *A. niger*) cause the vast majority of invasive infections. *A. fumigatus* is by far the predominant species that causes IPA [2, 17, 18]; therefore, its pathogenic traits have been studied in greatest detail. Disagreement exists as to whether this disparity amongst *Aspergillus* species is simply a consequence of the relative prevalence of conidia of different species in the environment or whether it reflects differences in the inherent pathogenicity of these species [18]. *A. fumigatus* was the predominant environmental *Aspergillus* species in some studies [20, 21], but others have found it to be less prevalent in air than other species [22–24]. For example, over a 7-month period of surveillance, *A. niger* was found in 56% of hospital air samples but only 17% of patient samples, whereas *A. fumigatus* was isolated from only 0.3% of air samples but 44% of patient samples [24]. Therefore, the relative success of *A. fumigatus* as a pathogen cannot be explained solely on the basis of its prevalence in the environment but rather should be viewed as the product of inhaled inoculums and intrinsic pathogenicity, which stems from small conidial size, the ability to grow at elevated temperatures, adaptation to the mammalian host environment, and possibly the production of mycotoxins.

4 Interaction of Inhaled *Aspergillus* Conidia with the Respiratory Epithelium

The majority of inhaled *Aspergillus* conidia are prevented from entering the lower respiratory tract by anatomical and mechanical barriers in the upper airways. Specifically, the respiratory epithelium lining the nasopharynx, oropharynx, trachea, and bronchi contains ciliated and mucus-producing cells, which together form the mucociliary apparatus. The labyrinthine structure of the upper airways, including the nasal turbinates and the sharp-angled branching of the main airways, favours entrapment of inhaled conidia by secreted mucus. Impacted conidia are either expelled from the airways by ciliary action and coughing or swallowed.

The conducting airways (trachea and bronchi) allow the passage of particles 2–10 μm in diameter. However, only particles ≤2 μm in diameter are able to reach the terminal airways and alveoli. Therefore, the small conidia of *A. fumigatus* (2.0–3.5 μm) and *A. terreus* (2.0–2.5 μm) readily traverse the terminal airways, whereas the larger conidia of *A. flavus* (3–6 μm) and *A. niger* (4–5 μm) are primarily deposited in the upper airways. Accordingly, *A. fumigatus* and, to a lesser degree, *A. terreus* are the major causes of IPA, whereas *A. flavus* and *A. niger* less commonly cause infection of the lower respiratory tract [2, 25]. In contrast, *A. flavus* has been implicated in a disproportionate number of infections of the paranasal sinuses and skin [18, 20], and *A. niger* primarily causes superficial disease such as otitis.

Conidia that reach the terminal airways come into direct contact with the epithelial cells lining the alveoli and are deposited on the epithelial surface or engulfed and internalised by these cells (Fig. 1). Moisture conditions and temperatures within the alveolar spaces favour germination of *Aspergillus* conidia, a process that initiates with rapid swelling (hydration) of conidia and continues with production of a germ tube, which extends to form hyphae, the tissue-invasive form of the fungus [26, 27]. Resident alveolar macrophages (AMs), which efficiently phagocytise inhaled conidia, form the first line of innate immune defence against aspergillosis. IPA develops if conidia and germlings evade immune effector cells and soluble components of innate immunity and traverse the alveolar-capillary barrier, which consists of alveolar epithelial cells (types I and II), a basement membrane, and capillary endothelial cells.

Epithelial cells themselves are important components of the innate immune system. The respiratory epithelium is able to resist fungal invasion and foster an inflammatory cytokine response through Toll-like receptor (TLR) 4 signalling in the presence of polymorphonuclear leukocytes (PMNLs) [28]. Epithelial cells produce a wide variety of natural antimicrobial peptides such as defensins, lysozyme, and lactoferrin [29–31]. Specifically, lactoferrin, an iron-scavenging molecule, inhibits conidial growth at concentrations comparable with those present in respiratory secretions [32]. The osmotic milieu of the respiratory airways may significantly influence respiratory mucosal resistance to *Aspergillus* infection. High salt

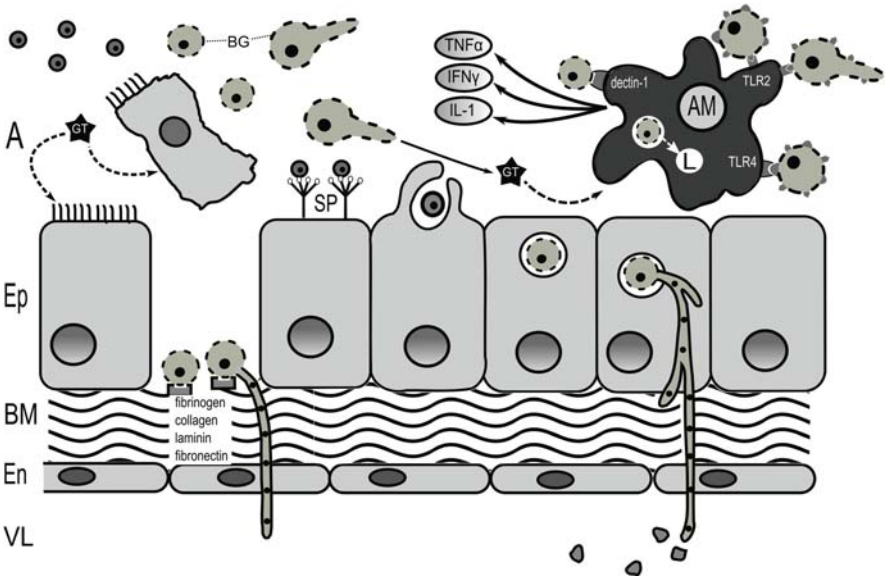

Fig. 1 Interaction of *Aspergillus* species with host defence mechanisms at the respiratory epithelial surface. Inhaled *Aspergillus* conidia face a number of innate defence mechanisms at the respiratory epithelial surface, including alveolar macrophages (AMs) and antimicrobial peptides such as surfactant protein (SP). Two routes of epithelial invasion by *Aspergillus* species are shown. In the first route, damaged epithelial cells are denuded, allowing conidia to bind to extracellular matrix proteins. In the second, more common route, conidia induce their own uptake by epithelial cells, where they can survive and be protected against immune effector cells. Following conidial swelling, a germ tube is formed, which penetrates through the epithelial and endothelial layers into the vascular lumen. Hyphal fragmentation and vascular dissemination may then ensue. *Aspergillus* species employ a number of tactics to evade the immune system, including phenotypic switching, inhibition of phagosome-lysosome fusion, masking of immunoreactive epitopes, and production of gliotoxin (GT), which has multiple immunosuppressive actions. A, alveolar airspace; BG, β-glucan; BM, basement membrane; Ep, respiratory epithelium layer; En, vascular endothelium layer; IFNγ, interferon γ; IL, interleukin; L, lysosome; TLR, Toll-like receptor; TNF, tumour necrosis factor; VL, vascular lumen

concentrations, as found in the airways of patients with cystic fibrosis, as well as exposure to steroids reduce the activity of epithelium-associated antimicrobial peptides, thus contributing to *Aspergillus* colonisation and infection in this susceptible population [31].

The pulmonary surfactant proteins SP-A and SP-D are collagen-containing C-type lectins (collectins) produced by type II pneumocytes. Surfactants contribute to airway mucosal resistance to infection by acting as opsonins, stimulating phagocytic cells and modulating inflammatory cytokine release [33]. Surfactant proteins enhance phagocytosis and killing of *A. fumigatus* conidia by AMs and PMNLs in vitro [34] and intransal administration of SP-D protectes mice against fatal IPA [35].

Patients with pre-existing bronchopulmonary disorders, such as chronic obstructive pulmonary disease (COPD) [36], bronchiectasis, and pulmonary cavities caused by tuberculosis or cystic fibrosis, are particularly prone to *Aspergillus* colonisation. These conditions alone are permissive yet insufficient to allow the development of full-blown IPA unless some degree of immune-suppression is present in conjunction with epithelial damage.

Aspergillus conidia are readily internalized by type II pneumocytes, endothelial cells, and, less efficiently, by tracheal epithelial cells [27, 37, 38]. Conidia induce their own endocytosis by type II pneumocytes. When conidia come into contact with type II pneumocytes, they trigger the formation of pseudopods from the pneumocyte cytoplasmic membrane, a process that requires intact host cell microtubules and microfilaments [38]. These pseudopods then proceed to engulf and internalize conidia. Internalized conidia are transported to late-stage acidic lysosomes, where the majority of conidia are destroyed. However, a small fraction of internalized conidia (~3%) survives within pneumocytes, and these conidia may serve as latent reservoirs of infection [39]. When germination of conidia occurs, it causes remarkably little damage to the host cell. In fact, conidia inhibit apoptosis of type II pneumocytes, an active process because it only occurs if the internalised conidia are viable [40]. The mechanism of inhibition of apoptosis in type II pneumocytes remains unknown. The intracellular compartment may act as a sanctuary for conidia, concealing them from immune surveillance. Therefore, the ability of *Aspergillus* conidia to survive within the intracellular compartment whilst maintaining the integrity of the host cell may represent a key virulence trait that facilitates evasion of immune destruction.

Invasion of the respiratory epithelium by *Aspergillus* hyphae has been studied in in vitro cultures of bronchial mucosa and cell culture models of the alveolus. Invasion can occur by three routes: penetration of hyphae through intercellular gaps, germination of internalized conidia within epithelial cells, and direct invasion through the intracellular space following destruction of the cytoplasmic membrane (Fig. 1) [41]. The last two processes probably predominate [27]. *A. fumigatus* conidia inoculated onto a bilayer of epithelial and endothelial cells in vitro penetrate to the endothelial side after 14 to 16 hours [27]. These rapid dynamics of *Aspergillus* germination and invasion suggest that the alveolar epithelium is penetrated well before the earliest clinical manifestations of IPA become apparent.

The integrity of the epithelial lining and mucociliary apparatus is a mainstay of pulmonary resistance to infection. Epithelial damage by *Aspergillus* infection may facilitate invasion by additional germinated conidia as well as co-infection by other pathogens. Germinated *Aspergillus* conidia produce secondary metabolites that adversely affect the integrity and function of the respiratory epithelium [42–44]. These effects include separation of intercellular junctions, inhibition of ciliary action, and detachment of ciliated cells. The most potent ciliostatic toxin of *A. fumigatus* is gliotoxin (GT) [43]. Other toxins, including helvolic acid and fumagillin, suppress ciliary beating at much higher concentrations. Fungal proteases cause epithelial cell detachment from respiratory epithelium [44]. Verruculogen, another

mycotoxin produced by *A. fumigatus*, modifies the electrophysiological properties of epithelial cells [45].

Although the factors that determine the attachment of *Aspergillus* conidia to epithelial cells are incompletely understood, conceptually, activation or alteration of these cells by noxious agents may facilitate binding of *Aspergillus* conidia to respiratory epithelium. Thus, patients who have undergone haematopoietic stem cell transplantation (HSCT) frequently experience damage to the respiratory epithelium as a result of irradiation, cytotoxic drug use, graft versus host disease (GVHD), or respiratory viral infection [46]. Adherence of conidia to respiratory epithelium is a critical early step in airway colonisation and subsequent invasion. Conidia and hyphae adhere to a variety of host substrates, including fibrinogen [47, 48], laminin, collagen types I and IV, fibronectin, and pulmonary epithelial cells [49]. Of note, binding to fibrinogen has been demonstrated in pathogenic *Aspergillus* species but not in strictly saprophytic or phytopathogenic species [48]. The fungal adhesins that mediate these interactions remain largely undefined. Studies have identified only two proteins that likely mediate adherence of *Aspergillus* conidia to the extracellular matrix: rodA and Asp f2.

4.1 RodA

The outer layer of the *Aspergillus* conidial cell wall is composed of clustered microfibrils called rodlets [50], which are involved in both conidial adherence to respiratory epithelium and conidial hydrophobicity and dispersal [51]. The *rodA* gene encodes a small hydrophobic cysteine-rich polypeptide. RodA-deficient mutants produce conidia that lack the external rodlet layer, are hydrophilic, and have reduced adherence to collagen and bovine serum albumin but not to fibrinogen, laminin, or primary rat pulmonary epithelial cells [51]. Nonetheless, *ΔrodA* mutants exhibit normal virulence in an immunosuppressed murine model of IPA [51], suggesting redundancy in the genes that control adhesion.

4.2 Asp f2

Asp f2 is a 37-kDa protein produced in large amounts by hyphae of *A. fumigatus* (Asp f2 represents 20–40% of the protein in these cells). Asp f2 is a major allergen produced by *A. fumigatus* and is thought to play a role in the pathogenesis of ABPA [52] (an overview on *Aspergillus* allergens is presented in the chapter by Dr Crameri et al.). In addition, significant homology exists between Asp f2 and Pra1, a surface mannoprotein of *Candida albicans* that binds fibrinogen [53]. Recombinant Asp f2 binds to immobilized laminin [49, 52]. Because researchers have yet to construct an Asp f2-deficient mutant, the role of this protein in the virulence of *Aspergillus* is unknown.

4.3 Sialic Acids

Sialic acids on the surfaces of conidia play a role in their adhesion to the extracellular matrix [54]. Sialic acids are negatively charged derivatives of the monosaccharide neuraminic acid. The majority of sialic acids on conidia are unsubstituted *N*-acetyl-neuraminic acid and are α-2,6-linked with galactose. Enzymatic removal of sialic acid from conidia significantly attenuates binding of conidia to fibronectin as well as their uptake by type II pneumocytes and macrophages [54]. *A. fumigatus* conidia have higher sialic acid densities than do conidia of non-pathogenic *Aspergillus* species [55].

5 Innate Immunity Against *Aspergillus*: Detection and Evasion

AMs, the major resident phagocytic cells in the lung, bear the brunt of elimination of inhaled conidia. AMs rapidly phagocytise conidia before germination can occur and destroy them within their phagosomes using NADPH oxidase-dependent mechanisms [56]. Germinated conidia that escape phagocytosis by AMs are targeted by PMNLs recruited to the lungs by chemotactic signals. PMNLs aggregate around germinated conidia and damage fungal cells by releasing granule content and reactive oxygen intermediates (ROI) [57, 58]. In addition to their action against hyphae, PMNLs also inhibit conidial germination thus supplanting the conidiacidal activity of AMs when these cells are overwhelmed by a large inhaled load of conidia [57].

Activation of AMs and PMNLs by *Aspergillus* conidia results in release of pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon (IFN)-γ, prime professional phagocytes. The equilibrium between these defensive cytokines and anti-inflammatory cytokines such as IL-4 and IL-10 is essential for competent innate immunity against IA [59].

The interaction between innate immune effector cells and *Aspergillus* species is mediated by pattern recognition receptors (PRR) on AMs and PMNLs, which recognise and bind to conserved fungal structures, collectively termed pathogen-associated molecular patterns (PAMP). Beta-1,3-linked and β-1,6-linked glucans, carbohydrate polymers present in the *Aspergillus* cell wall, are important fungal PAMP that are ligated by dectin-1, a C-type lectin-like receptor on AMs, PMNLs, and dendritic cells [60]. Engagement of dectin-1 by β-glucan mediates phagocytosis of swollen conidia by AMs, as evidenced by the redistribution of dectin-1 from the macrophage cell membrane to phagosomes following conidial ingestion [61, 62]. In addition, dectin-1 mediates pro-inflammatory cytokine release in response to β-glucans, an effect that is independent of the MyD88 signalling pathway common to TLRs [61]. Phagocytosis is reduced but not abrogated following inhibition of conidial β-glucan display, suggesting that fungal ligands other than β-glucans are involved in phagocytosis [62].

Expression of TLR2 and TLR4 in AMs and PMNLs plays an important role in innate immunity against *Aspergillus*. TLR4 knockout has been associated with

increased mortality in mice challenged intranasally with *Aspergillus* conidia [63], whereas TLR2 knockout was associated with increased mortality rates in one of two studies [63, 64]. Mice deficient in either TLR2 or TLR4 have higher pulmonary fungal burdens than do wild-type mice [63]. TLR signalling is mediated by the adapter protein MyD88 and results in activation of nuclear factor (NF)-κB and release of TNF-α and other pro-inflammatory cytokines [65–69]. The *Aspergillus* ligands involved in activation of TLRs have yet to be characterized.

There appear to be differences between the responses of macrophages and PMNLs to activation of TLR2 and TLR4. In macrophages, TLR4 signalling mediates a robust pro-inflammatory phenotype, whereas TLR2 activation is associated with production of the anti-inflammatory cytokine IL-10 [70]. In contrast, in PMNLs, TLR2 signalling activates fungal killing through release of gelatinases and pro-inflammatory cytokines, whereas TLR4 activation is associated with oxidative killing through release of azurophil myeloperoxidase-positive granules and IL-10 [71]. In addition, *Aspergillus* conidia and hyphae interact differently with TLRs, although seemingly conflicting results have been published concerning this point. Results from two studies indicate that *A. fumigatus* conidia activate both TLR2 and TLR4, whereas *A. fumigatus* hyphae activate TLR2 but not TLR4 [66, 70]. In contrast, other groups have found that *A. fumigatus* hyphae activate both TLR2 and TLR4 [67] or only TLR4 [69]. These discrepancies have been ascribed to experimental variation, possibly stemming from the use of differently treated fungal preparations or contamination by lipopolysaccharide [65].

Taken together, these results suggest that TLR4 recognises PAMP expressed on the conidial surface early in the course of IPA and that TLR4 signalling in AM is involved in the inflammatory response to inhaled *Aspergillus* conidia. TLR2 signalling likely plays an important role later in the course of IPA, when PMNLs are recruited to the lung and engage extracellular hyphae. Netea et al. [70] suggested that loss of TLR4 signalling in AM following germination may constitute an escape mechanism for *Aspergillus* whereby phenotypic switching shifts the pro-inflammatory TLR4-associated response to a predominantly anti-inflammatory TLR2-associated response. The innate response to *Aspergillus* conidia involves additional soluble and cell-associated receptors such as pentraxin 3, which binds galactomannan [72]; a Langerhans cell galactomannan-specific C-type lectin [73]; and dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), which mediates internalisation of conidia by DCs and macrophages [74]. Much remains to be learned about the complex interplay between PRR and the different fungal developmental stages before one can construct a full picture of the innate immune response to *Aspergillus* infection.

Alteration in surface PAMP expression in innate immune cells can profoundly influence the inflammatory response to *Aspergillus* and dynamics of fungal killing by effectors of innate immunity (Fig. 2). Resting *Aspergillus* conidia are relatively resistant to phagocytosis and killing by macrophages because of the under-expression of the immunoreactive β-glucans on their surfaces [61]. In dormant *Aspergillus* conidia, β-glucans are concealed from immune recognition by an outer proteinaceous rodlet layer composed of hydrophobins and pigment [75]. Electron

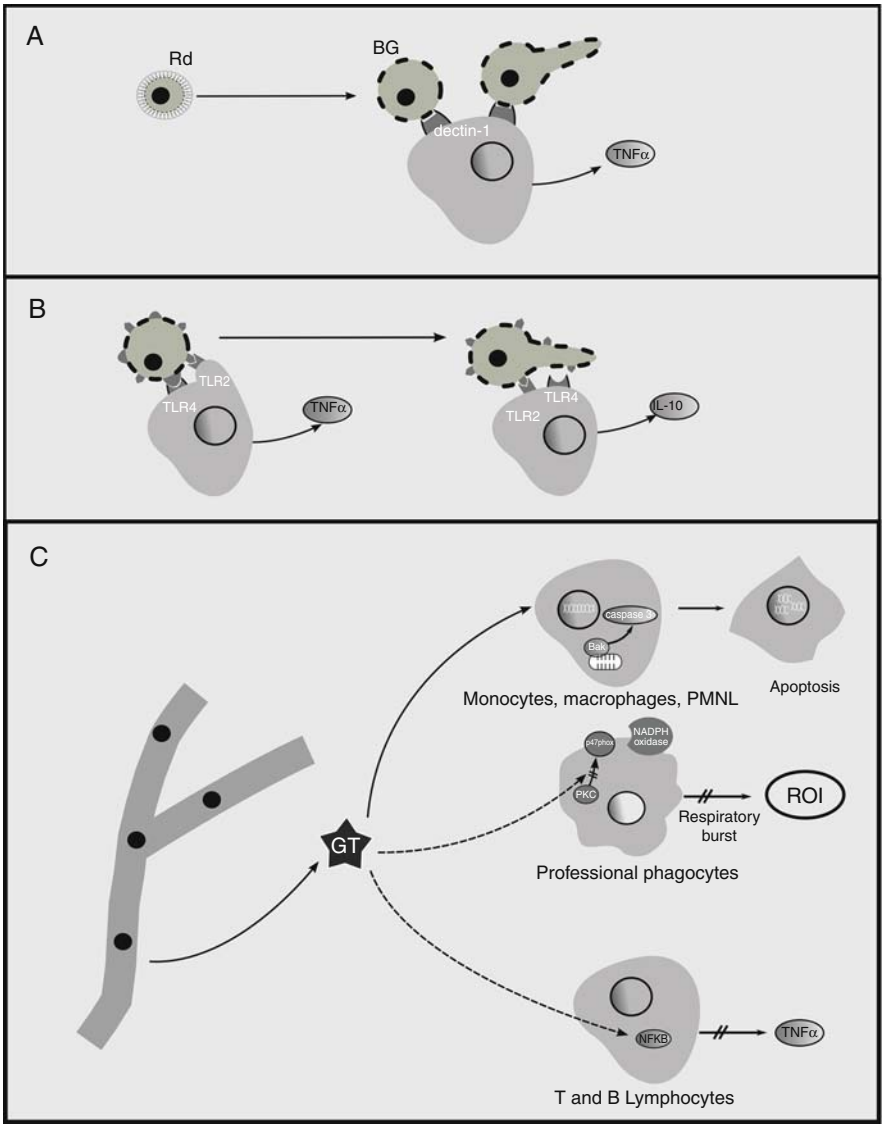

Fig. 2 Immune modulation and evasion by *Aspergillus*. *Aspergillus* species modulate the host immune response in a stage-specific manner. (**A**) The cell wall of a dormant *Aspergillus* conidium is composed of an outer layer of rodlets (Rd) that conceals β-glucan (BG) from recognition by dectin-1 – bearing innate immune cells. Conidial maturation into swollen conidia and early germlings is associated with cell wall remodelling and exposure of β-glucan, which then stimulates inflammatory cytokine release from innate immune cells by ligating dectin-1. (**B**) *Aspergillus* conidia are recognised by both Toll-like receptors (TLR)-2 and TLR4 on macrophages, leading to production of pro-inflammatory cytokines. As conidia germinate, signalling through TLR4 is lost, and TLR2 signalling induces a more anti-inflammatory cytokine profile marked by IL-10 production. (**C**) *Aspergillus* mycelia produce a host of secondary metabolites, such as gliotoxin (GT). Three immunosuppressive actions of GT are shown: induction of apoptosis in immune cells, inhibition of respiratory burst in phagocytes, and inhibition of NF-κB in lymphocytes. The *solid arrows* indicate stimulation, and the *dashed arrows* signify inhibition. PKC, protein kinase C; ROI, reactive oxygen intermediates

microscopy studies have shown that during conidial swelling and germination, the conidial cell wall undergoes extensive remodelling, which involves loss of the external rodlet layer and exposure of underlying ligands [76]. Exposed β-glucan on the surfaces of swollen conidia and early germlings triggers a robust pro-inflammatory response by engaging dectin-1 on AM [61, 62, 77, 78]. This stage-specific regulation of the inflammatory response to *Aspergillus* conidia may be advantageous in that immune effector functions are recruited against germinating conidia that present an imminent risk of invasive infection, whereas the large numbers of dormant conidia that are inhaled daily fail to induce a potentially harmful inflammatory response.

In contrast with our understanding of β-glucan masking in *Aspergillus* conidia, little is known about the expression of β-glucan on hyphae, the tissue-invasive form of *Aspergillus*. Recent studies suggested that *Aspergillus* species evade immune recognition at the hyphal stage by modulating surface expression of β-glucan. Wheeler and Fink [79] uncovered in *C. albicans* a conserved set of genes that are responsible for concealing β-glucan from innate immune cells by keeping it under an external coat of mannoprotein. Interestingly, subinhibitory concentrations of the echinocandin drug caspofungin increased surface exposure of β-glucan and, consequently, dectin-1 signalling and pro-inflammatory cytokine release by these cells. Results from two independent groups have shown that, similar to *C. albicans*, the baseline surface exposure of β-glucan on *A. fumigatus* hyphae is low [80, 81]. Exposure of hyphae to subinhibitory concentrations of echinocandins upregulates β-glucan expression as well as release of pro-inflammatory cytokines by macrophages. For additional information on the interactions between *Aspergillus* and the host the reader is referred to the chapter by Dr Romani, in this book.

6 Angioinvasion

Aspergillus hyphae have marked tropism for blood vessels. Angioinvasion is a central feature of IPA, especially in patients with quantitative or functional deficits in PMN [82]. Angioinvasion leads to vascular thrombosis, tissue infarction, and dissemination of *Aspergillus* to distant organs. In addition, tissue necrosis limits the penetration of immune cells as well as antifungal agents to the site of infection.

Two types of angioinvasion occur over the course of IPA. The first, more prevalent type occurs in the lungs as *Aspergillus* hyphae traverse the alveolar-capillary barrier and penetrate endothelial cells from their abluminal sides. The second type, which takes place in the most profoundly immunosuppressed hosts, occurs following haematogenous dissemination of hyphal fragments that lodge in peripheral capillary beds and penetrate endothelial cells from their luminal sides to form distant foci of infection [83].

The interaction between *Aspergillus* and endothelial cells likely plays an important role in angioinvasion. Although *Aspergillus* conidia and hyphae can induce their own endocytosis by endothelial cells, the interaction of hyphae with endothelial cells is most significant, because the hyphal form is the one most likely to come into contact with endothelial cells during invasive infection. Endocytosis is associated

with actin polymerisation around the organism. Endocytosis of both *Aspergillus* conidia and hyphae induces endothelial cell damage. However, the mechanisms underlying endothelial damage are probably different for conidia and hyphae as indicated by the fact that fungal viability is required for damage associated with conidia, whereas viable and non-viable hyphae induce similar damage [82].

Aspergillus hyphae but not conidia induce expression of tissue factor on the surface of endothelial cells [83]. Tissue factor (thromboplastin, CD142) is a transmembrane glycoprotein expressed by endothelial cells as well as leukocytes and platelets. Tissue factor binds to factor VII, forming a complex that initiates coagulation by the extrinsic pathway [84].

The features of angioinvasion described above may vary if *Aspergillus* hyphae penetrate the endothelium from the luminal or abluminal side. Luminal penetration is associated with greater endothelial damage than abluminal penetration is. However abluminal invasion, as primarily occurs in the lung, is associated with greater upregulation of expression of genes involved in inflammatory response (e.g., cytokines, leukocyte adhesion molecules) and thrombosis (tissue factor) than is luminal penetration [83].

Tissue injury and infarction, as occur during IPA, are intimately linked with angiogenesis, the formation of new blood vessels. An early response to tissue injury consists of vasodilatation and increased vascular permeability followed by angiogenesis, which facilitates tissue healing and regeneration. Essential mediators of this response are adenosine and adenine nucleotides, which rapidly accumulate at sites of tissue ischemia and act as "danger" signals [85]. Adenosine acts synergistically with specific TLR ligands, resulting in synthesis and release of vascular endothelial growth factor (VEGF). Modulation of angiogenesis, either by interaction between endothelial cells and *Aspergillus* metabolites or by the effects of the various immunosuppressive agents that are almost invariably present in the background of IPA, is likely to significantly impact the progression and outcome of IPA. Thus far, four secondary metabolites produced by *Aspergillus* species have been found to have potent anti-angiogenic activity: gliotoxin, fumagillin, actibind and cytochalasin E.

6.1 Gliotoxin

In addition to its immunosuppressive activity (described later in this chapter), gliotoxin was found to inhibit H2O2-induced angiogenesis as a result of thioreduxin-dependent reduction of H2O2 [Choi HS, Shim JS, Kim JA, Kang SW & Kwon HJ. (2007) Discovery of gliotoxin as a new small molecule targeting thioredoxin redox system. Biochem Biophys Res Commun, 359, 523-8]. These effects occur at gliotoxin concentrations not associated with cytotoxic activity.

6.2 Fumagillin

Fumagillin, a small molecule produced by *A. fumigatus* [86, 87], blocks VEGF-induced endothelial cell migration by inhibiting methionine aminopeptidase

(MetAP)-2, a dimethaloprotease that catalyses the removal of N-terminal methionine from newly formed polypeptide chains [87]. Although MetAP-2 is a common target of other anti-angiogenic agents (e.g. ovalicin), its precise role in angiogenesis has yet to be defined.

6.3 Actibind

Actibind, a T2 ribonuclease produced by *A. niger*, is a glycoprotein with anti-carcinogenic and anti-angiogenic properties [88]. Actibind interferes with intracellular actin networks and competes with the angiogenic factor angiogenin.

6.4 Cytochalasin E

Cytochalasin E is an epoxide containing metabolite produced by the rarely pathogenic species *A. clavatus*. Although structurally similar to fumagillin, cytochalasin E does not inhibit MetAP-2, and is therefore postulated to inhibit angiogenesis by other mechanisms [89].

Although the potential of anti-angiogenic *Aspergillus* metabolites to act as parent drugs for the development of anticancer agents is being explored, little is currently known about their role in the pathogenesis of aspergillosis.

7 Melanin

The pigment of *A. fumigatus* contains 1,8-dihydroxynaphthalene (DHN)-like melanin, a polyketide polymer that forms a dense layer on the conidial surface adjacent to the rodlets, conferring mature colonies with a grey-green colour. Like rodlets, melanin is hydrophobic and negatively charged [90]. Melanin has multiple functions in protecting conidia against adverse environmental conditions outside the mammalian host. Specifically, melanin acts as a natural sunscreen and protects conidia from ultraviolet radiation, provides thermal insulation, augments the structural rigidity of the conidial cell wall, prevents desiccation, binds metals, and attenuates oxidative damage by acting as a redox sink and trapping unpaired electrons [90].

A. fumigatus pigment is synthesized through the DHN-melanin pathway. Six genes involved in this pathway have been identified, and mapped as a gene cluster. The *pksP* (*alb1*) gene in *A. fumigatus* encodes a type I polyketide synthase homologous to white A in *A. nidulans*. Unlike the grey-green conidia of wild type *A. fumigatus* which have characteristic surface ornamentation, *pksP*-deficient *A. fumigatus* mutants produce smooth white conidia [91–93]. Studies have found *A. fumigatus pksP* deletion mutants to be hypovirulent in mice, pointing to a role for melanin in the pathogenesis of IPA [91–93]. Three mechanisms have been proposed for the effect of melanin production on virulence: quenching of ROI, interference with intracellular trafficking of phagocytised conidia, and β-glucan masking.

7.1 Quenching of Reactive Oxigen Intermediates (ROI)

In the mammalian lung, the ability of melanin to act as a scavenger of ROI is a defensive mechanism against oxidative damage caused by AM and PMN. *Δalb1* mutants are more sensitive to hydrogen peroxide and sodium hypochlorite and are more susceptible to damage from macrophages in vitro compared with wild-type *A. fumigatus* [91, 94].

7.2 Interference with Intracellular Trafficking of Phagocytised Conidia

The presence of a functioning *pksP* gene in *A. fumigatus* is associated with inhibition of phagosome-lysosome fusion following conidial phagocytosis [95, 96]. The conidia of *pksP*-deficient *A. fumigatus* mutants are transported to acidic phagolysosomes at a rate two to three times higher than that of wild-type *A. fumigatus* conidia [95]. This supports the notion that a product of the *pksP* gene, which has yet to be characterized, acts as an immune suppressant. Interestingly, because *A. nidulans* synthesises melanin through a non-DHN pathway, it does not possess the *pksP* gene. The fact that both of these species produce melanin but only *A. fumigatus* is associated with IPA suggests that *pksP* affects virulence in ways unrelated to the synthesis of melanin.

7.3 β-Glucan Masking

Because melanin, together with rodlets, comprises part of the outer conidial layer, it has a role in the masking of β-glucan from recognition by macrophages bearing the dectin-1 phagocytic receptor. Resting conidia of the *pksP* deletion mutant express β-glucan abundantly on their surfaces and are thus ingested by macrophages as efficiently as germinating conidia of wild-type *A. fumigatus* [62].

pksP is linked with the cyclic AMP (cAMP) signal transduction pathway in *Aspergillus* species. This pathway, which includes a G protein α subunit (Gs α), adenylate cyclase, and protein kinase A, controls growth and sporulation as well as *pksP* expression in *Aspergillus* [96, 97]. Deletion of any of the three elements of the cAMP signalling pathway results in nearly avirulent *Aspergillus* strains [97–99]. Adenylate cyclase- and protein kinase A-deficient mutants (*ΔacyA* and *ΔpkaC1*) are slow growers, but the G protein-deficient mutant (*ΔgpaB*) has normal growth characteristics [97]. Signalling through a cAMP-protein kinase A pathway regulates the virulence of other fungi that are pathogenic in humans, such as *C. albicans* and *Cryptococcus neoformans*, as well as that of some plant pathogens [100]. Therefore, the involvement of cAMP signalling in the virulence of *Aspergillus* species may be a common theme amongst pathogenic fungi.

8 Non-melanin Antioxidants

A. fumigatus produces several metabolites with antioxidant function, including Cu/Zn superoxide dismutases, catalases, and mannitol. These products protect *A. fumigatus* against ROI released by phagocytes and may contribute to its pathogenicity. A Cu/Zn superoxide dismutase of *A. fumigatus* has been purified and cloned. Antibodies against this enzyme are found in the sera of patients with aspergillosis [101]. However, because no gene deletion studies of Cu/Zn superoxide dismutase are reported in the literature, its contribution to virulence remains unknown.

The genome of *A. fumigatus* contains at least seven genes for different catalases, the products of three of which have been purified: two mycelial catalases (Cat1 and Cat2, also known as slow and fast, respectively, for their electrophoretic mobility) and one conidial catalase (CatA) [102–104]. This redundancy in antioxidant activity has made assessment of the individual contributions of these genes to the virulence of *A. fumigatus* difficult. CatA is a heat-stable monofunctional catalase with a dimeric structure. Deletion of CatA resulted in increased susceptibility of *A. fumigatus* conidia to hydrogen peroxide in vitro but no change in the sensitivity to macrophage respiratory burst or virulence in a murine model [104].

Cat1 has a tetrameric structure common to many catalases in nature [102]. Cat2 is a heat labile bifunctional catalase (i.e., it also has peroxidase activity) that is unusual amongst microbial catalases for its monomeric structure. Disruption of either Cat1 or Cat2 does not affect the sensitivity of the mycelium to hydrogen peroxide or the virulence of the disruptants in animals [102, 104]. Surprisingly, deletion of both Cat1 and Cat2 results in only a mild increase in sensitivity to hydrogen peroxide, indicating a role for other antioxidants [104]. The Cat1(–/Cat2(–)) double mutant exhibits reduced virulence in immunosuppressed rats.

9 Thermotolerance

The ability to grow at 37°C is a prerequisite for any human pathogen. Indeed, thermotolerance is thought to be a defining feature of *A. fumigatus* that sets it apart from non-pathogenic *Aspergilli* [105]. As an inhabitant of self-heating compost piles, *A. fumigatus* has developed mechanisms for surviving temperatures of up to 55°C, making it the dominant organism in the high-temperature phase of the compost cycle [106]. Transcription profiling of *A. fumigatus* at elevated temperatures is a promising strategy for identifying genes relevant to pathogenicity [5, 107]. Nevertheless, the molecular basis for thermotolerance is poorly understood. To date, two genes with roles in thermotolerance have been identified: *cgrA* and *thtA*.

9.1 CgrA

CgrA is an orthologue of a *Saccharomyces cerevisiae* nucleolar protein that functions in ribosome biosynthesis. An *A. fumigatus* mutant with a deletion of *cgrA*

grows and germinates normally at room temperature but exhibits poor growth and impaired germination at 37°C [108]. Consistent with these observations, *ΔcgrA* has reduced virulence in immunosuppressed mice (which have a body temperature of 37°C) but is only marginally less virulent than the wild-type strain of *A. fumigatus* in *Drosophila* flies, which are maintained at 25°C [108].

9.2 thtA

A second gene involved in thermotolerance, *thtA*, was discovered by studying temperature-sensitive mutants of *A. fumigatus*. A mutant *A. fumigatus* with impaired growth at 48°C but normal growth at 42°C was isolated, and *thtA* was identified as the deficient gene by complementation. The function of the putative 141-kDa *thtA* product is unknown. A *thtA* deletion mutant was as virulent in mice as the wild type *A. fumigatus* strain, suggesting that *thtA* does not affect the fitness of *A. fumigatus* at physiological temperatures [109].

10 Proteinases

Aspergillus hyphae produce a large array of proteinases, including elastase, alkaline proteinase, metalloproteinase, intracellular metallopeptidase, and aspartic proteinase [110–114]. Of these, only deletion of the elastase gene was found to result in attenuated virulence in a neutropenic mouse model [115]. Elastase is produced during infection as demonstrated by the presence of anti-elastase antibodies in the serum of patients with IPA [116] and immunostaining of lungs of experimentally infected mice for elastase [115]. Elastase activity is significantly more prevalent in strains of *A. fumigatus* than in strains of *A. flavus* [115] and in invasive strains of *A. fumigatus* than in colonising strains of *A. fumigatus* [117, 118]. In one study, histopathological examination of the lungs of mice inoculated with an elastase-producing *A. fumigatus* strain revealed extensive alveolar necrosis, whereas the alveolar architecture was preserved in the lungs of mice infected with strains lacking elastase activity [119]. However, despite the abundance of preclinical evidence of a role for elastase in the pathogenesis of aspergillosis, examination of lung tissue blocks obtained from patients with IPA revealed no loss of elastin from blood vessel walls [120].

11 Calcineurin

Calcineurin is a highly conserved calcium/calmodulin-regulated protein phosphatase. The gene encoding calcineurin A (*cnaA*) was first identified in *A. nidulans* and found to be essential to the viability of this species [121]. Disruption of *cnaA* resulted in *A. nidulans* cell-cycle arrest at the G1 phase as well attenuation of conidial germination to less than half of the rate in *A. nidulans* wild-type for *cnaA*.

Calcineurin is expressed in *Aspergilli* during stressful environmental conditions, such as alkaline pH, heat shock, and salt-induced stress [122].

Analysis of the role of *cnaA* in *A. fumigatus* revealed that, in contrast with *A. nidulans*, calcineurin is not essential for fungal survival but plays a crucial role in virulence by polarising hyphal growth and, consequently, tissue invasion. A *ΔcnaA A. fumigatus* mutant exhibited defective germ-tube formation and mycelial growth with blunted and malformed hyphae [123, 124]. A similar phenotype was evident in a calmodulin-deficient (*ΔcalA*) *A. fumigatus* mutant [125]. Surprisingly, *ΔcnaA* also had a defect in conidiation, as the mutant strain produced long chains of conidia that lacked rodlets [124]. Furthermore, the *ΔcnaA* mutant strain was significantly hypovirulent in immunosuppressed mice. Histopathological examination of lungs of animals infected with *ΔcnaA* was notable for a complete absence of hyphae [124]. In sum, compelling evidence indicates a role for calcineurin in the pathogenicity of *A. fumigatus*. The results of these studies have prompted interest in calcineurin as a target for novel drugs for the treatment of aspergillosis [126, 127].

12 Siderophores

The ability to acquire iron from the environment is common to most pathogenic organisms. Iron is a co-factor for catalases, oxygenases, and peroxidases and therefore plays an important part in resistance against oxidative stress [128]. *A. fumigatus* employs two high-affinity iron uptake systems: reductive iron uptake and siderophore-assisted iron uptake. Both systems are induced by iron deprivation, but only siderophore-assisted iron uptake appears to be essential for *Aspergillus* virulence [129]. Reductive iron uptake involves reduction of iron from the ferric (Fe^{+3}) to the ferrous (Fe^{+2}) state. Ferrous iron is taken up by the FtrA/FetC complex. Siderophores, on the other hand, are low-molecular-weight chelators that bind ferric iron. *A. fumigatus* synthesises four siderophores: two are extracellular (fusarinine C and triacetylfusarinine) and two are intracellular (ferricrocin and hydroxyferricrocin) [130]. The enzyme SidA catalyses the first step common to the synthetic pathways of all of these siderophores. SidC is a non-ribosomal peptide synthase that catalyses the production of ferricrocin and hydroxyferricrocin. Additionally, SidD, SidF, and SidG are involved in the synthesis of triacylfusarinine. Deletion of the genes for any of these five enzymes results in a strain of *A. fumigatus* that is significantly hypovirulent in comparison with the isogenic wild-type *A. fumigatus* strain, indicating that both intracellular and extracellular siderophores are required for *A. fumigatus* virulence [130].

13 Secondary Metabolites

Analysis of the *Aspergillus* genome indicates the potential for this fungus to produce an impressive array of mycotoxins (30–50 per species), more than any other

filamentous fungus studied to date [131, 132]. The mycotoxin repertoire is strikingly diverse at both the species and strain level. Genes involved in the synthesis of secondary metabolites in *Aspergillus* species are often found in co-regulated clusters. These clusters tend to be located in subtelomeric regions of the genome, which are prone to gene rearrangement. In its natural soil environment, this repertoire of mycotoxins provides *Aspergillus* with an advantage over other soil inhabitants that compete for nutrients. Therefore, these secondary metabolites are thought to have evolved by selection. However, many of these same genes play important roles in the pathogenesis of aspergillosis in the mammalian host. Genome-wide analysis of *Aspergillus* gene expression in a murine aspergillosis model revealed a coordinated program that controls metabolic adaptation to the mammalian niche [133]. Expression of 30% of subtelomeric and lineage-specific genes is higher in vivo than in laboratory culture. Upregulated genes include those in clusters involved in mycotoxin biosynthesis (pseurotin and GT), transport (amino acids, carbohydrates, and endocytosis), catabolism, iron acquisition (siderophores), transcriptional regulation, and other metabolic pathways [133].

Mycotoxins are secreted secondary metabolites that are harmful to humans and animals [134]. Two families of toxins have been studied to determine their roles in IPA: ribonucleotoxins and GT.

13.1 Ribonucleotoxins

Ribonucleotoxins are ribosome-inactivating proteins that are highly active against the phosphodiester bond of the sarcin/ricin domain, which is universally preserved in 28S ribosomal RNA. In nature, they are likely defence mechanisms for *Aspergillus* fungi against insects. Mutagillin, restrictocin, and Asp f1 antigen, a major *A. fumigatus* allergen, are closely related 18-kDa proteins with ribonucleotoxin activity that are produced by *A. fumigatus* during infection [135]. Disruption of either the *res* gene or the *aspf1* gene, despite abolishing all extracellular ribonucleolytic activity, did not alter survival or histopathological manifestations in a murine model of IPA [116, 136]. Hence, ribonucleotoxins are not presently considered to be important to the pathogenesis of *Aspergillus*.

13.2 Gliotoxin (GT)

GT is a mycotoxin belonging to the epipolythiodioxopiperazine class of secondary fungal metabolites. These compounds are likely synthesized by non-ribosomal peptide synthetases [137]. GT has pleiotropic immunosuppressive effects, which theoretically facilitate evasion of *Aspergillus* species from immune-mediated damage (Fig. 2). GT interferes with essential functions of professional phagocytic cells, including chemotaxis [138] and phagocytosis [139]. In addition, GT suppresses respiratory burst and oxidative killing by inhibiting the assembly of the NADPH

oxidase complex [138–141]. This effect is mediated by defective membrane translocation of protein kinase C βII, leading to reduced phosphorylation of $p47^{phox}$, which is crucial to the assembly of NADPH oxidase. Nanomolar concentrations of GT have been shown to inhibit activation of NF-κB, the central transcriptional regulator of inflammatory response genes, in T and B lymphocytes [142]. GT also inhibits ciliary beating in the respiratory epithelium as described above.

At higher concentrations, GT induces apoptosis in peripheral blood mononuclear cells, macrophages, and PMN [143–145]. Injection of GT into mice following irradiation resulted in delayed recovery of leukocytes and apoptosis of cells in the thymus, spleen, and mesenteric lymph nodes [146]. The proapoptotic activity of GT is mediated by direct activation of the mitochondrial protein Bak, which leads to generation of ROI and activation of caspase 3 [144, 147].

Evidence from studies of animals and patients with aspergillosis suggests a link between GT production and virulence of *Aspergillus* species. GT can be detected in the lung tissue and sera of animals with IPA and in the sera of patients with IPA [148, 149]. Amongst isolates collected from patients with IPA, *A. fumigatus* was associated with the highest rates of GT production (93%) followed by *A. niger* (75%) and *A. terreus* (25%) [150]. These rates contrast with the much lower rate of GT production found in environmental strains of *A. fumigatus* (11%) [151]. The virulence of *A. fumigatus* in invertebrate models of aspergillosis, such as *Galleria* and *Drosophila* species, correlated with GT production in vitro [152, 153].

Recent studies of *A. fumigatus* mutants deficient in GT production as a result of deletion of the *gliP* gene have partially elucidated the interplay between immune suppression and the contribution of GT to *Aspergillus* pathogenesis. Results of these studies indicated that GT is not required for *Aspergillus* virulence in mice rendered neutropenic because of exposure to cyclophosphamide [137, 154] but plays an important role in the pathogenesis of IPA in non-neutropenic mice immunosuppressed with hydrocortisone [140, 153]. Thus, the clinical relevance of GT may depend on the nature of the immune deficiency in the host.

GT production is controlled by both specific and non-specific regulators.

13.2.1 LaeA

The *laeA* gene encodes a nuclear protein homologous to arginine and histone methyltransferase [153, 155]. Loss of *LaeA* function silences GT production, whereas overexpression of *LaeA* upregulates GT biosynthesis [156]. Morphologically, *laeA* deletion mutants appear to be nearly identical to wild-type *A. fumigatus* strains except for loss of pigment production in the mycelium. Examination of organic extracts from these strains using thin-layer chromatography showed that they were deficient in the production of many secondary metabolites [155]. These findings led to a model of *LaeA* as a global regulator of secondary metabolism gene clusters [155]. Interestingly, a study showed that *leaA* deletion mutants produced conidia that were more susceptible than wild type *A. fumigatus* conidia to phagocytosis by macrophages [156]. Because GT is produced by *Aspergillus* hyphae

but not conidia, resistance of conidia to phagocytosis is likely caused by other secondary metabolites. *laeA* expression is regulated by protein kinase A and Ras protein. Intranasal inoculation of neutropenic mice with the *laeA* deletion mutant resulted in lower mortality and fewer colony-forming units in the lungs compared with infection with the isogenic wild-type *A. fumigatus* strain [156].

13.2.2 GliZ

GliZ is a binuclear transcription factor with a Zn(II)2Cys6 structure that is a specific GT synthesis pathway regulator. Disruption of *gliZ* results in a non-GT-producing mutant, whereas overexpression of *gliZ* results in upregulation of GT production [157]. Both deletion and overexpression mutants were similarly virulent in a neutropenic mouse model (however, as noted above, the effect of GT production on *Aspergillus* pathogenicity may only be detectible in steroid-suppressed non-neutropenic animals).

14 Host-Specific Characteristics of *Aspergillus* Pathogenesis

IPA is a major cause of morbidity and mortality in patients who are severely immunosuppressed, most notably those with profound and prolonged neutropenia; recipients of allogeneic HSCT, especially those who have GVHD; and patients who receive large doses of steroid formulations for extended periods of time [2]. However, it is becoming increasingly apparent that the patterns of tissue injury, inflammation, and fungal growth differ substantially amongst these high-risk groups. In animals and patients with chemotherapy-induced neutropenia, IPA is associated with the presence of numerous *Aspergillus* hyphae invading blood vessels and tissue, thrombosis, coagulative necrosis, haemorrhage, a scant mononuclear inflammatory infiltrate, and eventual dissemination [158, 159]. In contrast, in steroid-treated subjects, IPA is associated with tissue infiltration by PMN, areas of pneumonia and bronchiolitis, inflammatory necrosis, and few fungal elements [158–161]. Recipients of HSCT with IPA have a pattern of tissue injury similar to that of neutropenic patients with IPA, probably because of impaired trafficking of PMN to the lungs [159]. Thus, whereas a high fungal burden, tissue invasion, and dissemination seem to be the key elements of IPA pathogenesis in patients with absolute or functional neutropenia, a dysregulated inflammatory response is responsible for much of the tissue injury in steroid-treated hosts.

Steroids suppress host defences against *A. fumigatus* by impairing the capacity of both AM and PMN to contain the invasive process [162]. Specifically, PMN that have been exposed to steroids are unable to generate respiratory burst oxidants [163]. Also, AM obtained from steroid-treated animals challenged with *Aspergillus* conidia fail to mobilize lysosomes to the phagocytic membrane [164]. In addition, steroids have direct stimulatory effects on *A. fumigatus* growth in vitro [165]. Detailed kinetic analysis of the inflammatory events in the lungs of steroid-treated mice inoculated with *A. fumigatus* conidia identified the following three stages [161]: (1) altered clearance of inhaled conidia, leading to intense germination

(0 to 12 hours); (2) invasive infection, accompanied by increased neutrophil trafficking to the lungs and pulmonary expression of pro-inflammatory cytokines (12 to 48 hours); and (3) major tissue destruction, necrosis, and regeneration (48 hours to death). Compared with immunocompetent mice, steroid-treated mice develop a delayed pro-inflammatory cytokine response that is likely related to the action of steroids through their cytoplasmic receptors, which translocate to the nucleus and block inflammatory gene transcription [166]. However, by 72 hours after infection with *A. fumigatus*, the cytokine response in steroid-treated mice becomes persistent and deregulated [161]. This ongoing production of pro-inflammatory cytokines can be attributed to continuing stimulation of host immune cells by fungal elements that steroid-exposed phagocytes fail to clear; it is also likely related to other, poorly defined immunomodulatory effects of corticosteroids.

The genetic makeup of the host may significantly influence the host's susceptibility to IA. Analysis of single-nucleotide polymorphisms (SNPs) in genes that govern innate immunity is a novel method of identifying groups at risk for IPA in a population of patients undergoing allogeneic HSCT. For example, an SNP in the promoter region of the IL-10 gene that is associated with reduced levels of expression of this cytokine in white individuals is linked with protection against IPA [167]. Similarly, SNPs in the chemokine (C-X-C motif) ligand 10 (CXCL10) gene are associated with reduced expression of CXCL10 by immature DCs and increased risk of IPA following allogeneic HSCT [168]. Preliminary studies evaluating TLR gene polymorphisms described associations between SNPs in the TLR1, TLR4, and TLR6 genes and increased risk of IPA [169, 170]. Recently, a haplotype containing two cosegregated SNPs in the donor TLR4 gene (1363T and 1063G) was associated with the development of IA in HSCT recipients [Bochud PY, Chien JW, Marr KA, Leisenring WM, Upton A, Janer M, Rodrigues SD, Li S, Hansen JA, Zhao LP, Aderem A, Boeckh M. (2008) Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med, 359, 1766–77]. An association between TLR4 SNPs and chronic cavitary pulmonary aspergillosis (CCPA) has also been recently described [171]. One should interpret the results of such genetic association studies with caution because the linkage between a given SNP and IPA may be specific to certain ethnic groups.

Mannose-binding lectin (MBL), an opsonin and complement pathway activator, is another important component of innate immunity [172]. The MBL pathway is activated by binding of MBL to carbohydrates in the fungal cell wall which leads to cleavage of C2 and C4 by MBL-associated serine protease. MBL facilitates uptake of *A. fumigatus* conidia by PMN in vitro and is associated with significantly reduced mortality when administered therapeutically in a murine model of IPA [173]. MBL deficiency is one of the most common human immune deficiencies. Low serum levels of MBL are found in association with SNPs in the promoter and structural gene regions of MBL2 [172]. Analysis of HSCT recipient-donor pairs for MBL pathway polymorphisms identified a low MBL phenotype in donors and an MBL-associated serine protease 2 variant (Asp105Gly) in recipients as independent predictors of invasive fungal infection (the majority of which were attributed to *Aspergillus* species) [174]. In addition, MBL gene polymorphisms are linked with susceptibility to CCPA and ABPA [175, 176].

Acquisition of iron is an important determinant of *Aspergillus* growth and virulence as described above. Iron overload is not uncommon in patients with haematological malignancies, especially those with underlying myelodysplastic syndrome, because of the requirement for multiple blood transfusions. Increased bone marrow iron stores were found to be associated with IPA in a case control study [177]. Prospective data are needed to determine whether iron overload is yet another host-specific risk factor for IPA.

Finally, data regarding the diverse interactions between *Aspergillus* species and other human viral and bacterial pathogens are accumulating. The risk of IPA in recipients of HSCT increases more than 2-fold following infection by respiratory viruses, such as influenza, parainfluenza, and respiratory syncytial virus (RSV) [178, 179]. IPA has even been reported in apparently immunocompetent individuals after infection with the influenza virus [180, 181]. The association between IPA and respiratory viral infections is incompletely understood but is likely related to alteration of immune function and local airway defence in the context of respiratory viral infection. RSV inhibits phagocytosis and ROI release by AM [182]. Infection with influenza virus induces a state of sustained hyporesponsiveness to TLR ligands, which may result in susceptibility to post-influenza pulmonary aspergillosis [183]. In addition, influenza virus has multiple effects on the function of T and B lymphocytes, which may be relevant to immunity against *Aspergillus* infection. Infection of DCs by the influenza virus differentially polarises the T-cell response depending on the viral dose: a low viral dose polarises toward a Th1 response, whereas a high viral dose induces a Th2 response, which may facilitate subsequent *Aspergillus* infection [184]. In addition, influenza virus promotes secretion of IFN type I, which causes trapping of B lymphocytes in regional lymph nodes and inhibits the activation of these cells [185]. Cytomegalovirus (CMV), another important viral pathogen, is a risk factor for IA in recipients of solid organ transplantation and HSCT [178, 186]. Treatment of CMV infection with ganciclovir may cause neutropenia, but the risk of aspergillosis is independent of this complication and probably related to the inhibitory effects of CMV on the function of innate immune cells, including PMN, AM, and DCs [187, 188].

Bacterial sepsis may induce a transient state of immunosuppression, which facilitates the development of IPA. In one study, otherwise immunocompetent mice that had recently recovered from experimentally induced sepsis were exquisitely susceptible to IPA following intratracheal challenge with conidia [189]. In contrast, non-septic control animals were resistant to IPA. Gene expression analysis showed downregulation of the expression of several components of the innate immune response following sepsis, which may have resulted in impaired resistance to IPA [189].

15 Summary

Aspergillus exemplifies the paradigm of an opportunistic organism, the pathogenesis of which is inextricably tied to host immunity. Recent advances have begun to

Table 2 Future areas of research of *Aspergillus* pathogenesis

1. Expansion of the list of PRR and their downstream signals in innate immune cells involved in defence against *Aspergillus* infection.
2. Cross-talk amongst respiratory epithelial cells, AM, PMN, and DC in orchestrating efficient defence against aspergillosis.
3. Regulation of an appropriate inflammatory response to *Aspergillus* in infected lungs.
4. Polymorphisms in innate immunity genes that influence the risk of aspergillosis.
5. *Aspergillus*-induced immune perturbation.
6. Modulation of angiogenesis and its potential effect on the extent of tissue damage and fungal dissemination in patients with IPA.
7. Determinants of interstrain and interspecies differences in *Aspergillus* pathogenicity.
8. Optimisation of conventional animal models and development of alternative in vivo models to study *Aspergillus* pathogenesis. For example, site-specific models for the study of infection at extrapulmonary locations, such as the central nervous system and soft tissues.

Legend: AM, alveolar macrophages; DC, dendritic cells; IPA, invasive pulmonary aspergillosis; PMN, polymorphonuclear cells; PRR, pattern recognition receptors.

define the components of the innate immune system that are charged with restraining the pathogenic growth of *Aspergillus* species in the lung, as well as the mechanisms by which these fungi may evade them. In the process, it has become clear that even within the immunosuppressed patient population, IPA is a disease with a heterogeneous pathogenesis that is shaped by specific deficits in host immunity. The range of known genetic and acquired deficiencies in host defence against aspergillosis is expanding. Novel immunosuppressive drugs that modulate defined aspects of innate immunity, such as TNF-α antagonists, are accounting for a growing number of IPA cases [190]. Other targeted therapies for cancer, such as endothelial growth factor inhibitors, may affect the progression of IPA by inhibiting angiogenesis and increasing the extent of tissue infarction caused by vascular invasion and thrombosis (Table 2). Our understanding of host defence against aspergillosis must continue to advance in tandem with research of the fungal mechanisms that subvert it.

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Clinical Manifestations of Invasive Pulmonary Aspergillosis

John R. Wingard and Jack Hsu

Abstract The clinical manifestations of invasive pulmonary aspergillosis (IPA) are protean and may vary over time as IPA progresses. Early, fever may be the sole manifestation. Later, cough, pleuritic pain, dyspnoea, haemoptysis, rales, and friction rub may occur. Several clinical findings are strongly suggestive of the diagnosis of IPA, but most are non-specific and other aetiologies may account for the constellation of clinical findings. Accordingly, pursuit of a microbiological diagnosis remains vital to establishing the correct diagnosis. Today, with the growing emphasis on early diagnosis to optimise treatment success, reliance on radiography and serologic biomarkers of IPA are making the usefulness of clinical manifestations for diagnosis less important. However, understanding of the evolution of clinical and radiographic findings over time and how these changes are associated with treatment outcome is important for sound decision making during treatment to avoid needless or misguided changes in therapy.

Keywords Aspergillosis · Clinical manifestations · Haemoptysis · Pleuritic pain · Pneumonia

Contents

J.R. Wingard (✉)
Division of Hematology/Oncology, University of Florida College of Medicine, Gainesville FL, USA
e-mail: wingajr@medicine.ufl.edu

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1 Introduction

The most common clinical syndrome of invasive aspergillosis (IA) is pneumonia. Invasive pulmonary aspergillosis (IPA) constitutes more than half of *Aspergillus* disease [1]. Sinusitis, haematogenously disseminated infection, cerebral infarction/haemorrhage, and cellulitis are other manifestations that may occur either with or without pneumonia.

The predominance of pneumonia is not surprising since *Aspergillus* species are widely prevalent organisms in the environment and their natural portal of entry is inspiration of exogenous organisms into the nasal passages and respiratory tract. It has been estimated that ordinarily each individual inspires 100–200 organisms daily. *Aspergillus* has been found in every continent and in a wide range of different climatic conditions. However, climates of heat and humidity are often mentioned as associated with a greater risk for exposure. Similarly, the late summer and early autumn, times when decaying vegetation presents ideal conditions for growth of organisms, have been implicated as times when infections are more frequent. Even with geographic and seasonal differences, IPA is a risk to immunocompromised patients at all times of the year and in all geographic locations.

2 Clinical Characteristics of IPA

The major influence on how IPA presents is the immune status of the patient and when during the course of infection the diagnosis is established. Most patients who develop IPA are immunosuppressed. Two thirds of patients are patients with acute leukaemia or undergoing haematopoietic stem cell transplantation (HSCT) [1]. Thus, clinical manifestations early in the course of disease are often muted because of inadequate inflammatory responses. Over time, as IPA progresses, the clinical manifestations evolve and become more pronounced.

The clinical presentation of IPA is protean. Moreover, many of the clinical manifestations of IPA are not specific for IPA and may be present in a variety of other infectious syndromes. However, the presence of certain individual findings, certain combinations of factors, and the duration of some factors are more likely to be found in patients with IPA than in patients without IPA.

In many patients, especially neutropenic patients, the sole initial symptom may be fever. IPA is rarely the cause of fever early in the course of neutropenia. More commonly, IPA is a cause of persistent or recurrent fever later during neutropenia. Onset of IPA most frequently occurs after 2 weeks of neutropenia [2], substantially later than most bacterial infections. The association of IPA with prolonged neutropenia explains why IPA is less frequent in treatment regimens or underlying disease conditions that result in shorter duration neutropenia.

Cough is a frequent symptom. Dyspnoea and sputum production are variable accompaniments. None of these are specific for IPA. More specific symptoms are haemoptysis and pleuritic pain. Although present in only a minority of patients early

in the course of infection, they are more suggestive of IPA than bacterial pneumonia and are noted in the majority of patients with more advanced IPA [3].

Clinical signs that may be present in IPA include elevated temperature and signs of pulmonary consolidation on auscultation, especially rales. A pleural friction rub is a more specific sign although present only in a minority of cases.

Various clinical findings were evaluated in patients with acute myelogenous leukaemia to determine which factors distinguished patients with IPA from those without [3]. Factors more common in patients with IPA were pleuritic pain, acute sinus tenderness and nasal discharge, epistaxis and eschar (denoting the presence of concomitant sinusitis in some patients), rales, development of multilobar infiltrates after the fourteenth day, and the presence of nodular or cavitary infiltrates (Table 1). Further, patients with IPA had a longer duration of neutropenia, more febrile days, more febrile episodes without an aetiology established, and more febrile days on antibiotics [3].

From these initial observations, a discriminant scorecard was developed to assist clinicians in recognizing IPA [4]. Eleven parameters were distinctive (Table 1). The presence of four or more of the individual factors was found to be present in patients likely to be infected compared to those not infected with high degrees of sensitivity and specificity. Shortcomings of such a discriminant scorecard include lack of validation studies by multiple institutions, lack of evaluation in other patient populations, the fact that treatment and supportive care practices have changed in the more than two decades since this algorithm was developed, and newer diagnostic tests have been introduced since. Moreover, this scorecard is most useful in patients with advanced IPA rather than early in the course of infection.

In non-neutropenic patients, symptoms are less frequent, especially fever, cough, and chest pain [5]. Co-infection with other microorganisms is more predominant in non-neutropenic patients.

Today, with increased emphasis on early diagnosis and with the widespread availability of high resolution computed tomography (CT) scanning and the use of serologic biomarkers, IPA is often detected earlier in the course of infection. Thus, many

Table 1 Clinical criteria characteristic of *Aspergillus* pneumonia in neutropenic patients with acute leukaemia

Elevated temperature
Neutropenic days > 30
Two or more febrile episodes without a source
Fourteen or more febrile days without a source
Nineteen or more days of fever during antibiotic therapy
Rales in the absence of volume overload
Nasal eschar, ulcer, or discharge plus epistaxis plus sinus tenderness
Pleuritic chest pain
Onset of pulmonary infiltrate after 14th day
Multi-lobed pulmonary infiltrate on radiography
Cavity or nodules on chest radiography

Source: Adapted from references [3, 4].

Table 2 Clinical and radiographic findings likely to be found early and later during invasive pulmonary aspergilosis

	Early IPA	Advanced IPA
Likely clinical findings	Fever	Fever Cough Dyspnoea Sputum Haemoptysis Pleuritic pain Pleural friction rub
Likely radiographic findings	Macronodule(s)	"Non-specific" nodular/localised infiltrates, often multi-lobar
	Halo sign	Air crescent sign

Legend: IPA, invasive pulmonary aspergillosis.

of the "classic" clinical findings are not present at time of diagnosis (Table 2). For example, in one series that used a systemic screening with CT scanning at the time of initial diagnosis, fever was present in 84% of patients, chest pain in 60%, and haemoptysis in 39% [6]. In another series that used serial galactomannan screening, the serologic marker was positive before the development of new pulmonary infiltrates in 68% of IPA cases [7]. In a third series in which a systematic screening by both serial galactomannan testing and CT scans was performed, most episodes were detected without the presence of prolonged fever. Clinical signs or symptoms, new pulmonary infiltrates or isolation of moulds from respiratory secretions were not initial clinical clues in any of the patients [8]. Thus, it is clear from these various observations that reliance on clinical manifestations will not optimise detection of IPA in its earliest and most treatable phase.

3 Radiographic Manifestations

Radiography is important in detecting and monitoring IPA. The chest CT scan is much more sensitive than plain radiography and is the preferred imaging technique for both early diagnosis and monitoring the course of treatment. Nodules, consolidation, cavities, air bronchograms, pleural effusions, ground-glass opacifications, and small-airway lesions have all been described as radiographic features of IPA. In a large imaging study of IPA, the most common radiographic finding at diagnosis was one or more macronodules (>1.0 cm in diameter), found in 94% of patients. Multiple nodules were noted in 79% of IPA cases (Table 3) [9]. The nodules were equally distributed in all lobes of the lung. Twenty seven percent of the nodules abutted the pleura and were infarct shaped, not surprising in view of the angioinvasive nature of IPA. The halo sign, a dense nodule surrounded by a perimeter of ground-glass opacity (due to oedema or haemorrhage) extending for at least 75% of the circumference of the nodule, was noted in 61% of cases. The nodules, found in a preponderance of patients with IPA, were such a striking finding that the authors suggested that

Table 3 Radiographic manifestations of invasive pulmonary aspergillosis at diagnosis

Imaging finding	Percentage of patients with finding
One or more macronodule (>1.0 cm in diameter)	94
Two or more macronodules	79
Halo sign	61
Consolidation	30
Infarct shaped macronodule	27
Cavitary lesion	20
Air bronchograms	16
Clusters of small nodules (<1.0 cm in diameter)	11
Pleural effusion	11
Air crescent sign	10
Non-specific ground-glass opacification	9
Infarct shaped consolidation	8
Small-airway lesions	7
Atelectasis	3
Hilar/mediastinal lesion	2
Pericardial effusion	1

Source: From reference [9]

the absence of at least one macronodule should raise the question as to whether another aetiology is explanatory for the pulmonary syndrome. It should be noted however that most patients included in this study had an underlying haematological malignancy or had been submitted to HSCT. As discussed in other chapters in this book, macronodules are less frequently seen in other populations such as patients on steroids, with chronic obstructive pulmonary diseases or undergoing solid organ transplantation.

Consolidation was found in 30% of patients with IPA at the time of diagnosis. Eight percent of consolidations were infarct-shaped. Cavities were found in only 20% of patients and only 10% had an air crescent sign (in which a sequestrum resided in a crescentic pocket of gas surrounded by a rim of viable lung). Other radiographic findings were found in only a small proportion of patients.

The imaging findings vary over time. In one study in which serial CT scans were performed in neutropenic patients treated for haematological malignancies, a typical halo sign was found in all patients at the time of onset of IPA [6]. Three days later, a halo sign was noted in 68% of patients. After one week, the halo was observed in only 22% of cases and after two weeks in only 19%. Thus, it is clear that the halo sign is a transient sign and appears most commonly early in the course of IPA, mostly in neutropenic individuals. The finding of the halo sign early in the course of IPA has been observed in other studies, and institution of antifungal therapy at the time of a halo sign being present is associated with better treatment outcomes [9]. As the likelihood of the halo diminished over time as IPA progressed, the occurrence of non-specific lesions, such as air-space consolidation, increased: 31, 50, and 18% at 3, 7, and 14 days, respectively. CT scans performed during the second week of IPA were least helpful in providing features characteristic of IPA. The air crescent sign

was not present initially, but was more likely to be observed later in the course of IPA: 8, 28, and 63% at 3, 7, and 14 days after onset of IPA. Especially notable was the association of the air crescent sign with recovery of the neutrophil count. Additional radiographic findings during the temporal evolution of IPA were progressive increases in the volume of the infiltrate during the first 7–14 days. This increase occurred even in the face of antifungal therapy, was independent of the type of treatment, and its increase during the first week did not appear to be a reliable marker of failure of treatment response (see Section 4, below).

Although most radiographic studies of IPA have been conducted in neutropenic patients, similar findings have been described in non-neutropenic HSCT patients in whom the halo sign was noted in up to 60% of patients [10]. Detailed radiography in other patient populations has not been reported. In one series, the infiltrates of IPA in non-neutropenic patients other than HSCT patients were less likely to exhibit segmental areas of consolidation and the halo sign was not seen [5].

4 Influence of the State of the Host Immunity on Clinical and Radiographic Findings

Host immunity plays an important role in the clinical manifestations of IPA. In neutropenic patients IPA is characterised mainly by angioinvasion, haemorrhagic infarction, and intra-alveolar haemorrhage [11]. In contrast, in non-neutropenic patients, inflammatory necrosis and granulomas were more prevalent although intra-alveolar haemorrhage also was seen. Interestingly, allogeneic HSCT patients even after engraftment (with neutrophil recovery) had patterns more like neutropenic patients than patients without neutropenia, suggesting a profound deficiency in neutrophil function. These pathologic features correlate with the radiologic findings: the halo represents haemorrhage and/or oedema surrounding infracted tissue which constitutes the nodule. Thus, one would anticipate the halo sign to be a more likely feature in neutropenic patients and allogeneic HSCT patients than in other patient populations.

The clinical and radiographic findings vary over time during the course of IPA in part due to the progress of microbial proliferation and resulting tissue damage, but also in part due to the host responses to the microbial invasion. Since in many cases the immune status changes over time, the host responses vary. The air crescent sign occurs late in the course of IPA and is more commonly associated with neutrophil recovery than as a marker of microbial progression [6]. Similarly, an immune reconstitution syndrome, characterised by either radiographic or clinical progression (or both), has been described [12]. This is most commonly seen during the course of treatment and poses a dilemma to clinicians struggling to determine if the patient is failing antifungal therapy and requires a change to a second-line therapy. Biomarkers such as galactomannan can be useful in distinguishing clinical worsening due to microbial progression, since some data indicate a reduction of serum galactomannan in patients who are actually responding to antifungal therapy in the face of

clinical worsening due to immune reconstitution [13]. Such patients apparently not responding clinically and/or radiographically ultimately have successful treatment outcomes without any change in therapy [13].

5 Differential Diagnosis

IPA is the explanation for the majority of nodular pulmonary infiltrates in neutropenic leukaemia patients late in the course of neutropenia. However, it is not the only diagnostic consideration. Further, in other scenarios, such as non-neutropenic HSCT patients, solid organ transplant recipients, and other patients with immunosuppressive medical conditions, the differential diagnosis includes a wider array of pathogens which can cause quite similar clinical syndromes (Table 4). Especially notable is Zygomycosis. Infections by this mould pathogen have been increasing in recent years. Several reports suggest that Zygomycetes infections may be more frequent in centres that widely use voriconazole (an antifungal agent with excellent activity against *Aspergillus* species but no activity against the Zygomycetes), although multicentre reports have not confirmed this at present. Zygomycetes can produce a syndrome indistinguishable from IPA. In one report there was a suggestion that patients with pneumonia due to Zygomycetes had more pulmonary nodules (10 or more) and were more likely to have a pleural effusion than patients with IPA [14]. In addition, they were also more likely to have concomitant sinusitis and to have been on voriconazole. Radiographically, a "reversed" halo sign has been described [15]. The "reversed" halo sign is a dense nodular ring with a round central ground-glass core. Although it may be present in IPA, the preponderance of fungal pneumonias in which it has been seen are due to Zygomycetes.

Other fungal pathogens of particular note are species of *Fusarium* and *Scedosporium* which can cause pulmonary syndromes similar to IPA. Of note, in disseminated infections by these fungi, organisms frequently can be isolated from the bloodstream; thus, blood cultures may be useful in the diagnostic assessment in contrast to both *Aspergillus* and Zygomycetes which are rarely isolated from blood

Table 4 Organisms that cause syndromes that may masquerade as invasive pulmonary aspergillosis and/or co-infecting/super-infecting organisms

Organisms that cause syndromes similar to invasive pulmonary aspergillosis	Zygomycetes
	Fusarium
	Scedosporium
	Nocardia
	Staphylococcus aureus
	Pseudomonas aeruginosa
Organisms that may be co-infecting pathogens	Bacteria, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*
	Viruses, such as cytomegalovirus and community respiratory viruses
	Zygomycetes

even during disseminated infection. *Fusarium* also has a predilection for entry via breaches in skin. Cellulitis or nail-bed infections should lead to the suspicion of *Fusarium*. A bacterial pathogen that can cause a similar pulmonary syndrome with nodular infiltrates that progress to cavitation is *Nocardia*. *Nocardia* tends to occur more frequently in scenarios with profoundly compromised cell-mediated immunity (e.g., HSCT patients with persistent chronic graft versus host disease) than in neutropenia, but the clinical syndromes are indistinguishable.

It is also important to note that IPA is often associated with both co-infection by other pathogens and super-infection by other pathogens can occur during the course of treatment of IPA (Table 4). Co-infecting organisms frequently noted include cytomegalovirus, community respiratory viruses (e.g., respiratory sincicial virus, influenza, parainfluenza), bacteria such as *Staphylococcus aureus* and *Pseudomonas* spp., and occasionally Zygomycetes.

Because of the lack of clinical and radiographic clues to reliably distinguish IPA from other infectious aetiologies and because of the possibility of multiple infectious pathogens, establishment of a microbiological diagnosis is paramount. Diagnostic studies and strategies are discussed elsewhere in this book. Since early therapy is important in optimising outcomes, treatment should be initiated presumptively whilst the diagnostic testing is being performed. If the disease appears to be progressing during therapy, the diagnostic assessment should be repeated. It is important to note that concomitant infection by other viral and bacterial pathogens occasionally occurs and although treatment may be gaining control of IPA, other infections may be the cause of apparent treatment failure.

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Diagnosing Aspergillosis: The Role of Invasive Diagnostic Interventions

Cornelia Lass-Flörl and Martin C. Freund

Abstract The diagnosis of invasive aspergillosis remains challenging and current conventional methods are limited for the diagnosis of invasive fungal infections. The gold-standard for the definite diagnosis of proven invasive pulmonary aspergillosis remains either histopathologic, cytopathologic or direct tissue examination, based on the documentation of typical hyphae and a positive culture of *Aspergillus* spp. This chapter reviews the yield of invasive procedures in the diagnosis of aspergillosis. Biopsies may be taken percutaneously, bronchoscopically, via open surgery or video-assisted thorascopic surgery. Computed tomography-guided percutaneous, bronchoscopic and open lung biopsies have been performed with a diagnostic yield of 80–100, 51 and 70%, respectively. Bronchoscopy with bronchoalveolar lavage is generally helpful in diagnosing invasive pulmonary aspergillosis, especially in immunocompromised patients with diffuse lung involvement. The sensitivity and specificity of a positive result of bronchoalveolar lavage fluid are ∼50 and 97%, respectively. All specimens obtained via invasive interventions including bronchoalveolar lavage should be processed for microscopy and cytology, fungal cultures and by means of any other available procedure (e.g., *Aspergillus* PCR and galactomannan). Combining microscopy and culture will increase the diagnostic yield by 15–20%. Combining culture, microscopy, serology, and *Aspergillus* PCR in lung tissues and/or bronchial samples will increase the diagnostic yield by 99%.

Keywords Aspergillosis · Biopsies · Invasive diagnosis · PCR

Contents

C. Lass-Flörl (✉)
Divison of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria
e-mail: cornelia lass-floerl@i-med.ac.at

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1 Introduction

Despite the availability of new antifungal drugs, the overall survival for immunocompromised patients with invasive fungal diseases remains low, with variations according to underlying disease [1–3]. Case fatality rates range from 30 to 80% in neutropenic patients and results at least partly from difficulties in obtaining a reliable diagnosis in an early stage of disease [1, 2].

Pulmonary infiltrates in immunocompromised patients may arise from infectious or non-infectious causes (Table 1). Infection may be due to bacterial, mycobacterial, fungal, protozoal, helminthic, or viral pathogens. Non-infectious processes may mimic infection. The diagnosis of invasive aspergillosis (IA) remains challenging and current conventional methods are limited for adequate diagnosis [3]. The gold-standard for the definite diagnosis of invasive pulmonary aspergillosis remains

Table 1 Most frequent pulmonary infections in immunocompromised patients

Bacterial pneumonias
Gram positive bacteria
(e.g., *Staphylococcus aureus,* enterococci)
Gram negative bacteria
(e.g., *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*)
Viral pneumonias
Cytomegalovirus
Herpes simplex virus
Human herpesvirus 6
Respiratory syncytial virus
Parainfluenza virus
Influenza virus A and B
Adenovirus
Protozoa
Toxoplasma gondii
Fungi
Aspergillus spp.
Candida spp.
Pneumocystis jirovecii

either histopathologic, cytopathologic or direct tissue examination. In clinical practice, the diagnosis of proven invasive pulmonary aspergillosis is rarely established ante-mortem.

Invasive procedures are frequently necessary to obtain reliable specimens. Specimens can be obtained by biopsies (taken percutaneously, bronchoscopically, via open surgery or video-assisted thorascopic surgery) and via bronchoscopic interventions (bronchoalveolar lavages, protected brushes). Tissue examination is performed for definite diagnosis or exclusion of invasive pulmonary aspergillosis (IPA). Furthermore, it enables the distinction between colonisation and invasive infection to be made, and allows other invasive infections requiring different therapy to be diagnosed [4, 5].

2 Biopsies

2.1 Percutaneous Transthoracic Lung Biopsies

Percutaneous transthoracic lung biopsy is performed with imaging guidance and usually aims to diagnose a defined mass near the chest wall [6]. Imaging modalities are fluoroscopy, multidetector computed tomography (MDCT), and ultrasound [7]. Ultrasound is useful only where the tissue mass is in contact with the chest wall since the ultrasound beam does not pass through air and, hence, the aerated lung. Magnetic resonance imaging (MRI) currently has a limited use because of expense, difficulty accessing the patient within the magnet, the relatively poor visualisation of lung lesions, and difficulties with ferromagnetic instruments within the magnetic field. In general, CT-guided percutaneous lung biopsy has been performed with a diagnostic yield of 80–100% [8–10]. Similar excellent data were obtained for the diagnosis of invasive aspergillosis (IA). In different studies, infection was documented in 70% [11], in 78% [12], in 80% [4], and in 100% [13]. Overall, the procedure is safe and reliable in obtaining diagnostic materials [9]. This intervention requires platelet counts of >60,000/ml [11] which is frequently not possible in profoundly neutropenic patients. Platelets transfusion might be required just before the procedure. Additionally, CT-guided percutaneous lung biopsy carries a moderate risk of pneumothorax, reported up to 18% [10] and haemoptysis in 3% [7].

Kuhlman et al. [14] described the appearance of a nodular density with a surrounding halo of low attenuation, the so-called “halo sign” on thoracic CT. It has been reported that the halo sign is indicative of focal invasive pulmonary aspergillosis (IPA) during the first 2–3 weeks of infection [14–20]. Later, these lesions progress to larger nodules with central cavitation (consolidations or cavitary masses), which in turn may form a peripheral crescent-shaped lucency termed an “air crescent sign”. This radiographic appearance occurs due to connection of the nodule to a peripheral subsegmental bronchus with peripheral absorption of necrotic tissue within (from the centre of) the lesion, forming a sequestrum of necrotic lung tissue. The presence of lesions suggestive of *Aspergillus* had a 90% predictive value

for the diagnosis of IPA in one recent series involving 87 immunosupressed patients [21]. Similar results were obtained by others [15–20]. However, the halo has been described also in patients with several other lung diseases [22–25]. Therefore, tissue examination is needed for definite diagnosis or exclusion of IPA. Presently, only limited data are available on the diagnostic value of CT-guided biopsies in diagnosing IA [12, 13]. In our centre, we scan the whole chest using a multidetector CT technique with 1.0 mm or even submillimeter slice thickness and high-resolution reconstruction algorithm [4]. Biopsies are performed with an automated biopsy gun with a detachable coaxial cutting needle system (Bard®) as described by Lucidarme et al. [26]. In our study an outer coaxial needle with 17-gauge diameter and an inner biopsy needle with 18-gauge diameter were chosen. In 61 patients processed we lack important complications [4].

2.2 Bronchoscopic Lung Biopsies

Biopsy via a bronchoscope is useful for proximal endobronchial lesions and may be assisted by imaging guidance. The diagnostic yield increased from 35 to 51% in peripheral pulmonary lesions, especially in lesions >3 cm [27]. Transbronchial biopsy (TBB) is an established method in the diagnosis of pulmonary infiltrates in immunocompromised patients [10, 28, 29] in ventilated patients [30] and also in patients with diffuse lung disease [6]. In one study, TBB allowed detection of all fungi infecting immunocompromised patients [29]. False-negative results are frequently seen with TBB, which may be related to the size of the fragments obtained and the focal nature (at least in early stages) of *Aspergillus* infections. This procedure requires a platelet count of >50,000/ml to avoid a risk of bleeding – which limits its utility in many pancytopenic patients. According to Cazzadori et al. side effects occur at a low rate (2.5%) [29].

2.3 Open Lung Biopsies

Open lung biopsy and video-assisted thoracoscopic surgery in patients with diffuse pulmonary infiltrates provide larger samples of tissue with improved accuracy and specificity [31]; mortality was not significantly increased in patients with haematological malignancies. By contrast, others described a mortality rate of 39% and a diagnostic benefit of 46% [32]. Haematological patients are high-risk candidates for perioperative complications, and surgery for diagnostic reasons alone is not advisable. The role of open lung biopsies in diagnosing IPA is somewhat contradictory: Kim et al. [33], Won et al. [34], Al-Nassar et al. [35], and Cheson et al. [21] recommended this intervention as an accurate procedure for appropriate therapy and clinical improvement; McCabe et al. [36] reported of only little help in directing medical therapy or influencing clinical outcome in patients with acute leukaemia. In 15 patients, representing with neutropenia, fever and pulmonary infiltrates, open lung biopsy detected fungi in only 4 patients. Presently, limited data are available to draw definitive conclusions. Data from the literature show a complication rate of

10–15% [37]. Severe thrombocytopenic patients who urgently require histological identification should undergo open lung biopsy [37].

In summary, for peripheral pulmonary lesions, the procedure of choice is needle biopsy or surgical resection of the lesion [38, 39]. For patients with bilateral alveolar or multifocal disease in whom surgical resection is impossible, the best procedures are bronchoscopy and brochoalveolar lavage [38, 39]. For focal lesions near the hilum and the great vessels in the lung, particularly in neutropenic patients, urgent thoracotomy and resection should be considered because of the risk of life-threatening haemoptysis.

3 Bronchoscopy

Bronchoscopy with bronchoalveolar lavage (BAL) is generally helpful in the diagnosis of IPA [40–44], especially in patients with diffuse lung involvement and is an established tool for the diagnosis of infectious complications in neutropenic patients [45] as well as after haematopoietic stem cell transplantation (HSCT) [40, 46–48]. The sensitivity and specificity of a positive result of BAL fluid are about 50–97%, respectively. Yet the diagnostic is not consistent and much lower yields have been reported [27, 49–51]. Its value in the diagnosis of IPA requires critical analysis. *Aspergillus* species can be detected in the BAL or in bronchial washings by culture, by microscopy with detection of mould hyphae, by detection of the *Aspergillus* antigen, or by polymerase chain reaction (PCR).

The value of *Aspergillus* culture and microscopy in BAL displayed a sensitivity of 43% and a specificity of 100% in histologically-proven cases of IPA [27]. BAL was more often positive in multilobular IPA than in localised disease, irrespective of the duration of pre-treatment with amphotericin B. The sensitivity for detecting IPA increases with multiple cultures [52]. It has been reported that a positive fungal culture from secretion indicates a poor prognosis.

4 Spectrum of *Aspergillus* Species

The most common species of *Aspergillus* causing invasive disease include *A. fumigatus*, *A. flavus* and *A. terreus* [38]. The specific identification of the fungus isolated from culture specimens can help in determining clinical significance. *A. niger* is rarely a pathogen [53], whereas *A. terreus* and *A. flavus* have been shown to be statistically associated with IA [38, 54–56].

5 Specimen Procedures

All specimens obtained from sterile body sites including BAL and bronchial lavages should be processed for microscopy and cytology, fungal cultures and any other procedures (*Aspergillus* PCR and galactomannan) appropriate for ruling out differential diagnoses (Table 2).

Table 2 Laboratory methods used for the diagnosis of invasive aspergillosis

Conventional microbiologic methods
Direct microscopy (KOH/calcofluour stains, Giemsa)
Culture
Histopathologic methods
Conventional microscopy
Direct immunofluorescence
Biochemical methods
Galactomannan test
Molecular methods

Overall, cultures of respiratory tract secretions may lack sensitivity in the diagnosis of IA. *Aspergillus* grows from sputum in only 8–34%, and from BAL in 45–62%. *Aspergillus* recovery from the respiratory tract usually represents colonisation in immunocompetent patients but may strongly suggest invasive disease in the immunocompromised host. Combining microscopy and culture will increase the diagnostic yield by 15–20% [43, 48, 57–59] over that with culture alone (Tables 3 and 4).

Confirmation of the diagnosis requires microscopic examination, particularly via histopathology and direct evaluation. While microscopy is moderately sensitive and absolute specific for fungal infection, it does not allow unequivocal confirmation of the diagnosis of IPA. Several other fungi may have similar microscopic and histopathological appearances such as *Scedosporium apiospermum, Fusarium* spp., *Scopulariopsis* spp. and others [60]. Immunohistochemistry is useful for

Table 3 Bronchoalveolar lavages for diagnosis of invasive aspergillosis

	Positive results (%) in patients with proven/probable aspergillosis			
Patients	BAL culture	BAL cytology	Both	References
Acute leukaemia	–	–	50	[41]
Leukaemia	23	53	59	[43]
Leukaemia	0	0	0	[44]
Leukaemia	40	64	67	[48]
HSCT				
Oncology				
HSCT	0	0	0	[42]
Focal pneumonia	100	0	100	
focal				
Diffuse				
pneumonia				
Allogenic BMT	41	83	100	[40]
Leukaemia	2	4	23	[40]
HSCT				
Oncology				
Allogenic HSCT	17	0	17	[47]

Legend: BAL, bronchoalveolar lavage; HSCT, hematopoietic stem cell transplantation.

Table 4 Laboratory methods and their contribution to the diagnosis of invasive aspergillosis

Specimens	No. of specimens	Histology[a] positive, culture positive	Histology positive, culture negative	Cytology positive, culture negative	Culture positive only	References
Pulmonary	23	23	7	8	39	[38]
Extrapulmonary	22	7	2	1	6	[38]
Autopsy tissue	23	12	23	–	0	[40]
Surgical and biopsy tissues	1,683	9	30	–	17	[40]
BAL[a]	1,185	28		48	68	[40]

Legend: BAL, bronchoalveolar lavage; IA, invasive aspergillosis.
[a]Includes histocytologic findings.

distinguishing organisms seen in tissue but not grown in culture [61]. Monoclonal and polyclonal fluorescent-antibody reagents have been developed for differentiating the genera of *Aspergillus, Fusarium,* and *Scedopsorium* in situ. Unfortunately, the high degree of antigenic relatedness amongst these pathogens has resulted in a significant cross-reactivity and low specificity [62, 63]. At this time, there are no commercially available fluorescent-antibody reagents for in situ identification of *Aspergillus* spp.

5.1 Microscopic Examination

All tissues from patients with suspected invasive fungal disease should be processed with fungal stains [4, 38, 62]. In direct preparations, Gram staining or haematoxylin-eosin staining, fungi can easily be missed or misinterpreted as artifacts, fibrin filaments or necrotic fibres. Hyphae are best visualized by special fungal stains: bronchoscopic material and/or tissue biopsies should be examined with periodic acid Schiff reaction (PAS), Gridley′s fungus, or silver-methenamine technique [38, 62]. Necrotic fungal structures become better visible with Grocott staining than with the PAS reaction, but at least one of these two staining procedures should be performed. The use of an optical brightener such as Calcofluour white (also in combination with 10% KOH) or Blankophor BA is an important method and may allow rapid diagnosis [4]. These whitening agents selectively bind to cellulose and chitin and display to be a very sensitive method. By means of direct microscopic examination, important parameters include septal formation, diameter or ramification of hyphae. *Aspergillus* proliferates as septate hyphae with 2.5–4.5 μm in diameter and can be characterized as branching dichotomously (approximately 45° angle). Yet, it is not possible to specify the fungus (Fig. 1).

The histopathological findings associated with IPA have been recently shown to differ according to the underlying host. In patients with allogeneic HSCT and graft versus host disease (GVHD), there is intense inflammation with neutrophilic infiltration, minimal coagulation necrosis, and low fungal burden. IPA in neutropenic

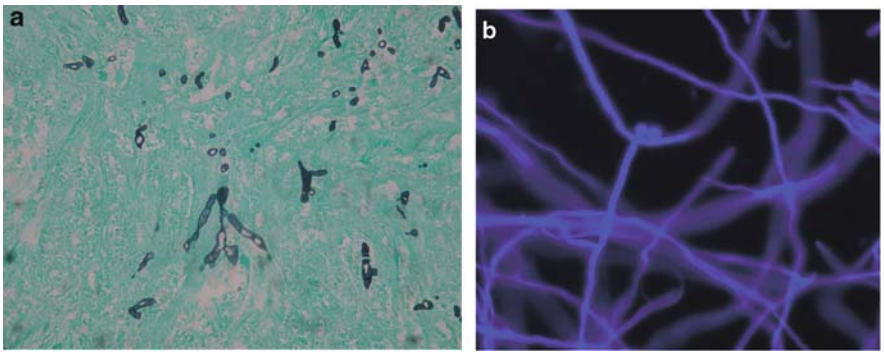

Fig. 1 Invasive aspergillosis shows variable morphologic features. (**A**) *Aspergillus fumigatus* in a lung biopsy specimen showing dichochtomous, acute branching septate hyphae (Haematoxylin & Eosin, ×1000). (**B**). *Aspergillus terreus* with calcoflour white stain displaying typical septate hyphae and aleurioconida

patients is characterized by scant inflammation, extensive coagulation necrosis associated with hyphal angioinvasion, and a high fungal burden. Dissemination to other organs is equally high in both groups [64].

5.2 Culture Techniques

Respiratory cultures of *Aspergillus* from expectorated sputum, bronchial washings, or BAL have a variable low sensitivity for IA but a high positive predictive value (>60%) in immunocompromised patients [43]. Blood cultures have little diagnostic value for invasive aspergillosis but may reflect true disease in *A. terreus* [65, 66].

A*spergillus* species are well recognised as common environmental airborne contaminant, therefore a positive culture from a non-sterile specimen, such as sputum, is not proof of infection [3]. Reliable results can be obtained from normally sterile body fluids and biopsy material. All fungi obtained from sterile sites should be identified to the species level. Materials should be cultured on special media enriched by antibiotics, preferably at 26 and 37°C over at least 7days. The use of enriched media, e.g. Sabouraud bouillon, may be helpful for the isolation of *Aspergillus.*

5.3 Serology Assays

Fluids and homogenised tissues may also be tested by serological methods such as specific *Aspergillus* ELISA and/or by molecular diagnostics. Detection of galactomannan antigen, an exoantigen of *Aspergillus*, has recently been shown to be a useful screening test for early diagnosis of IA. Galactomannan testing is discussed in more detail elsewhere in this book. Platelia *Aspergillus* EIA (Bio-Rad Laboratories) is a commercially available kit used to detect galactomannan antigen in body

Table 5 *Aspergillus* galactomannan testing in bronchoalveolar lavage or tissue biopsies with a cut-off of ≥1.0

Population (patients)	Cases	Sensitivity (%)	Specificity (%)	References
Haematology	26	100	100	[67]
HSCT	50	61	98	[46]
Haematology	67	87	96	[70]
Solid organ transplant	5	100	91	[71]
Solid organ transplant	67	67	98	[72]
Non-immunosuppressed patients	73	100	88	[73]
Haematology	65	98	97	[4]
ICU patients	89	88	87	[69]
Haematology	200	100	88	[68]

Legend: HSCT, haematopoietic stem cell transplantation; ICU, intensive care unit.

fluids. This method can detect as little as 1 ng/ml of galactomannan in the tested sample. Serum is the most frequently tested specimen and appears to provide high sensitivity in neutropenic patients. Use of other samples such as BAL [46, 67–73] and minced tissues [4] also appears to provide promising results (Table 5). There is evidence that galactomannan may become positive prior to clinical and radiological findings suggestive for IPA [46, 74–76]. Amongst HSCT recipients and patients with haematological malignancies, BAL galactomannan testing added to the sensitivity of both BAL culture and serum galactomannan testing [46, 76, 77].

Since galactomannan serum testing has low sensitivity in the diagnosis of IPA in non-neutropenic patients, BAL testing has been advocated in these individuals.

There was no conclusive benefit of determining BAL GM levels in the diagnosis of IPA amongst non-immunocompromised hosts. However, false-positive results with BAL galactomannan testing may occur due to colonisation, as has been demonstrated for lung transplant recipients [71]. Caution is therefore needed when interpreting these results. BAL specimens were also evaluated in intensive care units patients [69]. Culture and direct examination was positive in 60% of cases, while serum antigen was positive in 42%, BAL GM antigen was detected in 88%.

5.4 PCR Assays

Primers from either of the 18ssu-rRNA subunit genes, the 28S rRNA genes or mitochondrial genes have been studied as "panfungus PCR". The PCR using a sequence from the 18ssu-rRNA gene followed by species-specific hybridisation with probes for *Aspergillus* species has been studied most extensively in several clinical studies in haemato-oncological patients [78, 79] (Table 6). The specificity is around 65–75% depending on the number of tests needed to establish the diagnosis with a sensitivity of 100%. The species-specific approach using a nested PCR has been studied in BAL [78, 79]. The additional use of PCR detection methods increased

Table 6 *Aspergillus* PCR of bronchoalveolar lavage samples for diagnosis of invasive aspergillosis

Population	No. of patients	Sensitivity (%)	Specificity (%)	References
Immunocompromised	23	100	94	[82]
Neutropenic	12	100	100	[81]
Haematology	134	63	98	[83]
Immunocompromised	22	36	33	[86]
Haematology	74	100	96	[89]
Haematology	67	100	100	[91]
Haematology	141	94	94	[90]
Haematology	249	64	93	[87]
Haematology	24	90	100	[84]
Organ transplantation	66	73	93	[85]
Haematology	36	76	98	[88]
HSCT	49	67	100	[46]
Haematology	95	82	96	[80]

Legend: HSCT, haematopoietic stem cell transplantation recipients.

the diagnostic yield from 56 to 73%, especially concerning viral and fungal infections in BAL samples [80]. A positive *Aspergillus* PCR in BAL fluid has estimated sensitivity of 67–100% and specificity of 68–100% for IPA [46, 80–91]. However, until now, many test assays do not differentiate between colonisation and infection. Although PCR sensitivity may be improved by testing BAL samples, improved specificity must be questioned. Indeed, 25% of BAL samples from healthy donors are PCR positive through inhalation of airborne *Aspergillus* spores [79].

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Optimising the Use of Non-invasive Tests: From Blood to Radiology

Marcio Nucci and Elias J. Anaissie

Abstract Invasive pulmonary aspergillosis (IPA) is a leading cause of morbidity and mortality in immunocompromised patients. The main determinant of the poor outcome of IPA is the severity of immunosuppression associated with this infection. However, a delay in the diagnosis may be an important contributor. Therefore, attempts to early diagnose IPA are crucial. Non-invasive methods for the diagnosis of IPA include clinical manifestations, radiology and serology (particularly serum galactomannan and 1,3-β-D-glucan). Each of these tools has different performances, according to the severity of immunosuppression. The group with more severe immunosuppression is represented by patients with marked (<100/mm^3) and prolonged (>15 days) neutropenia. In these patients, clinical manifestations are more evident, thoracic computed tomography scan may reveal early and highly suggestive signs (i.e., the halo sign), and serum galactomannan may be of help, especially at ruling out the diagnosis. A second group is represented by non-neutropenic allogeneic haematopoietic stem cell transplant recipients, and the third group comprises patients with T-cell immunodeficiency such as solid organ transplant recipients. In the later 2 groups, clinical manifestations are subtle or absent, radiology is frequently non-specific, and serology is of less help. The diagnosis of IPA is challenging, especially in these less-severely immunosuppressed hosts.

Keywords Aspergillosis · Beta-glucan · Diagnosis · Galactomannan · Halo sign

Contents

E.J. Anaissie (✉)
Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA
e-mail: AnaissieEliasJ@uams.edu

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1 Introduction

Invasive pulmonary aspergillosis (IPA) is a leading cause of morbidity and mortality in immunocompromised patients. This chapter attempts to optimise the use of different diagnostic tools for IPA, including clinical presentation, radiology and serology, in different clinical contexts. Details of specific diagnostic tools such as processing of biologic material, identification of *Aspergillus* species, histopathology and serology, are discussed in other chapters along this book.

IPA occurs in the setting of a variety of underlying conditions. In a series of 621 cases, some patients had underlying diseases such as alcoholism, chronic bronchitis, hepatitis, burns and prematurity. However, the large majority of cases (72.6%) occurred in patients with haematological malignancies, especially acute leukaemia (31.1%), chronic leukaemia (12.2%) and lymphoma (9%). Thirty-six percent of the patients had undergone haematopoietic stem cell transplantation (HSCT) and 10% were recipients of solid organ transplant (SOT). Allogeneic HSCT was the most frequent transplant (12.8%), followed by heart-lung transplant (11.1%) and small bowel/liver-small bowel transplant (10.7%). HIV infection (9%), solid tumours (2%) and chronic immunologic diseases (2%) completed the list of underlying diseases [1].

IPA is associated with poor outcome mainly because of the severe immunosuppression associated with this infection. However, a delay in the diagnosis may be an important contributor. Therefore, attempts to early diagnose IPA are crucial.

The main determinant of the clinical form of IPA is the immune status of the host. One side of the spectrum is represented by patients with severe ($<100/mm^3$) and prolonged (>15 days) neutropenia, notably patients with acute leukaemia receiving induction remission, and allogeneic HSCT recipients in the early post-transplant period. In these patients, acute IPA is characterised by angioinvasion, with intra-alveolar haemorrhage and pulmonary infarction [2]. On the other side of the spectrum are non-neutropenic patients who develop aspergillosis because of T-cell mediated immunodeficiency. This group is represented by patients with a variety

of clinical conditions, including SOT, solid tumours, chronic granulomatous disease, and an emergent group of critically ill patients, especially those with chronic obstructive pulmonary disease (COPD) receiving steroids [3]. IPA in these patients does not exhibit the classic angioinvasion observed in severely neutropenic patients. Instead, the histopathology is characterised by inflammatory necrosis [4]. Finally, a third group is represented by non-neutropenic allogeneic HSCT recipients. In these patients, IPA occurs in the context of severe T-cell immunodeficiency caused by graft versus host disease (GVHD) and its treatment. Although angioinvasion may be observed, it is less frequent than in severely neutropenic patients. However, although the number of neutrophils is normal, there is a paucity of inflammatory cells in lung tissue, indicating that these patients are functionally neutropenic [4].

In addition to the clinical and radiologic picture, the status of the immune system may also affect the performance of different diagnostic tests, such as serum galactomannan. For example, in a study in animals, the concentration of galactomannan in different tissues was high in chemotherapy-treated mice and lower in steroid-treated mice. By contrast, the inflammatory response was intense in steroid-treated mice, and the migration of neutrophils contributed to lung injury [5]. This phenomenon of migration of neutrophils to the lungs is also observed in neutropenic patients when they recover from neutropenia. This immune reconstitution that follows neutrophil recovery is accompanied by worsening of clinical conditions and radiologic parameters, and may be confused with antifungal therapy failure [6]. In this chapter we will review the clinical presentation of IPA in these different hosts, discuss the main radiologic features, and comment on the usefulness and limitations of ancillary diagnostic tests, especially serum galactomannan.

2 IPA in Severely Neutropenic Patients

2.1 Clinical Manifestations

The association between the duration of neutropenia and the risk of aspergillosis was defined more than 20 years ago. In patients with acute leukaemia, it was observed that early in the course of neutropenia, patients developed invasive aspergillosis (IA) at a rate of approximately 1% per day, increasing to 4.3% per day between the 24th and 36th days of neutropenia. Neutropenia persisting >3 weeks was the main risk factor [7]. In a large series of invasive fungal infections in patients with haematologic malignancies, 69% of cases of IA were observed in patients with acute myeloid leukaemia (AML), and 2/3 of the cases occurred during neutropenia that followed induction remission for newly diagnosed or relapsed patients, a situation that is associated with a longer duration of neutropenia [8]. In a series of 269 AML patients with IA, 267 had neutropenia with a duration >10 days [9]. In a study of IA in a haematology ward, 92% of cases occurred during neutropenia of a median duration of 101 days, ranging from 13 to 277 days [10]. Another evidence for the close association between prolonged neutropenia and IPA is the fact that cord blood

transplantation is a major risk factor for IPA in allogeneic HSCT recipients. Cord blood transplantation is associated with the longer duration of neutropenia of all HSCT [11].

The close association between prolonged neutropenia and IPA may help clinicians to interpret early clinical signs that may appear in IPA. The classic clinical picture is that of persistent or recurrent fever in patients with prolonged neutropenia, associated with signs of pulmonary infection: dry cough and pleuritic chest pain [12]. Therefore, this triad occurring in the appropriate host (severe and prolonged neutropenia) should be interpreted as indicative of IPA until proven otherwise. On the other hand, fever, cough and chest pain occurring early in the course of neutropenia is unlikely to represent IPA. The poor specificity of these findings may be illustrated by a study that compared 47 cancer patients with autopsy-proven IPA and 49 consecutive controls that died of non-fungal pneumonia during the same study period. None of the 3 clinical manifestations (fever, cough and pleuritic chest pain) was significantly associated with IPA, although there was a trend favouring a higher frequency of cough and chest pain in patients with IPA [13]. Therefore, only the combination of the 3 clinical findings in the appropriate host (severe and prolong neutropenia) is indicative of IPA.

Despite the close association between prolonged neutropenia and IPA, there is no cut-off for the duration of neutropenia above which one might suspect the diagnosis, or below which the diagnosis is ruled out. We have recently developed an index that combines duration and intensity of neutropenia, based on the area over the neutrophil curve. The index (called D-index) was predictive of invasive mould infections, and for a cut-off of 5,800, the negative predictive value was between 97 and 99% [14]. A prospective validation of this tool is needed.

Frequent clinical manifestations other than fever, cough and chest pain include shortness of breath and dyspnoea. In a series of 52 neutropenic patients with IPA, fever was present in 96%, cough in 67%, dyspnoea in 60%, chest pain in 33%, and haemoptysis in 6% of cases [15]. Haemoptysis is more frequently reported after bone marrow recovery, and occasionally evolves to death, as a consequence of invasion of a large blood vessel [16]. A rare complication is spontaneous pneumothorax or pneumomediastinum, with sharp chest pain and dyspnoea [17].

2.2 Radiology

The radiologic features of IPA in severely neutropenic patients reflect the angioinvasive nature of the disease. The chest X-ray is not useful in the early diagnosis of IPA. It is often abnormal, but the appearance is non-specific, with patchy segmental or lobar consolidations and less frequently nodular opacities [18]. Later in the course of the disease, when neutropenia resolves, the chest X-ray may demonstrate the air crescent sign, a nodular opacity with a crescent or circumferential cavitation that is formed as a consequence of the retraction of the infracted lung. The air crescent, although not specific of IPA, is highly characteristic [19].

In contrast with the limited utility of the chest X-ray, the computed tomography (CT) scan is very useful in the early diagnosis of IPA. The typical image is the halo sign. It is a nodule surrounded by a ground-glass opacity, and represents pulmonary oedema and haemorrhage surrounding an area of coagulative necrosis [20]. The halo sign was evaluated as a diagnostic tool for IPA. Chest CT scans and autopsy findings were retrospectively reviewed in 48 patients with haematologic malignancies. The halo sign was present in 13 of 17 patients with IPA, but in none of the patients with other diagnoses. Other findings in the CT scan observed in patients with IPA included nodules (14 patients), consolidations (9 patients), ground-glass opacities (15 patients), mass, air bronchogram and effusions (6 patients each), and cavitation (2 patients), but other than the halo sign, the only lesions that were significantly more frequent in patients with IPA were nodules and mass [21]. The halo sign in the context of patients with prolonged neutropenia is not exclusive of IPA, and may be observed in other mycoses [22]. For example, in a retrospective study comparing the clinical characteristics of patients with pulmonary zygomycosis and IPA, the halo sign was present in 25% of 16 patients with zygomycosis and in 21% of 24 patients with IPA [23]. The halo sign may be present in a variety of non-infectious (such as Wegener granulomatosis, Kaposi sarcoma and haemorrhagic metastases) and infections conditions (such as viral pneumonias, tuberculosis and invasive fungal infections [24]). However, in patients with severe and prolonged neutropenia, IPA is by far the most frequent cause of the halo sign.

Although the halo sign is very useful in the early diagnosis of IPA, it is not present for long period of time, and after a few days it is replaced by non-specific infiltrates [25]. Later in the course of the infection, another typical lesion appears, the air-crescent sign, but its recognition has a much lower impact on the prognosis of IPA, since it usually appears only when the patient recovers from neutropenia [19].

By magnetic resonance imaging (MRI), the earliest image appears as a “target” lesion, with a hypodense or isodense centre, represented by cavitation or coagulative necrosis, a higher signal intensity in the periphery, corresponding to sub acute haemorrhage or haemorrhagic infarction, and peripheral enhancement with gadolinium diethylenetriamine penta-acetic acid, which represents an area of inflammation and hyperaemia [26].

The importance of the CT scan in the early diagnosis of IA was demonstrated in a study that showed that the halo sign was present in 92% of cases when a CT scan was performed early and routinely in persistently febrile neutropenic patients, compared to 13% when CT scans were performed only after the appearance of signs and symptoms suggestive of IPA. More importantly, the early recognition of the halo sign allowed the early institution of appropriate antifungal therapy, with a significant reduction in the mortality rate [27].

The prognostic importance of the halo sign was also evaluated in a large prospective study that compared voriconazol to deoxycholate amphotericin B as initial treatment. Amongst 235 patients with IPA, the most frequent lesion in the baseline CT scan was nodule with $\geq$1 cm in diameter (including those with halo sign and infarct-shaped), present in 94.5% of cases. The halo sign was present in 60.9% of patients,

cavitary lesions occurred in 20.4%, infarct-shaped lesions in 20%, and air crescent signs in 10%. The presence of the halo sign was associated with better response to treatment and survival [28].

In neutropenic patients, a rapid immune reconstitution following resolution of neutropenia is associated with transient clinical and radiologic deterioration. The pulmonary immune reconstitution inflammatory syndrome (PIRIS) of IPA is typically, but incorrectly, considered evidence of progressive infection on the basis of which patients may undergo additional diagnostic procedures and treatment modifications including enrolment on investigational agents. These patients are in fact achieving control of their infection and do not require any treatment modifications [6]. Serum galactomannan is of help in diagnosing PIRIS and guiding management (see below).

2.3 Serology and Antigen Detection

Antibody detection has limited usefulness in the diagnosis of IPA in neutropenic patients [29], but may be of help in establishing the diagnosis of sub-acute IPA in non-neutropenic patients with some immunodeficiency, such as chronic granulomatous disease [30].

2.3.1 Galactomannan

The most important serologic test for the diagnosis of IPA is serum galactomannan. The sensitivity of the test in neutropenic patients, as reported in a meta-analysis, was 70% (95% confidence interval 62–77%), whereas the specificity was 92% (95% confidence interval 90–93%) [31]. The high sensitivity of the test in neutropenic patients is in accordance with the pathogenesis of IPA, with angioinvasion, intense hyphal growth and high fungal burden [5]. The test may be used as screening of high-risk patients, or to confirm the diagnosis in patients with clinical manifestations suggestive of IPA. It may be positive 5–8 days before the appearance of the first clinical manifestation of IPA [32, 33]. The test has a high negative predictive value (95–98%), suggesting that it may be useful at ruling out the diagnosis of IPA [34]. For the diagnosis, the use of 2 consecutive samples with optical density index $\geq$0.5 gives a high accuracy, with an overall sensitivity of 92% and specificity of 97.5% [35]. A good performance of the test in the early diagnosis of IPA in neutropenic patients is obtained when the test is performed 2–3 times per week and supported by suggestive chest CT scan findings, usually taken early in the course of febrile neutropenia [35, 36]. Serum galactomannan is also useful in the diagnosis of PIRIS. While progressive disease is associated with increasing serum levels, clinical response is accompanied by a decrease in serum galactomannan [37–40]. Limitations of the test include false positive results in patients receiving some β-lactam antibiotics, especially piperacillin-tazobactam and amoxicillin-clavulanate, and false-negative results in patients receiving mould-active antifungal agents [41].

The utility of galactomannan testing in the bronchoalveolar lavage (BAL) in patients with long term neutropenia has also been demonstrated [42]. In neutropenic patients with lung infiltrates, the accuracy of galactomannan testing is improved when BAL is tested instead of sera.

2.3.2 β-Glucan

Another serologic test that has been evaluated in the diagnosis of IPA is serum 1,3-β-D-glucan (BG), a component of the cell wall of a variety of fungi, including *Aspergillus* species. The 2 tests commercially available (Fungitec-G assay, Seikagaku, Japan; and Fungitell assay, Associates of Cape-Code, USA) use different reagents, resulting in different cut-off values.

BG was tested in 190 episodes of neutropenia in 95 patients with acute leukaemia. A median of 16 tests were performed per neutropenic episode, and there were 62 episodes of invasive fungal infection, including 15 cases of proven or probable IPA. The sensitivity, specificity, positive and negative predictive value of 2 consecutive tests with $\geq$7 pg/ml of BG in the serum was 63, 96, 79 and 91%, respectively. BG was negative in 4 cases of IPA [43].

Because BG is present in different fungi, its detection is not diagnostic of IPA. Like serum galactomannan, BG has high negative predictive value and seems to be good at ruling out an invasive fungal infection (not only IPA). The negative predictive value of twice-weekly sampling is 100%, and test results are apparently not influenced by the use of mould-active antifungal agents [44]. By contrast, there are various factors that may lead to false-positive results, including the use of albumin or immunoglobulins, exposure to glucan-containing gauze, haemodialysis and some antibiotics, such as amoxicillin-clavulanate [45].

2.3.3 Polymerase Chain Reaction (PCR)

In the past several years different PCR assays have been developed with the intent to detect *Aspergillus* DNA in different clinical specimens. The lack of a consensual technique has limited its usefulness in the diagnosis of IPA [46]. More recently, real-time PCR and automated DNA extraction has brought hope to the molecular diagnosis of IPA. In a prospective study, a nested PCR assay was used to detect *Aspergillus* in blood during 95 episodes of neutropenia in high-risk haematologic patients. The sensitivity, specificity, positive and negative predictive values of 2 positive PCR results were 100, 75.4, 46.4 and 100%, respectively. More importantly, PCR positivity preceded standard diagnosis by a mean of 14 days [47]. In another study, real-time quantitative PCR of blood and serum samples of 83 neutropenic patients was performed. Taking 2 consecutive positive results as diagnostic criterion, the PCR detected 11 of 12 cases of IA, with sensitivity, specificity, positive and negative predictive values of 91.6, 94.4, 73.3, and 98.5%, respectively. The combination of serial PCR and galactomannan detected all cases of aspergillosis, with PCR being positive earlier than galactomannan [48].

3 IPA in Non-Neutropenic Allogeneic HSCT Recipients

3.1 Clinical Manifestations

IPA in non-neutropenic allogeneic HSCT recipients occurs almost exclusively in patients with GVHD receiving high doses of steroids. These patients usually have signs of severe immunosuppression, including lymphopenia, cytomegalovirus (CMV) reactivation or other viral infections, or receipt of T-depleted or CD34-selected stem cell products [11, 49, 50]. The clinical manifestations are subtle and non-specific. In a study, IPA was the most common (83% of patients) cause of late-onset community-acquired pneumonia amongst hospitalised HSCT recipients with chronic GVHD. Of importance, in 6 of the 10 cases of IPA, a Gram-negative bacterial agent was concurrently isolated from respiratory secretions, and in 5 patients the initial clinical presentation and radiologic picture was that of bacterial pneumonia [51]. In a series of 22 cases of invasive mould infections (18 of them caused by *Aspergillus* species), only 32% presented with fever, chest pain was observed in 14% and cough in 23%. The most frequent clinical manifestation was shortness of breath, occurring in 64% of cases [52].

3.2 Radiology

IPA in non-neutropenic allogeneic HSCT recipients may be characterised by angioinvasion (like in neutropenic patients), or airway invasion. In the later form, the radiologic picture is that of a bronchopneumonia. The X-ray shows patchy air space consolidation, with or without small nodules, and the CT scan exhibits air space consolidations or nodules.

The radiologic picture of late-onset IPA in allogeneic HSCT recipients was described in a series of 27 patients, with a total of 111 chest CT scans. Halo sign and centri-lobular nodules were present in 12 patients each. On the basis of the initial CT scan, 11 patients were classified as having the angioinvasive type, 8 presented with the bronchopulmonary type, 4 exhibited a combination of the two patterns, and 4 presented non-specific radiologic findings [53].

3.3 Serology and Antigen Detection

Like in neutropenic patients, serum galactomannan may be used in the diagnosis of IPA in non-neutropenic HSCT recipients. However, its performance may not be as good as in neutropenic patients, with false-negative and false-positive results. A retrospective study evaluated 157 adult allogeneic HSCT recipients who had at least 2 galactomannan tests performed after transplant. Fifty patients (32.2%) had positive galactomannan results, and 25 developed IA. The positive predictive value was lower during the first 100 days post-transplant, and gastrointestinal chronic GVHD

was the only factor associated with false-positive galactomannan [54]. In another study, none of 3 non-neutropenic HSCT recipients with IPA had positive serum galactomannan, as opposed to 8 of 10 neutropenic HSCT recipients [55]. This differences in the performance of the test may be related to the immune status of the host, with more galactomannan being released to the blood in severely immunosuppressed patients [56].

4 IPA in Other Settings

As mentioned earlier, acute leukaemia and HSCT account for the large majority of underlying diseases in IPA. Amongst the other hosts that may develop IPA, two groups are worth mentioning: solid organ transplant (SOT) recipients, especially recipients of lung transplant, and intensive care unit (ICU) patients.

4.1 IPA in Solid Organ Transplant Recipients

4.1.1 Lung Transplantation

Amongst SOT, the incidence of IPA is higher in lung transplant recipients, occurring in 3–15% of cases [57]. A review of 159 reported cases of IA in lung transplant recipients showed that infection occurred in a median of 3.2 months post transplant, with 72% of cases being diagnosed until 6 months post transplant. The majority of infections were tracheobronchitis (37%), 32% had invasive pulmonary disease, 21% had bronchial anastomotic infections and 10% had disseminated IA [58]. Tracheobronchitis may be characterised by ulceration, cartilage invasion and pseudomembrane formation. Fever is insidious or absent. Other clinical findings include dry cough and dyspnoea. CT findings are usually subtle, with focal plaques along the tracheal wall, with or without thickening of the tracheal mucosa [59]. Complications involving the anastomosis site, infection of airway stenoses and development of a bronchial-pulmonary artery fistula have been described [60]. In invasive pulmonary disease, fever occurs in about 30% of cases, and CT scan may reveal nodules or cavitary lesions. A remarkable finding of IPA in lung transplant recipients is the high frequency of colonisation of the airways by *Aspergillus* species [57].

Galactomannan testing in the BAL fluid has shown to be a useful tool for the diagnosis of IA in lung transplant recipients [61, 62], which has been associated with a high negative predictive value (>90%). The best cut-off for galactomannan testing in BAL (i.e., whether 0.5, 1.0 or 1.5) is still a matter of debate, with some studies showing that false-positive results may occur in patients colonised with *Aspergillus* species [62, 63]. The sensitivity of serum galactomannan testing in lung transplant recipients is too low (30%) to be of any value in clinical practice [64]. Other diagnostic strategies such as PCR or BG have not been evaluated for lung transplant patients.

4.1.2 Other Organ Transplants

IPA occurs in 1–8% of liver transplant recipients. Most infections develop within 1 month from transplant, and risk factors include renal dysfunction and retransplantation [57]. Clinical presentation is non-specific with fever (may be absent), cough and dyspnoea, or patients may have no clinical findings. Nodules are frequently present in the thoracic CT scan.

In a series of 844 heart transplants recipients from a single centre, 82 had *Aspergillus* species isolated, and 25 presented with IPA, at a median of 46 days post-transplant. Fever was present in 52% of patients, followed by dyspnoea and cough (12% each). Thoracic CT scans were available in 8 cases. Nodules (mean of 4.4 per patient) were present in all patients, but only 1 presented with the halo sign. Other findings included cavitation and pleural effusions (4 cases each). Compared with patients with colonisation, those with IPA were more likely to have fever, CMV co-infection and rejection [65].

Serum galactomannan has been increasingly evaluated in SOT recipients. A meta-analysis polled data of a few studies in SOT recipients and reported sensitivity and specificity of 22 and 84%, respectively [31]. Amongst 70 lung transplant recipients (12 of whom developed IPA), the sensitivity was only 30% and the specificity was 93% [64]. In a study in 154 liver transplant recipients, positive tests were observed in 21 patients but only one developed IPA. Patients with autoimmune disease and those requiring dialysis were more likely to have positive galactomannan tests [66]. In another study, serum galactomannan was performed in 85 liver transplant recipients. The test was repeated once a week in the first month after transplant. False-positive results were observed in 9.6% of 414 serum samples. The number of false-positive results was significantly higher in the first week post-transplant, and exposure to ampicillin was the only independent factor associated with false-positive results [67].

4.2 IPA in Intensive Care Unit Patients

IPA has been increasingly reported in ICU patients [68]. Although many of these cases are represented by severely immunosuppressed patients that require advanced supportive care, less-severe immunosuppressed patients have been diagnosed with IPA. These include COPD in combination with prolonged steroid use, high-dose systemic steroids, chronic renal failure, liver cirrhosis, transfusion-associated haemosiderosis, acute hepatic failure and diabetes mellitus [69]. Immunodeficiency in these patients is thought to result from a combination of steroid therapy, septic shock, sepsis-associated immunoparalysis, malnutrition, transfusions of allogeneic blood products, haemodialysis and poor glycemic control [70].

The diagnosis of IPA in non-neutropenic ICU patients is difficult. Signs and symptoms are non-specific, *Aspergillus* species are frequently recovered from respiratory secretions (especially in COPD and in mechanically ventilated patients) and not necessarily represent true infection, radiologic manifestations are non-specific (alveolar infiltrates, atelectasis), and serology (galactomannan) has not been

extensively tested in ICU patients. In a retrospective study, serum galactomannan was positive in only 53% of ICU patients with IA [71]. More recently, a prospective study evaluated 110 adults with chest CT scan, serum galactomannan and culture and GM from BAL. Only 22% of patients were neutropenic and 67% had underlying diseases other than haematologic malignancy, including cirrhosis and COPD. Twenty-six patients (24%) developed IPA, confirmed by histopathology. Chest CT was of limited diagnostic value and no patient demonstrated a halo sign. The sensitivity of serum galactomannan was only 42%, but the sensitivity and specificity of galactomannan in the BAL was 88 and 87%, respectively. Serum galactomannan and culture of the BAL remained negative in 11 patients (42%) in whom galactomannan in the BAL was positive [72]. As for lung transplant recipients, no study has evaluated the utility of PCR and BG testing in ICU patients developing IA.

It is suggested that in ICU patients with underlying conditions reported to be associated with IPA, the presence of persistent or rapidly developing pulmonary infiltrates should trigger additional tests that may include direct staining and culture of respiratory secretions and biopsy [69].

Table 1 Diagnosis of invasive pulmonary aspergillosis in different hosts

		Non-neutropenic patient		
	Neutropenic patient	HSCT recipient	Lung transplant recipient	Other settings
Clinical picture	Fever, cough, pleuritic chest pain	Fever (∼30%), cough, dyspnoea	Dyspnoea, cough	Fever (occasionally), Dyspnoea, cough
Radiologic pattern	Angioinvasion, with pulmonary infection	Angioinvasion, bronchopneumonia	Tracheobronchitis, unspecific infiltrates	Bronchopneumonia
Halo sign	Present, early	May be present (∼40%)	Typically absent	Rare
Other radiologic findings	Wedge-shaped infiltrates, nodules, cavitation (air crescent sign)	Centrilobular air space consolidations or nodules	Focal plaques along the tracheal wall, with or without thickening of the tracheal mucosa	Non-specific lung infiltrates, nodules
Serum galactomannan	Sensitivity 70%; specificity 92%. Good at ruling out the diagnosis	Likely lower sensitivity than in neutropenic patients. False-positives reported in patients with gastrointestinal (GVHD)	Sensitivity 30%, specificity 93%[a]	In SOT recipients sensitivity 30%, specificity 93%; sensitivity 42% in ICU patients[a]

Legend: GVHD, graft versus host disease; HSCT, haematopoietic stem cell transplantation; ICU, intensive care unit; SOT, solid organ transplantation.

[a]Testing galactomannan in the BAL is preferred for the non-neutropenic patient.

5 Conclusions

The diagnosis of IPA is based on the proper identification of susceptible hosts, the knowledge of the main clinical form of disease in each host, and on the correct interpretation of clinical and radiologic signs (Table 1). Neutropenic patients have clinical signs of pulmonary infarction, and the halo sign is frequently present. In non-neutropenic HSCT recipients, IPA may present without fever or have an initial diagnosis of bacterial pneumonia. The halo sign may be absent. IPA in lung transplant recipients is characterised by involvement of the tracheobronchial tree, and the diagnosis may be difficult because the airways are frequently colonised by *Aspergillus* species. Finally, non-neutropenic patients may develop IPA with non-specific clinical and radiologic findings. Serum galactomannan also have different performances in these different hosts, and it is more useful in neutropenic than in non-neutropenic patients. Galactomannan testing in the BAL is particularly useful for non-neutropenic patients with IA.

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Defining Invasive Aspergillosis: What the Revised EORTC/MSG Definitions Have in Store

J. Peter Donnelly

Abstract The EORTC/MSG definitions are being widely used for clinical trials of therapy and prophylaxis, evaluating diagnostic tests, and for epidemiology studies. However shortfalls were obvious hence a consensus group was initiated in 2003 with the clear goal of building on what the original definitions had achieved whilst improving the definitions to address the known issues. The revised definitions were finally published in 2008 and contain important changes. Host factors now include solid-organ transplants, hereditary immunodeficiencies and connective tissue disorders, and "major" and "minor" clinical features have been replaced by medical imaging that indicates invasive fungal disease. The detection of the galactomannan has been joined by tests for β-D-glucan as markers for probable invasive fungal disease. Nucleic acid detection, such as achieved by polymerase chain reaction (PCR) testing is not included as there is no standardisation or validation as yet. Though imperfect, the revised definitions should help set a benchmark for the epidemiology, diagnosis, and management of aspergillosis.

Keywords Aspergillosis · Beta-D-glucan · Consensus · Diagnosis · EORTC · Galactomannan · MSG

Contents

J.P. Donnelly (✉)
Department of Haematology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
e-mail: p.donnelly@usa.net

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1 Introduction

In the 6 years since the publication of the consensus definitions of the Invasive Fungal Infection group of the European Organisation for Research and Treatment of Cancer and the Mycoses Study Group, now known as the EORTC/MSG definitions, it was obvious that they were being widely used for their intended purpose namely, for clinical trials of therapy [1, 2] and prophylaxis [3, 4], evaluating diagnostic tests [5–9] and for epidemiology studies [10–13]. However there were also critical notes and cracks had appeared. For instance Subira et al. reported that only 36% of cases of invasive aspergillosis (IA) met the EORTC/MSG criteria in life the remainder only being found at autopsy [14]. Others have reported a similar experience [15].

Shortfalls were obvious insofar as the range of hosts encompassed by the original definitions was restricted to patients who developed invasive fungal infection as a result of cancer and its treatment. Clearly invasive fungal infections were occurring outside this narrow ambit and so there was a keen explicit desire to widen the scope of the definitions. The distinction between probable and possible invasive fungal infections hinged upon the presence of mycological evidence but the term "possible" did not seem suited to allogeneic haematopoietic stem cell transplant (HSCT) recipients with nodular pulmonary infiltrates with or without a halo sign for which mycological support was either not found or couldn't be established. In the clinic these cases were treated anyhow not unreasonably on the assumption that invasive pulmonary aspergillosis (IPA) was the most likely explanation for the illness. This was reinforced by clinical trials of antifungals that allowed these cases to be included by modifying the EORTC/MSG definitions and regarding them as "probable IPA" [1, 2]. Clearly this was not a satisfactory state of affairs. There was also wider experience with the Platelia galactomannan EIA test which was approved in 2003 by the Food and Drug Administration (FDA) of the United States of America for diagnostic use. Tests for β-D-glucan had also become available outside Japan, their land of origin and one, Fungitell was cleared in 2004 by the FDA for diagnostic use. Finally, there was a growing clinical experience with respect to nucleic acid tests particularly polymerase chain reaction (PCR) for *Aspergillus* species giving rise to the hope that the technique could be incorporated into a revised set of definitions to provide early indirect evidence of IA [16–18].

2 Revising the Definitions

The process of revising the definitions began in 2003 with the clear goal of building on what the original definitions had achieved to refine and improve them so as to capture as many cases of infections due to invasive fungal infection as possible

whilst excluding those cases for which the likelihood of invasive fungal infection was low. The journey was sometimes tortuous and took longer than had been originally anticipated but finally culminated with the publication of the revised definitions in June 2008 [19].

It is worth describing the nature of the consensus process as there are several ways in which this might be achieved. The consensus group in this case consisted of experts drawn from both sides of the Atlantic who had been, or were, members of the EORTC Invasive Fungal Infection group on the one hand, and the Mycoses Study Group on the other hand. Hence it was essentially North America-European Alliance. It was also established early on that the lifeboat principle would apply, namely, "once on board, stay on board until the harbour is reached". This meant retreating a step or two when a crucial issue failed to gain complete support. Hence the resulting definitions were the best that could be achieved by the group as a whole. It was not a review committee nor did it follow a formal process of gathering and weighing evidence. Other constraints included the aim of preserving the framework for defining and distinguishing entities for which there was less certainty of diagnosis by integrating host factors, clinical features and mycological evidence.

3 From Infection to Disease

The group adopted the term "invasive fungal disease" to reflect the fact that clinically we are dealing with a disease process resulting from a fungal infection. This also entails undertaking every reasonable effort to exclude alternative causes of the infectious disease as a necessary prerequisite to classifying it as an invasive fungal disease.

4 Expansion of Host Factors

Neutropenia and receipt of an allogeneic HSCT were retained as host factors whilst those related to fever of any description were abandoned (Table 1). The host factors were also expanded to included solid-organ transplants, hereditary immunodeficiencies and connective tissue disorders. Graft versus host disease as such was replaced by receipt of immunosuppressive agents such as steroids and the host factors were completed by including treatment with T cell immunosuppressants, including calcineurin inhibitors, anti-TNF-α drugs, anti-lymphocyte antibodies, and purine analogues.

5 No More Major and Minor Clinical Features

The distinction between "minor" and "major" clinical criteria was discarded in favour findings on medical imaging that indicated a disease process consistent with invasive fungal disease (Table 1). For example, patients with IPA tend to have focal

Table 1 EORTC/MSG definitions for invasive apsergillosis – comparison of the old and the new

	EORTC/MSG definitions	
	Original	Revised
Host factors	– Neutropenia	– Neutropenia
	– GVHD	– GVHD
	– >3 weeks steroids	– Allogeneic HSCT recipient
	– <36°C or >38°C and	– Inherited severe immunodeficiency
	Prior mycosis	– Treatment with other recognised T-cell immune suppressants
	– AIDS	
	– Immunosuppressive drugs	
	>10 days neutropenia	
	>4 days unexplained fever despite broad spectrum antibiotics	
Clinical features	*Lower respiratory tract infection*	
	– Major (any 1 of the following)	– No major or minor criteria
	Halo sign	– The presence of 1 of the following 3 signs on CT:
	Air-crescent sign	Dense, well-circumscribed lesions(s) with or without a halo sign
	cavity within an area of consolidation	Air-crescent sign
	– Minor (any 2 of the following)	Cavity
	Cough, chest pain, haemoptysis, dyspnoea	
	Physical finding of pleural rub	
	Any new infiltrate not fulfilling major criterion	
	Sinonasal infection	
	– Major (any 1 of the following)	– No major or minor
	Suggestive radiological evidence of invasive infection in sinuses (i.e., erosion of sinus walls or extension of infection to neighboring structures, extensive skull base destruction)	– Imaging showing sinusitis plus at least 1 of the following 3 signs:
	– Minor (any 2 of the following)	Acute localized pain (including pain radiating to the eye)
	Nasal discharge. stuffiness	Nasal ulcer with black eschar
	Nasal ulceration, eschar of nasal mucosa or epistaxis	Extension from the paranasal sinus across bony barriers, including into the orbit
	Periorbital swelling	
	Maxillary tenderness	
	Black necrotic lesions or perforation of the hard-palate	
	CNS infection	
	– Major (any 1 of the following)	– No major or minor

Table 1 (continued)

	EORTC/MSG definitions	
	Original	Revised
	Radiological evidence suggesting CNS infection (e.g., mastoiditis or other parameningeal foci, extradural empyema, intraparenchymal brain or spinal cord mass lesion)	– 1 of the following 2 signs:
	– Minor (any 2 of the following) Focal neurological symptoms and signs: Focal seizures Hemiparesis Cranial nerve palsies Mental changes Meningeal irritation findings Abnormalities in CSF biochemistry and cell count (provided that CSF is negative for other pathogens by culture or microscopy and negative for malignant cells)	Focal lesions on imaging Meningeal enhancement on MRI or CT
	Tracheobronchitis	
	– Not included	– Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis
Mycological criteria	– Positive result of culture for mould (including *Aspergillus*, *Fusarium*, or *Scedosporium* species or Zygomycetes) or *Cryptococcus neoformans* or an endemic fungal pathogen from sputum or BAL fluid samples	– Direct test (cytology, direct microscopy, or culture)
	– Positive result of culture or findings of cytologic/direct microscopic evaluation for mould from sinus aspirate specimen	– Mould in sputum, BAL fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:
	– Positive findings of cytologic/direct microscopic evaluation for mould sputum or BAL fluid samples	Presence of fungal elements indicating a mould
	– Positive result for *Aspergillus* antigen in specimens of BAL fluid, CSF, or 2 blood samples	Recovery by culture of a mould (e.g., *Aspergillus*, *Fusarium*, Zygomycetes, or *Scedosporium* species)
	– Positive findings of cytologic or direct microscopic examination for fungal elements in sterile body fluids	– Indirect tests i.e. detection of antigen or cell-wall constituents

Table 1 (continued)

EORTC/MSG definitions	
Original	Revised
	Aspergillosis: galactomannan antigen detected in plasma, serum, BAL fluid, or CSF Invasive fungal disease other than cryptococcosis and zygomycoses: β-D-glucan detected in serum

Legend: BAL, bronchoalveolar lavage, CSF, cerebrospinal fluid, CNS, central nervous system, CT, computed tomography, GVHD, graft versus host disease, HSCT, haematopoietic stem cell transplantation, MRI, magnetic resonance imaging.

rather than diffuse pulmonary infiltrates on chest computed tomography (CT) radiography showing one or more nodules some with and some without a halo sign [20]. Hence the emphasis on imaging to detect and delineate the nature of lesions that can be consistent with invasive fungal disease.

6 Indirect Mycological Tests

As with the original definitions, indirect tests were considered for inclusion only if they were validated and standardised. Furthermore, it was decided to rely entirely on the thresholds recommended by the manufacturer of commercial tests as this was a requirement for approval by the FDA and the group considered it beyond its remit to re-examine these. Having had a place in the original definitions, the detection of galactomannan (GM) was extended to CSF and bronchoalveolar lavage fluid to provide evidence of *Aspergillus* infection (Table 1). The β-D-glucan assay (Fungitell) was also included as a marker for probable invasive fungal disease though it is not specific for *Aspergillus*. Disappointingly, methods for detecting fungal nucleic acids, such as PCR, were not included in the definitions because there is, as yet, no standard, and none of the techniques has been formally validated.

7 Improvements

As mentioned earlier, two of the largest studies ever conducted on the treatment of invasive aspergillosis included a substantial number of patients on the basis of a host factors namely receipt of an HSCT and radiology – specifically the presence of pulmonary nodules on high resolution CT scan surrounded by a halo sign without there being any mycological support [1, 2]. A recent report on the radiography of patients entered into the study of Herbrecht et al. emphasised the high sensitivity and specificity of the halo sign provided it appeared whilst the patient was neutropenic [20]. The studies of Herbrecht et al. [2] and Cornely et al. [1] modified the

EORTC/MSG criteria to allow the term "probable" to allow such cases to be evaluated for treatment. The revised definitions now address this issue this by assigning the category of "possible invasive fungal disease". Other cases that might have been considered hitherto as being possible aspergillosis will no longer be classified as such. Examples are show in Table 2. Simply put, the assignment of probable aspergillosis depends upon there being mycological evidence alongside clinical evidence. Cases with the same clinical features but for which there is no mycological evidence found after testing or when no tests are done for whatever reason, will be assigned the category of possible aspergillosis.

Sinonasal and central nervous system (CNS) aspergillosis have been retained as clear entities (Table 1) and tracheobronchitis due to aspergillosis has also now been included in the revised definitions which will be of benefit to recipients of lung and other transplants by allowing therapy to be tested and diagnostic tests to be evaluated [21–23].

8 Known Issues

One of the principal shortcomings of the revised definitions is the omission of criteria for probable and possible invasive aspergillosis that develops in patients in the intensive care unit (ICU), or after surgery or in those whose immunity is not shown to be compromised in the classical sense [24]. These cases are currently required to meet the criteria for proven which is often a tall order as tissue is required. Recently, the issues arising from diagnosing IA in the ICU have been highlighted [25]. They include the difficulties arising from mechanical ventilation in the interpretation of clinical signs and radiographic images, the low sensitivity and specificity of respiratory cultures and the limited experience on testing GM and β-D-glucan in bronchoalveolar lavage fluid. This together with the lack of host factors that help recognise those most disposed to developing IA sets such cases outside the scope of the revised EORTC/MSG definitions.

The certainty of diagnosis is predicated upon obtaining appropriate specimens. Tissue is required to prove aspergillosis and the distinction between possible and probable aspergillosis hinges upon mycological evidence obtained from other specimens, whether by direct or indirect tests. Clinical experience with indirect tests such as the EIA for GM varies considerably from very good [26] to bad [27]. The reasons have been explored by Pfeiffer et al. [28] who showed that the best performance was found amongst patients who had undergone treatment for haematological malignancies and recipients of HSCT whilst the poorest results were found amongst children and solid-organ transplant recipients. There are also two other important considerations – sampling intensity and the definition of a positive test result. Screening is preferred by most centres and it has been recently shown that twice-weekly screening is as efficient as daily testing [29]. The issue of the cut-off value, at least for serum and plasma, is more protracted as lowering the optical density index to 0.5 undoubtedly improved sensitivity [6, 30]. However this is achieved at the expense of specificity. This deficit might be solved by adopting the so-called dynamic threshold

Table 2 Difference in the results of applying the original and revised EORTC/MSG definitions

Host factor	Clinical feature	Mycological evidence	EORTC/MSG definitions	
			Original	Revised
GVHD	Nonspecific bilateral pulmonary infiltrates on HRCT, dry cough	Plasma – *Aspergillus* antigen 0.8	Probable	Unclassified
GVHD	Nonspecific bilateral pulmonary infiltrates on HRCT, dry cough	None	Possible	Unclassified
Neutropenia after remission-induction chemotherapy	Persistent fever Dyspnoea, pulmonary infiltrates on plain X-ray	BAL culture *Aspergillus*	Probable	Unclassified
Neutropenia after remission-induction chemotherapy	Persistent fever	BAL culture *Aspergillus*	Possible	Unclassified
Neutropenia after HSCT	Bilateral pulmonary nodules some with halo signs on HRCT	Plasma – *Aspergillus* antigen 0.8	Probable	Probable
Neutropenia after HSCT	Bilateral pulmonary nodules some with halo signs on HRCT	Not done	Possible	Possible
Neutropenia after consolidation chemotherapy	Persistent fever, cough, diffuse bilateral pulmonary infiltrates on HSCT	BAL culture *Aspergillus* spp., BAL *Aspergillus* antigen <0.5	Probable	Unclassified
Neutropenia after remission-induction chemotherapy	Persistent fever, cough, diffuse bilateral pulmonary infiltrates on HSCT	None	Possible	Unclassified

Legend: BAL, bronchoalveolar lavage, GVHD: graft versus host disease, HRCT, high resolution computed tomography, HSCT, haematopoietic stem cell transplantation.

proposed by Maertens et al. namely optical index ≥0.8 for a single sample or >0.5 for two consecutive samples [30].

Similar considerations apply to the use of tests for β-D-glucan. Experience with this test is also more limited that is the case for GM assays. There are also several different tests each with its own threshold and characteristics. That of Fungitell uses enzymes from amoebocytes of *Limulus polyphemus*, the North Atlantic horse shoe crab, whereas the others, including the test used by Senn et al. [31] are based on amoebocyte enzymes from the Pacific horshoe crb, *Tachypleus tridentatus* [7]. The test has shown potential for the early diagnosis of invasive fungal disease among patients at high-risk [31, 32] but much is still required to firmly establish its place and utility. The panfungal nature of the test also poses a challenge to classifying potential cases of IA since a case that meets the criteria for possible IA would be classified a probable invasive fungal disease *possibly aspergillosis* if the mycological evidence rests solely on the detection of β-D-glucan.

The omission of PCR in the definitions has already been alluded to and necessary steps needed to rectify this are clear. A working party of the International Society of Human and Animal Mycology has taken on the task of establishing such a standard for *Aspergillus* PCR in blood or its components. The elements in PCR known to result in variable performance are well known and need to be addressed in order to achieve a standard [18]. These range from the nature and volume of the specimen to the method of extracting DNA, the type of primer target, the PCR format and the inclusion of appropriate controls. Validating the test clinically is also a necessary prerequisite and poses a major challenge in terms of study design, selecting the right population, employing a minimum standard of diagnosis such as proposed by the British Society of Medical Mycology [33] and funding.

Another potential limitation has been highlighted by Hope et al. [24]. These authors raised the issue of employing indirect evidence to implicate aspergillosis when direct evidence is impossible to obtain. For instance, when a cerebral lesion is found on CT scan and there is no direct evidence of *Aspergillus* infection available, the aetiology could none the less be surmised if proven or probable aspergillosis was discovered in another body site e.g. the lung and there was no better explanation. Other examples are given in which indirect evidence might be used to establish a working diagnosis of aspergillosis to justify therapy. Whilst this makes sound clinical sense, it is doubtful whether any definitions could accommodate these situations without sacrificing their primary aim – to serve scientific endeavour – as they were not intended to be employed in the clinic. Clearly, failure to meet the criteria for invasive fungal disease does not mean that there is no invasive fungal disease, only that there is no enough evidence to support the diagnosis.

Other aspergillosis entities are also excluded from the revised definitions as shown Fig. 1 reflecting the implicit scope to define invasive fungal disease associated with high mortality. Also, the definitions were designed to provide a coherent framework for diagnosis using the best tools available at the time. There was no ambition to make them comprehensive enough to encompass every sort of fungal disease. Besides, there is no impediment to complementing them with more detailed definitions of other forms of aspergillosis entities.

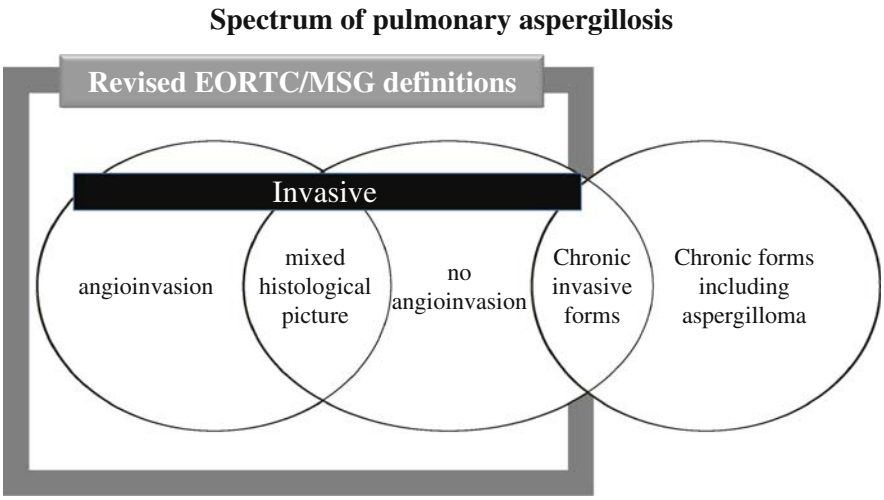

Fig 1 Spectrum of pulmonary aspergillosis. Adapted from reference [24]. This figure illustrates the scope of the revised EORTC/MSG definitions. Those entities which fail to meet the current criteria for probable and possible aspergillosis affect patients in the intensive care unit, those with chronic granulomatous disease and individuals who are not immunocompromised in the traditional sense but are none the less at risk of developing pulmonary aspergillosis. Allergic forms of aspergillosis are not considered in this figure

The revised EORTC/MSG definitions are not the final result but rather the product of a continual process that will evolve as more diagnostic tests become available and their performance is established. Nucleic acid detection in blood and tissues will likely soon earn a place in the definitions and may even be joined by the use of FDG-PET scan [34]. Perhaps even proteomics will find a role in diagnosis [35, 36]. Only time will tell whether or not the revised EORTC/MSG definitions will help or hinder advances against aspergillosis. One thing is certain, they and the original definitions have helped foster communication between mycologists, radiologists and clinicians alike, and have helped set a benchmark for the epidemiology, diagnosis, and management of aspergillosis.

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Treatment of Invasive Pulmonary Aspergillosis

Kieren A. Marr

Abstract Treatment of invasive aspergillosis requires consideration of appropriate antifungal therapy, potential surgical management, and modulation of immunity. In the past several years, advances in therapy include the availability of multiple new antifungal drugs that have activity against *Aspergillus* species (expanded spectrum azole antifungals and echinocandins), with some studies showing potentially improved outcomes compared to therapies that employ conventional formulations of amphotericin B. The first large randomised trial performed to evaluate therapy for aspergillosis demonstrated improved outcomes and survival in patients randomised to receive voriconazole as primary therapy. Other recent studies have demonstrated activity of the liposomal formulation of amphotericin B in treating aspergillosis, although the relative utility of this drug compared to voriconazole is unclear. "Salvage" therapy studies, results of which are limited by small numbers of patients and host-related biases in outcomes, demonstrate potential utility with echinocandins (caspofungin, micafungin), posaconazole and amphotericin B lipid complex. Most recently, use of multiple therapies in combination ("combination therapy"), especially use of regimens including echinocandin antifungals, has attracted much attention, although few data are available to support adoption as standard practice. A comprehensive approach to therapy should consider the potential necessity of surgical management and immune modulation, which are discussed.

Keywords Antifungal drugs · Echinocandins · Invasive aspergillosis · Voriconazole · Therapy

K.A. Marr (✉)
School of Medicine, Johns Hopkins University, Baltimore, MD, USA
e-mail: kmarr4@jhmi.edu

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Contents

1 Antifungal Treatment: Drugs of Choice and Alternatives

In the past two decades, pharmaceutical companies have been actively involved in the development of new antifungal agents. While some have not made it to market, marked success has been noted, with introduction of numerous new agents that have activity against *Aspergillus* species. Properties of these agents are discussed elsewhere in this textbook. In this chapter, discussion focuses on the data supporting (or refuting) use of these drugs for treatment of documented invasive pulmonary aspergillosis (IPA).

Antifungal polyenes target ergosterol in the cell membrane of fungi, resulting in impaired integrity of fungal cells, with broad applicability. Up until recent years, this class of drugs, particularly, the parent compound, amphotericin B deoxycholate (d-AMB), was considered the most appropriate "gold therapy" for IPA. However, results of most recent studies indicate that the potential toxicities of this drug tip the scale in favour of less toxic alternatives; this may be particularly true in the highest risk patients, such as those receiving other drugs that exert renal toxicities.

Amphotericin B deoxycholate, as the first systemically tolerated antifungal drug with activity against *Aspergillus* species, was approved for this indication based on essentially no controlled data. A case of aspergillosis treated successfully with amphotericin B was first reported in the literature in 1960 [1]. Increased recognition of disease led to increased use of the drug, although at that time, many patients were alternatively treated with surgical management alone. In one of the earliest series of patients reported with "pulmonary" aspergillosis, only one of 21 patients received medical therapy; the remaining exhibited good responses to surgical management [2]. However, it is notable that most of these early patients had fungal balls complicating pre-existing lung disease; patients with documented infections complicating malignancies in this series did uniformly poorly without medical management. Over time, and with reports of successful management of IPA using approaches that included amphotericin B [3, 4], this became accepted as the standard of care. However, it is notable that no adequately powered randomised trial was ever performed to document baseline efficacy of this drug.

Itraconazole, and lipid formulations of amphotericin B, introduced in the late 1980s and 1990s, were increasingly investigated, and used for therapy of IPA based on non-controlled, and/or underpowered comparative studies, and experience in the "salvage" setting in which patients had failed prior therapies. A small study

performed in neutropenic patients with both documented and "highly suspected" fungal infections compared outcomes of itraconazole (200 mg twice daily) to d-AMB (0.6 mg/kg daily or 0.3 mg/kg in combination with 5-flucytosine); results suggested good – potentially better – outcomes associated with itraconazole therapy [5]. One of the largest experiences, although not a controlled trial, was the multicentre open study evaluating outcomes of invasive aspergillosis (IA) with oral itraconazole therapy (600 mg daily for 4 days followed by 400 mg daily) [6]. Amongst 76 evaluable patients, 30 (39%) were considered to have had a positive response to therapy. Results of animal models and small case series suggest potential efficacy of itraconazole for therapy of IA complicating numerous disease states (e.g., neutropenia and organ transplant), however use of the drug has historically been largely limited to maintenance and salvage therapy.

Few randomised trials have been performed to definitively assess outcomes of IA. Figure 1 depicts results of selected comparative trials assessing different primary therapies in mixed populations of patients [7–12]. The first randomised trial that was adequately powered to compare outcomes of documented IPA compared d-AMB with voriconazole [8]. In this groundbreaking randomised trial, two strategies were compared: patients received primary antifungal therapy with either voriconazole (intravenously for the first 7 days, after which time patients could switch to oral voriconazole) or d-AMB (1.0–1.5 mg/kg once daily), for a 12-week period. Intolerant or non-responding

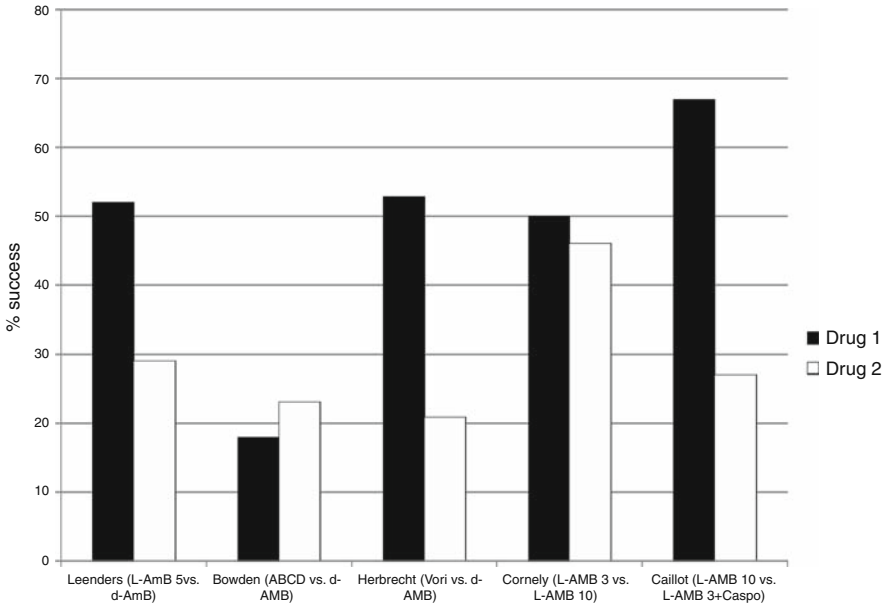

Fig. 1 Results of selected comparative prospective studies evaluating therapies for pulmonary aspergillosis. Specific agents studied are listed as drug 1 and drug 2, in respective order. Legend: L-AmB, liposomal amphotericin B (number equivalent to mg/kg/day); ABLC, amphotericin B lipid complex; vori, voriconazole; caspo, caspofungin

patients were allowed to switch to other licensed antifungal therapy. Superior outcomes were observed for patients randomised to receive voriconazole, with consideration of overall success using a composite endpoint, and overall survival. As expected by the high dose of d-AMB required to treat IA, 80.4% of patients in the d-AMB arm switched to other antifungal drugs, mostly due to intolerance. Importantly, patients who required a new antifungal regimen due to progression of infection had a poor response with in both study arms (d-AMB 19.0%, voriconazole 26.3%) [13]. This trial also demonstrated the feasibility of studying this disease in an adequately powered, blinded fashion, although resources required are considerable. Due to the results of this study, voriconazole monotherapy can be considered to be the new "gold standard" for the treatment of IA.

Other randomised trials evaluating therapies of IPA are worthy of discussion. One was the first prospective trial that was performed by the European Organization for Research and Treatment of Cancer (EORTC). In this study, 120 patients were randomised, with 87 patients ultimately evaluable for response analyses to either liposomal amphotericin B (L-AMB) administered at high (4 mg/kg/day) or low (1 mg/kg/day) doses [9]. Unfortunately, due to the few patients evaluated, one cannot make definitive statements with regards to comparative efficacy. However, results did show that some patients responded clinically and survived IPA with low dose therapy. The idea of dose escalation of liposomal amphotericin B has been subsequently studied in the more recent "AmBiLoad" trial, which compared higher dose L-AMB (10 mg/kg/day) to more standard dosing (3 mg/kg/day) [10]. Results also demonstrated efficacy with the lower dose, with no apparent improvement in outcomes (perhaps excessive toxicities) in the high-dose treatment arm. Since just a few patients with cerebral aspergillosis were included in the AmBiLoad trial, it is unknown whether 3 mg/kg of L-AMB is effective in these patients.

Another randomised trial compared outcomes of treating IA with amphotericin B colloidal dispersion (ABCD) to d-AMB [11]. Although there were relatively fewer evaluable patients in this trial ($n = 174$), there was no trend to suggest that ABCD was associated with better results. Moreover, infusion-related chills and fever occurred more frequently in patients who received this particular lipid formulation.

Non-controlled and "salvage" therapy studies evaluating monotherapy outcomes with lipid formulations of amphotericin B (e.g. amphotericin B lipid complex, ABLC), other azole antifungals (e.g. posaconazole) and echinocandin antifungals (e.g. caspofungin) suggest therapeutic efficacy, but the actual clinical utility compared to the strictly defined gold-standard (voriconazole) is not clear. With this in mind, L-AmB has an obvious role in therapy for IA, but lack of an adequately powered comparative trial clouds our ability to make definitive conclusions regarding comparative efficacy. Some have tried to make generalisations of comparative efficacy of L-AmB and voriconazole based on reasonably equivalent efficacy in the two randomised trials. However, comparing results of treatment groups in sequential studies for IA is fraught with hazards introduced by host variables that dictate overall outcomes [14]. Historically, response rates were very poor, especially in recipients of haematological stem cell transplantation (HSCT); a large review published in 1990 documented a 94% mortality rate in HSCT recipients

with IA [15]. Poor response to antifungal therapy in allogeneic HSCT recipients was also observed in both voriconazole and AmBiLoad trials [8, 16]. A more recent observational analysis of IA outcomes in a single large transplant centre noted improvement in outcomes since the early 1990s, with important variables involving the host (e.g. pre-transplant pulmonary function), transplant type, and therapies [17]. Thus, changes in transplant practices and hosts essentially invalidate comparisons of outcomes in sequential cohorts of patients.

Thus, a great deal of effort has been placed into defining optimal antifungal therapies for IA; results of data available suggest that there are several alternatives. Certainly the most powerful data come from the amphotericin B-controlled trial that demonstrated improved survival for patients on voriconazole monotherapy. Results of this study define this as the current "gold standard" to which other therapies should be compared. Although results of other studies suggest that alternatives include L-AmB, ABLC, posaconazole, and echinocandins, definitive statements with regards to the role of these drugs awaits results of other randomised trials.

2 Combination Antifungal Therapy

Despite advances in therapies, outcomes provide room for improvement. Recent efforts have been put into defining a potential role for antifungals administered in combination, with the intent of providing synergistic antifungal activity and additive exposure of antifungal drugs. Historically, results of animal models and in vitro studies combining azoles with amphotericin B have not generated encouraging results. For instance, the combination of ravuconazole and amphotericin B in a rabbit model of IPA resulted in potential antagonism [18]. While results of these studies may be dependent on models used, timing of drug administration, concentration of drugs, and outcomes measured, results of clinical observations have also generated little enthusiasm towards combined application of azole drugs with amphotericin B formulations; for instance, a large retrospective analysis of outcomes of IA in patients who received combined itraconazole with amphotericin formulations suggested no trend to improved outcomes with combined therapy [19].

The potential role of echinocandins administered with either azole drugs or amphotericin B has generated much more enthusiasm, given differences in microbial targets. Multiple in vitro and animal studies have shown potential additive or synergistic activities using different combinations of drugs, but few comparative clinical studies have been performed [12, 20–28]. Highlights come from comparative clinical studies that suggest improved outcomes with the combination of caspofungin with voriconazole or L-AMB for IPA, although none of the studies have been powered to show definitive differences in outcomes [12, 24, 28]. Results of small studies, especially those that use historical control groups or administer drugs for "salvage" therapy, are very difficult to interpret due to multiple sources of bias; the definitive role of combination antifungal agents for IA awaits results of prospective trials. An ongoing randomised double-blind trial is comparing the effectiveness and safety of anidulafungin in combination with voriconazole versus voriconazole monotherapy (ClinicalTrials.gov identifier NCT00531479).

Another combination approach that has attracted some attention has been the potential of administering therapy directly into the airways during systemic infusion of other compounds. The rationale is to deliver more drug to the pulmonary tissues, especially in the setting in which the patient has documented tracheobronchial disease. Few studies have evaluated outcomes using this approach; for the most part, only cases have been reported. However, aerosolised or nebulised administration of amphotericin B products have been studied in recent years for preventative therapies, and shown to be relatively safe [29, 30]; it is likely that this will gain more attention in a potential combination algorithm in the future. Many questions remain unanswered, including the pharmacokinetics of the inhaled drugs and the best inhalation system. It is also unknown how much of the nebulised drug will actually get to necrotic lung sites and how the drug will distribute in patients with abnormal lung architecture.

3 Surgical Treatment

Early treatment regimens for IPA aggressively utilised surgical resection as adjunctive therapy, however, the utility of surgical resection has not been formally studied, and it is associated with a significant potential of morbidity. General recommendations are to consider surgical therapy in the setting in which patients have severe haemoptysis, resectable lesions adjacent to large vessels or other critical structures, and in settings in which cavitary lesions are not responding to medical therapy when further immunosuppressive management is needed [31]. Recently, investigators reported results of operative management in a series of 10 patients and reviewed the literature [32]. Operative procedures included pneumonectomy (4%), lobectomy (66%), and wedge resection (29%). In the literature review, it is notable that <10% of all infected patients underwent surgical therapy. Results demonstrated that surgery was relatively safe and associated with reasonable outcomes. Still, its overall utility is unknown, with reporting bias in the literature and a lack of comparative studies, and surgical morbidity can include bleeding and infected pleural effusions, especially in the setting in which lesions are in close proximity to the pleura.

4 Immune Modulation

IPA generally occurs only in people who have relatively severe disruptions in host defences. Although it was originally noted to be an infection that complicates prolonged severe neutropenia in recipients of cytotoxic chemotherapy and/or HSCT, other studies have shown that cellular deficiencies imposed by organ transplantation or AIDS can also predispose to pulmonary infection [33, 34]. This provides functional proof of data coming from animal models, which demonstrate the complexity of innate and adaptive immune responses in providing protection against IPA. A general discussion of immunity to *Aspergillus* species is provided by Dr Romani elsewhere in the book.

Defining defects in *Aspergillus* immunity also provide the ammunition by which to "reverse" risks to augment therapy. The simplest method to modulate immunity is to withdrawal immunosuppressive therapies, and this is suggested in treatment algorithms [31]. However, this recommendation infrequently results in real improvement in immune function, as few patients are receiving immunosuppressive drugs without necessity. Withdrawing drugs such as steroids in long-term immunosuppressive regimens frequently results in organ rejection or worsening graft versus host disease, which ultimately triggers the need for increased "salvage" therapies and poor outcomes. Management of these patients thus requires a fine balance between providing enough immunosuppression to effective treat host conditions, yet not providing so much that infection morbidity is excessive.

The approach to the neutropenic patient is a little less complicated than in transplant recipients, as modulation involves increasing numbers and/or function of circulating polymorphonuclear phagocytes (PMN). Small case series and in vitro studies have reported that administration of granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon-γ can stimulate anti-*Aspergillus* PMN and/or monocyte killing, and potentially provide benefit, but a clear benefit has not been demonstrated in large clinical trials [35–39]. More recently, use of G-CSF stimulated granulocyte transfusions as augmented therapy has been suggested [40]; definitive statements regarding efficacy await results of a large clinical trial.

One of the most creative approaches to immune modulation that is being evaluated is with cellular immunotherapy. Small clinical case series and animal models have documented important roles of CD4+ T cells in mediating risks of pulmonary aspergillosis, potentially even with direct effector activities [41]. Several groups have taken these observations further in attempting to develop cellular therapies. Safety of adoptive immunotherapy using autologous dendritic cells or CD4+ T cells has been reported, although efficacy remains unknown [42, 43, 44]. One of the problems in doing these studies is that we have not yet identified "dominant" *Aspergillus* antigens; thus, assays and T cell therapies rely on exposure to whole cell antigen extracts, which can have problems with reproducibility in contents and potentially induce non-specific stimulation by virtue of polysaccharide components such as β-glucan. Although this is an active area of research today, much needs to be done before widespread adoptive immunotherapy for *Aspergillus* species becomes feasible.

While methods to improve immunity to *Aspergillus* species may well assist development of more effective therapeutic strategies, one must carefully consider the balance between augmented immunity and immunopathology. Recently, investigators have suggested that there is an immune-reconstitution syndrome to *Aspergillus* species, especially in the context of neutrophil engraftment [45]. My clinical experience supports this observation; I have noted both progressive focal infiltrates and more diffuse infiltrates in patients with documented IPA that appears to be otherwise well controlled with antifungal therapies (Fig. 2). This appears to be common late post-allogeneic HSCT, and complicates management as these patients have high risks for other underlying infections and non-infectious pathology (e.g.

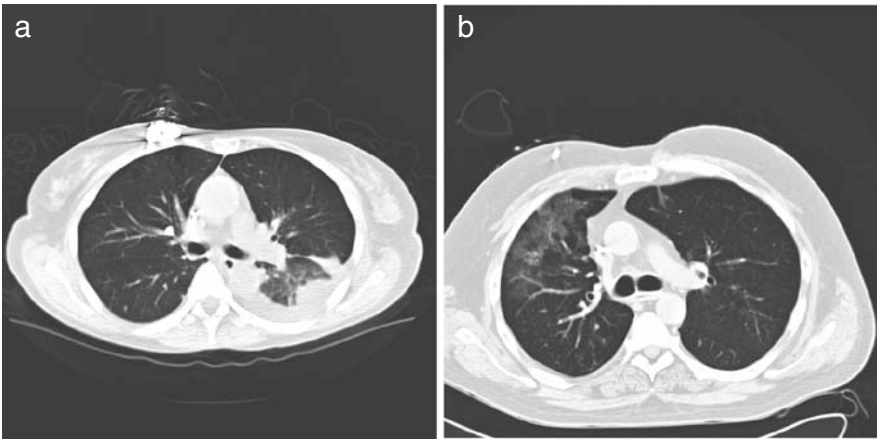

Fig. 2 Progressive focal (**A**) and diffuse (**B**) radiographic pattern in patients with documented pulmonary aspergillosis on appropriate therapies. Both patients were late after allogeneic haematological stem cell transplantation, underwent bronchoscopy with evidence of alternate microbial diagnosis, and resolved clinically (and radiographically) without additional management

cytomegalovirus infection and bronchiolitis obliterans organising pneumonia); for this reason, diagnostic diligence is still mandatory. The suggestion that pulmonary *Aspergillus* infection invokes non-specific immunopathology in the setting of relative reconstitution is consistent with the results of animal studies performed more than a decade ago, which suggested that the pulmonary progression of *Aspergillus*-invoked disease differs in the setting of exogenous neutropenia vs. steroid-mediated risks [46]. More information regarding the natural history of disease, especially in non-neutropenic hosts, is necessary in order to develop the most effective modulation strategies.

Finally, most recent studies have shown that there might be unexpected "immunomodulatory" effects of specific antifungal therapies. For instance, echinocandin antifungals have been shown to "unmask" cell wall β-glucan, effectively resulting in increased recognition and inflammatory activity of phagocytes that rely on the β-glucan (dectin-1) receptor to trigger activity (e.g. macrophages and PMNs) [47, 48]. Whether this results in significant clinical benefit- or event detriment, is unknown.

5 Summary

Introduction of several new antifungals has expanded our therapeutic armamentarium and resulted in improved outcomes of *Aspergillus* therapies during the last decade. Improvements in therapies will likely continue to be witnessed, with strategies that rely on combination antifungal therapies and adjunctive immunomodulation.

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Antifungal Prophylaxis in Haematology

Maria J.G.T. Rüping, Jörg J. Vehreschild, and Oliver A. Cornely

Abstract Invasive fungal diseases are a source of significant morbidity and mortality in patients with haematological malignancies, particularly those with prolonged and severe neutropenia. In view of the poor prognosis associated with tardy treatment initiation, antifungal prophylaxis has become increasingly popular. Until recently, fluconazole – a drug with no *Aspergillus* activity – and itraconazole, which has absorption-associated problems and is often poorly tolerated, used to be the preferred choices for antifungal prophylaxis. Clinical trials have now assessed the prophylactic use of posaconazole, a new-generation triazole with broad spectrum antifungal activity against *Candida*, *Aspergillus* and *Fusarium* spp., as well as the Zygomycetes. Results have further strengthened the evidence base for antifungal prophylaxis, showing a significant reduction of invasive fungal diseases and significantly improved overall survival in patients with acute myelogenous leukaemia and myelodysplastic syndrome. Moreover, improved attributable survival in patients with severe graft versus host disease on immunosuppressive therapy was documented. For patients undergoing allogeneic haematopoietic stem cell transplantation, fluconazole prophylaxis is still considered current standard. Considering the extended spectrum of posaconazole and its good tolerability, it seems warranted to replace fluconazole by posaconazole for this indication.

Keywords Antifungal prophylaxis · Fluconazole · Invasive aspergillosis · Itraconazole · Posaconazole

Contents

O.A. Cornely (✉)
Klinik I für Innere Medizin, Kinisches Studienzentrum 2 für Infektiologie
Klinikum der Universität zu Köln, Kerpener Str. 62, 50937
Köln, Germany
e-mail: oliver.cornely@ctuc.de

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1 Introduction

It has been widely accepted that treatment delays significantly reduce the chances of a successful outcome of invasive fungal diseases (IFD) [1, 2]. Particularly patients with haematological malignancies, who represent a major share of those contracting IFD, profit from antifungal prophylaxis [3–6]. In the absence of reliable diagnostic tools for rapid identification of fungal pathogens, diagnoses are tardy and thus associated with considerable fungal inoculums that require prolonged antifungal treatment courses. Subsequent delays of chemotherapy cycles often have a negative impact on the prognosis of the underlying disease. Using secondary prophylaxis [7, 8] adherence to a given chemotherapy schedule can often be assured, but the risk of relapse during consecutive periods of neutropenia always remains [7]. In the end, complete resolution before continuation of treatment or use of secondary prophylaxis may both be associated with prolonged hospitalisation, an increased number of adverse events and drug-drug interactions, as well as higher costs. Under these circumstances, prophylactic strategies have gained increasing popularity, even though they narrow therapeutic options in case of a breakthrough infection and lead to overtreatment of patients who would never develop IFD [4, 5].

2 Clinical Evidence

In order to provide good antifungal protection without compromising tolerability, prophylaxis should remain limited to those high risk patients who demonstrably profit from it.

In the past, numerous trials have assessed the use of antifungal prophylaxis, but most of them, failed to show a marked advantage over placebo [8–10]. Only fluconazole administration to patients undergoing allogeneic haematopoietic stem cell transplantation (HSCT) at 400 mg daily for at least 75 days after transplantation significantly reduced morbidity and mortality when compared to placebo [3, 6]. Since fluconazole lacks activity against *Aspergillus* spp., other azoles have been evaluated in the prophylactic setting. An overview of the most important prophylactic regimens with their corresponding strength of recommendation and quality of evidence is given in Tables 1 and 2 [11].

Marr et al. randomised 299 HSCT recipients to receive either fluconazole 400 mg or itraconazole oral solution 2.5 mg/kg three times daily or 200 mg intravenous itraconazole. As expected, itraconazole provided better protection against invasive mould infections, while protection against candidiasis and overall survival were similar [12]. In a second trial by Winston et al., 140 allogeneic HSCT recipients were randomised to receive either 400 mg itraconazole or 400 mg fluconazole. Itraconazole prophylaxis was associated with better protection against IFD due to yeasts and moulds, but overall survival was similar for both arms [13].

Table 1 Infectious Diseases Society of America – United States Public Health Service Grading System for ranking recommendations [11]

Category, Grade	Definition
Strength of recommendation	
A	Good evidence to support a recommendation for use
B	Good evidence to support a recommendation against use
C	Poor evidence to support a recommendation
D	Moderate evidence to support a recommendation for use
E	Moderate evidence to support a recommendation against use
Quality of evidence	
I	Evidence from ≥1 properly randomized, controlled trial
II	Evidence from ≥1 well-designed clinical trial, without randomisation; from cohort or case-controlled analytic studies (preferably from >1 centre); from multiple time-series; or from dramatic results from uncontrolled experiments
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees

Table 2 Regimens for antifungal prophylaxis with corresponding strength of recommendation and quality of evidence

Patient-population	Purpose	Drug	Dosage	Level of evidence
Conventional chemotherapy	Reduction of attributable mortality	Fluconazole	50–400 mg once daily PO	C I
		Itraconazole oral solution	2.5–7.5 mg/kg once daily	C I
		Itraconazole capsules	At any dose	C I
		Posaconazole	200 mg 8 hourly PO	A I
		Amphotericin B deoxycholate	0.5–1.0 mg/kg q48h IV	C II
			<0.5 mg/kg q48h IV	C II
			20 mg once daily inhalation	C I
		Liposomal amphotericin B	50 mg q48h	C I
Allogeneic bone marrow or stem cell transplant		Fluconazole	400 mg once daily PO	A I
		Fluconazole	50–200 mg once daily PO	C I
		Itraconazole oral solution	400 mg once daily	C I
		Posaconazole	200 mg 8 hourly PO	A I
		Liposomal amphotericin B	1.0 mg/kg once daily IV	C I

The use of itraconazole prophylaxis has also been evaluated for patients exposed to profound and prolonged neutropenia not related to allogeneic HSCT. In an open-label, randomised, parallel-group, multicentre trial, itraconazole oral solution 2.5 mg/kg twice daily ($n = 248$) was compared to fluconazole oral solution or capsules 400 mg once daily ($n = 246$). Most patients included received chemotherapy for acute leukaemia. No significant differences in terms of IFD and mortality rates were shown [14]. Even though itraconazole seems to have a slight advantage over fluconazole, its clinical usefulness remains limited by the poor tolerability of its oral cyclodextrine solution. In all trials cited here, gastrointestinal side effects and hepatic toxicity rates were substantially higher in the itraconazole than in the fluconazole group [12, 13]. The oral capsule formulation does not represent an acceptable alternative, due to its poor bioavailability [15]. In conclusion, early generation azoles are limited by their narrow spectrum of antifungal activity and reduced tolerability. These findings were confirmed by a recent meta-analysis evaluating the role of antifungal prophylaxis in cancer patients receiving chemotherapy and those undergoing HSCT [16].

With the availability of posaconazole, a new generation triazole, that exhibits antifungal activity against a wide range of fungal pathogens, including *Candida*, *Aspergillus* and *Fusarium* species, as well as the Zygomycetes [17], the situation has changed. In a randomised multicentre trial, oral suspensions of posaconazole 200 mg three times daily ($n = 304$), itraconazole 200 mg every 12 hours ($n = 58$) or fluconazole 400 mg once daily ($n = 240$) were administered to patients receiving chemotherapy for acute myelogenous leukaemia or myelodysplastic syndrome. The primary end-point, defined as the incidence of proven or probable IFD during prophylaxis, was met by 7 patients (2%) in the posaconazole arm and 25 patients (8%) in the pooled fluconazole or itraconazole arm. *Aspergillus* spp. was the most frequently identified cause of IFD, whereas significantly fewer cases of aspergillosis were associated with posaconazole prophylaxis than with fluconazole or itraconazole prophylaxis ($p < 0.001$). Posaconazole was also superior in overall efficacy and significantly improved survival rates ($p = 0.04$) [4].

It has been questioned whether these favourable results, obtained in the controlled setting of a clinical trial, can be transferred to the less artificial conditions of clinical practice. Treatment courses of patients with acute myelogenous leukaemia or myelodysplastic syndrome during first induction chemotherapy in the year following the introduction of posaconazole prophylaxis were compared to those of a historic control [18]. The incidence rates of lung infiltrates, typical signs of aspergillosis and probable or proven IFD were significantly reduced in the cohort receiving posaconazole prophylaxis. Even though the power of the trial was not sufficient to allow conclusions on a survival difference, it was shown that the effects of posaconazole were not restricted to the clinical trial populations.

As mentioned above, fluconazole prophylaxis at 400 mg daily PO for at least 75 days is considered standard in patients undergoing allogeneic HSCT [3, 6]. In these patients, severe graft versus host disease (GVHD) is a common complication, requiring administration of immunosuppressive

agents that further increase the risk of fungal infection. An international, randomised, double-blind trial, that included 600 patients in this particular situation, compared antifungal prophylaxis with posaconazole 200 mg every 8 hours PO to fluconazole 400 mg once daily PO for efficacy, safety, and tolerability. After a fixed treatment period of 112 days, posaconazole and fluconazole were shown to be equally efficacious in preventing invasive fungal infections (incidence, 5.3 and 9.0%, respectively). Posaconazole was, however, superior in preventing invasive aspergillosis (IA) ($p = 0.006$) and improving attributable mortality ($p = 0.046$) [5].

Other alternatives that have been evaluated in clinical trials are outlined to provide a complete overview of the issue. Voriconazole is another new generation azole whose ability to prevent IFD has been assessed in clinical trials. In the prevention of lung infiltrates during induction chemotherapy for acute myelogenous leukaemia efficacy and safety of oral voriconazole at 200 mg three times daily were compared to placebo. The trial had to be stopped prematurely, because another trial had demonstrated reduced mortality by antifungal prophylaxis with posaconazole. Under these circumstances, further randomisation against placebo was considered unethical. Only 25 patients were randomised to voriconazole ($n = 10$) or placebo ($n = 15$). Incidence of lung infiltrates until day 21 was 0% in the voriconazole compared to 5 (33%) in the placebo group ($p = 0.06$) [19]. In a subsequent multi-centre, randomised, double blind trial, fluconazole 400 mg daily was compared to voriconazole 200 mg bid for the prophylaxis of IFD in patients receiving full intensity conditioning regimens with standard risk allogeneic HSCT. No significant difference in the rate of IFD, overall survival and tolerability was reported [20]. These results are somewhat surprising, as voriconazole has often been associated with higher toxicity rates [21]. The results should be regarded with caution and a prophylaxis with voriconazole is not recommended for recipients of HSCT.

A small number of other trials assessed the efficacy and safety of prophylaxis with systemic antifungals. These regimens gain major importance amongst patients with severe anorexia, such as during post-transplant intestinal GVHD. In HSCT recipients, the efficacy of antifungal prophylaxis with micafungin is comparable to prophylaxis with fluconazole [22]. The combination of fluconazole with micafungin yielded no additional benefit [23]. Caspofungin prophylaxis proved similar protection as itraconazole and intravenous administration of voriconazole in high risk acute leukaemia patients significantly decreased the incidence of IFD, but was associated with an increased rate of adverse reactions, when compared to itraconazole [24]. While antifungal prophylaxis with amphotericin B colloidal dispersion was associated with an unacceptably high adverse event rate [25], liposomal amphotericin B prophylaxis was as efficient as the combination of itraconazole and fluconazole [26]. The results of the latter trial are of limited value, since this antifungal combination is uncommon in clinical practice.

Two randomised trials assessed the efficacy of inhalative amphotericin B as prophylaxis for pulmonary aspergillosis in haematological patients [27, 28]. In an open study, 382 patients with haematological malignancies or autologous HSCT and an expected neutropenia of >10 days were randomised to receive either inhalations

with deoxycholate amphotericin B 10 mg twice daily or no intervention [27]. There was no significant reduction in the frequency of IA in patients inhaling amphotericin B, but adverse events were reported in $^2/_3$ of these patients, leading to premature discontinuation in 31%. The second study, a placebo-controlled trial in which high-risk haematological patients received inhalations with liposomal amphotericin B 12.5 mg/2.5 ml or placebo twice weekly until neutrophil recovery, was recently published by Rijnders et al. [28]. There was a marked reduction in the incidence of IA, from 14% in the placebo group to 4% in the liposomal amphotericin B group ($p = 0.007$), with no impact on mortality. Cough was more frequent in patients inhaling lipid amphotericin B than placebo ($p = 0.002$). Of note, all amphotericin B formulations require a preserved lung architecture for optimum distribution, which limits there use in patients with prior respiratory diseases [29, 30]. Before amphotericin B inhalation can be recommended as prophylaxis, further studies on the pharmacokinetics, ideal dosage, frequency of administration, and inhalation system are warranted.

3 Conclusion

With regards to the evidence discussed in this chapter, it seems clear that antifungal prophylaxis benefits patients at high-risk for IFD. In order to prevent IA, the chosen drug has to be active against *Aspergillus* species. It has often been discussed whether pre-emptive treatment should be preferred to prophylaxis in these patients. Pre-emptive treatment is triggered by radiographic signs and/or laboratory test results that point at an IFD in the absence of definitive histopathological and/or cultural pathogen identification. In current clinical practice, typical infiltrates on chest computed tomography and an increased galactomannan are commonly used as surrogate markers for IFD. Even though this approach might appeal to centres with low rates of IA, it should always be considered that the clinical efficacy of this approach has not been satisfactorly demonstrated in controlled clinical trials [31]. Concerning antifungal prophylaxis, this evidence is readily available [3–6]. An overview of the most important prophylactic regimens with their corresponding strength of recommendation and quality of evidence is given in Tables 1 and 2 [11].

For centres going for prophylaxis, it seems warranted to replace fluconazole by posaconazole in patients undergoing allogeneic HSCT, based on posaconazole's extended spectrum, low rate of adverse events and limited drug-drug interactions. Prophylaxis with posaconazole could be initiated after completion of the conditioning regimen.

4 Future Perspectives

Concerning future research on antifungal prophylaxis, two questions are of special concern: two clinical trials defined the rate of lung infiltrates as an end-point [19]. Both trials reported a substantial reduction of the incidence of unspecific pulmonary infiltrates by administration of systemically active antifungal prophylaxis. This effect by far exceeded the expectations on grounds of current knowledge

on fungal epidemiology probably hinting at a high rate of otherwise undiagnosed pulmonary fungal infections.

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Part IIA
Particularities in Special Populations

Invasive Aspergillosis in Paediatric Patients

Andreas H. Groll, Emmanuel Roilides, and Thomas J. Walsh

Abstract Invasive aspergillosis is an important cause of infectious morbidity and mortality in children with innate or acquired deficiencies in phagocytic host defences and anatomical barriers. Similar to adults, it most commonly affects the lung, and remains difficult to diagnose. Prognosis depends on early recognition, prompt institution of appropriate treatment and restoration of host defences. Paediatric patients represent a distinct population in regard to clinical presentation and epidemiology, and in particular, the utility of diagnostic tools such as the serum galactomannan ELISA and high resolution computed tomography scan imaging. The disposition of antifungal agents with clinical efficacy against invasive aspergillosis is different, resulting in different dosages across age groups and differential therapeutic algorithms due to the lack of either published clinical experience or a paediatric dosage as in the setting of antifungal prophylaxis. This chapter reviews presentation and epidemiology of invasive aspergillosis in neonates, children and adolescents and discusses the value of currents diagnostics as well as options for treatment and prevention in these distinct populations.

Keywords Aspergillosis · Children · Diagnosis · Epidemiology · Neonates · Prevention · Treatment

Contents

A.H. Groll (✉)
Infectious Disease Research Program, Center for Bone Marrow Transplantation and Department of Pediatric Hematology/Oncology, University Children's Hospital, Muenster, Germany
e-mail: grollan@ukmuenster.de

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1 Introduction

Invasive aspergillosis (IA), which most commonly affects the lung, has evolved into an important cause of morbidity and mortality in immunocompromised children. It remains difficult to diagnose and prognosis depends on early diagnosis, appropriate treatment and restoration of host defences. Paediatric patients represent a unique population in regard to clinical presentation, and epidemiology, and in particular, the utility of newer diagnostic tools and the disposition of antifungal agents. This chapter reviews presentation and epidemiology of IA in children and adolescents and discusses the value of currents diagnostics as well as options for treatment and prevention in this population.

2 Populations at Risk and General Epidemiology

In children and adolescents, IA is generally associated with profound and prolonged deficiencies in the number or function of phagocytic cells as it commonly occurs following myelosuppressive antineoplastic chemotherapy, in the course of treatment with therapeutic doses of steroids, or, congenitally, in chronic granulomatous disease. T-cell dependent, acquired defence mechanisms are involved but appear to be of limited epidemiological relevance. Non-immunological factors also are important and include damage to protective surfaces and co-morbidities such as cytomegaloviral disease [1].

In extension of this classification, the predominant paediatric populations at risk for IA include those with acquired immunodeficiencies as sequelae of treatment for haematological cancer, bone marrow failure syndromes, allogeneic haematopoietic stem cell (HSCT) or solid organ transplantation (SOT), or immunosuppressive therapy with steroids, and those with congenital immunodeficiencies predominantly affecting phagocytosis (i.e., chronic granulomatous disease) (Table 1). Whilst IA has been reported in paediatric patients with solid tumours, advanced human immunodeficiency virus (HIV)-infection, chronic destructive lung disease and in severely ill hospitalized neonates, its occurrence in these populations is more of sporadic nature [1, 2].

Table 1 Paediatric populations at greater risk for invasive *Aspergillus* infections

Premature neonates
Children with primary immunodeficiencies
– Defects of phagocytic host defences
Children with acquired immunodeficiencies
– Iatrogenic immunosuppression
– Treatment for cancer
– Allogeneic haematopoietic stem cell transplantation
– Solid organ transplantation
– HIV infection
Children with acute illnesses or trauma
Children with chronic airway diseases

The general epidemiological features of IA in the paediatric population are well reflected by the results of two more recent studies conducted in the United States of America (USA), that have provided the first multi-institutional data ever on the epidemiology and outcome of the disease in paediatric patients [3, 4].

The first was a retrospective cohort study using the 2000 Kids Inpatient Database, a national database of hospital discharges during the year 2000. IA was defined as aspergillosis that occurred in a child with malignancy, haematologic/immunologic deficiency, or bone marrow–or solid organ transplantation. During 2000, there were an estimated 666 paediatric cases of IA, yielding an annual incidence of 437/100,000 (0.4%) amongst hospitalized immunocompromised children. Children with malignancy accounted for the majority (74%) of cases. The highest incidences were seen following allogeneic HSCT (4.5%), in acute myelogenous leukaemia (4%), and in congenital immunodeficiencies (3.2%). The overall in-hospital mortality of immunocompromised children with IA was 18%; children with malignancy and IA were at higher risk for death than children with malignancy and without IA (21 vs. 1%). Paediatric patients with IA had a longer median length of hospital stay (16 vs. 3 days) and higher total hospital charges ($49,309 vs. $9,035), compared with immunocompromised children without the disease [4].

The second study was retrospective review of 139 cases of proven and probable paediatric IA diagnosed at 6 major medical centres between 2000 and 2005. The majority of the children (63%) had a haematologic malignancy with or without HSCT, followed by inherited immunodeficiencies (11.5%), SOT (11.5%), solid tumours (6.5%), HIV infection (0.7%) and other disorders (7%); almost all (96%) had at least one immunosuppressive risk factor (granulocytopenia, treatment with steroids, immunosuppressive therapy) within the 30 days before diagnosis (Table 2). The lungs were the most common clinical sites (59%), followed by the skin (10%), the paranasal sinuses (9.6%) and the brain (5.9%). *Aspergillus fumigatus* was the species most frequently recovered by culture (52.8%) followed by *A. flavus* (15.7%). Outcome was dismal with an overall case fatality rate of 52.5% (73 of 139) during treatment [3].

Table 2 Immunosuppressive risk factors in 139 paediatric patients with probable or proven invasive aspergillosis (modified from reference [3])

Risk factor	Frequency (%)
Corticosteroid therapy	69
Neutropenia (>3 days)	59
Immunosuppressive therapy	43
Malignancy (non-HSCT)	38
Allogeneic HSCT	37
GVHD	12
Congenital immunodeficiency	12
Solid organ transplant	11
>1 of the listed risk factors	95

Legend: HSCT, Haematopoietic stem cell transplantation; GVHD, graft versus host disease.

3 Special Epidemiology and Presentation

3.1 Neonates

Infections by *Aspergillus* spp. are rare in the neonatal setting. They tend to have a predilection for the skin and the gastrointestinal tract, resulting in necrotizing skin lesions and devastating necrotizing enterocolitis, respectively. Potential sources of the organisms are contaminated water, ventilation systems and dressing materials or infusion boards [5–9]; institutional outbreaks from a common environmental source have been reported [7, 8]. A comprehensive literature review covering the period from 1955 to 1996 identified 44 reported cases of IA in children of $\leq$3 months of age. Most of these infants had invasive pulmonary (23%), primary cutaneous (25%) or disseminated aspergillosis (32%). Prematurity, chronic granulomatous diseases and a complex of diarrhoea, dehydration, malnutrition, and invasive bacterial infections accounted for the majority of underlying conditions (82%). Only few patients were neutropenic, but at least 41% had received steroids. Whilst all other forms of the disease mainly occurred in term infants, cutaneous and gastrointestinal aspergillosis occurred almost exclusively in preterm neonates. All cases of primary cutaneous aspergillosis were diagnosed during life, whereas disseminated infection was diagnosed almost exclusively at autopsy. The disease was uniformly fatal in untreated patients. In contrast, outcome was relatively favourable, with 73% survival in the 22 patients receiving systemic antifungal therapy and/or surgical treatment [6]. Up to 2008, further 20 cases of IA in children $\leq$3 months of age have been reported without new aspects in presentation and clinical course [9–11].

Thoughtful use of corticosteroids, avoidance of skin trauma, careful attention to ventilation systems and room care may prevent the occurrence of nosocomial aspergillosis in neonatal medicine. Any cultures obtained from a neonate that are positive for moulds should be considered seriously, and prompt empirical antifungal

treatment should be instituted until infection can be reliably excluded. In infants with skin or gastrointestinal aspergillosis, surgical debridement is essential in most cases [6]. More detail on cutaneous and gastrointestinal aspergillosis are given in separate chapters on this book.

3.2 Children with Primary Immunodeficiencies

Chronic granulomatous disease of childhood (CGD) is a genetically diverse congenital disorder of the NADPH oxidase complex that is associated with an inability of phagocytic cells to provide antimicrobial oxidants and to kill ingested microorganisms or extracellular fungal elements. It is the prime example for an inherited immune disorder with a high risk of invasive fungal infections; at the same time, it serves as a paradigm for the importance of phagocytic killing in the defence of infections by opportunistic moulds. A chapter on aspergillosis occurring in CGD patients is presented by Dr Segal in this book.

Invasive fungal infections, particularly IA, may repeatedly complicate the course of CGD, accounting for an estimated lifetime incidence of between 16 and 40% [12–14]. The most frequent sites of infection are the lungs; however, osteomyelitis is a particularly common form of invasive infection due to *Aspergillus* spp. and other filamentous fungi [13]. *A. fumigatus* and *A. nidulans* are amongst the most common fungi causing infection in CGD patients; of note, the latter is a distinct pathogen in CGD and its isolation carries more severe prognostic implications than that of *A. fumigatus* [15, 16]. Galactomannan detection assay, a helpful early diagnostic tool in adults with haematological malignancies, has low sensitivity in these children [17]. Whether interferon-gamma or prophylactic, mould-active antifungal triazoles may reduce the frequency of fungal infections in CGD patients is not yet well established; however, there are indirect evidences that continuous administration of interferon-gamma has in vivo antifungal activity. In addition, daily administration of itraconazole to 39 patients with CGD was safe and prevented fungal infections as compared to placebo. Key principles of management of fungal infections involve early recognition and aggressive treatment and appropriate surgical debridement of localized disease. Because CGD is a disorder of phagocyte stem cells in which the gene defects are well defined, it is a model disease to evaluate immune reconstitution through HSCT and gene therapy [12, 18–20].

Patients with the hyper-IgE syndrome with recurrent infections (Job syndrome) and lung cavities (pneumatoceles) are at significantly increased risk for invasive pulmonary infections by *Aspergillus* species [21], typically in their third decade of life [22]. Cysts and cavities appear to be a marker of susceptibility rather than a cause of the infection, as invasive infections do also occur outside of these lesions [22].

Other primary phagocytic disorders, in particular myeloperoxidase deficiency, B-cell deficiencies and T-cell disorders are uncommonly associated with IA in the absence of HSCT [12, 18].

3.3 Children with Acquired Immunodeficiencies

3.3.1 Iatrogenic Immunosuppression

Treatment with pharmacological dosages of steroids rapidly provides a functional impairment of phagocytosis by mononuclear and polymorphonuclear leukocytes. Similar to adults, such therapy is one of the most important reasons for the increased susceptibility to IA for children receiving immunosuppressive therapy for immunological disorders, following SOT, and following engraftment after HSCT [1, 23, 24].

3.3.2 Cancer

Whilst current treatment for paediatric cancer is curative in most instances, highly dose-intensive chemotherapy regimens and aggressive supportive care measures also result in profound impairments of host defences. Prolonged, profound granulocytopenia, therapeutic use of steroids and, to a lesser extent, chemotherapy-induced mucositis, are the most important risk factors for IA in children with cancer [15].

IA has emerged as important cause for morbidity and mortality in children and adolescents with cancer [3, 4, 25, 26]. Indeed, large national, multicentre and single centre epidemiological surveys demonstrate that 60–75% of cases of paediatric IA occur in children with cancer [3, 4, 27]. IA in paediatric cancer patients is associated with a dismal outcome as evidenced by case fatality rates of 69–85% in single centre surveys [25–27]; similarly, in the 2000 Kids Inpatient Database survey, the mortality rate for children with all types of malignancies and IA (21%) at first discharge was far greater than that in children with malignancy but without IA (1%) [4] (Fig. 1).

Not all paediatric cancer patients are at equal risk for developing IA. This is best exemplified by a prospective observational study in 346 unselected paediatric cancer

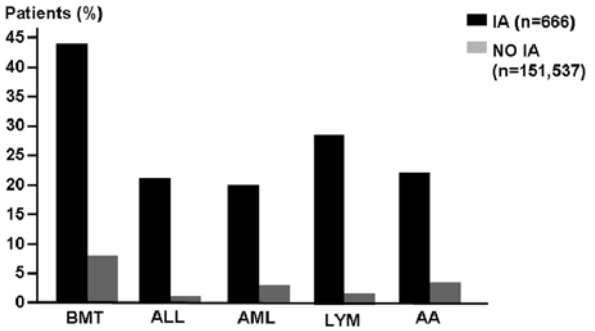

Fig. 1 In-hospital absolute and attributable mortality in patients with invasive aspergillosis (IA), United States of America, 2000. Depicted are cases with IA per underlying condition as compared to patients with the identical condition but no aspergillosis (NO IA). Modified from reference [4]. Legend: BMT, bone marrow transplantation; ALL, acute lymphoblastic leukaemia; AML, acute myeloblastic leukaemia; LYM, lymphoma; AA, aplastic anemia

patients receiving dose-intensive chemotherapy for newly diagnosed or recurrent malignancies and no mould-active antifungal prophylaxis. With an incidence rate of 6.8%, IA occurred exclusively in the context of haematological malignancies and was absent in patients with solid tumours; the highest incidence rates in this single-centre study were found in patients with de novo or recurrent AML (28% each), recurrent ALL (9%) and de novo ALL (2%) [26]. Similar proportions have been reported in larger settings [3, 4], underscoring the role of prolonged granulocytopenia and steroid therapy in the pathogenesis of the disease [28]: In the 2000 Kids Inpatient Database survey, incidence rates were 3.7% for AML, 0.6% for ALL, 0.4% for lymphoma, 0.3% following high-dose chemotherapy with autologous stem cell rescue, and 0.1% for solid tumours; the incidence for bone marrow failure syndromes, another paediatric haematological risk population, was 1.4% [4].

As in adults, *A. fumigatus* is the most frequent cause of IA, followed by *A. flavus* and *A. terreus* [3, 4]. The lungs are the most frequently affected sites, and disseminated disease is found in approximately 30% of cases. Whilst paranasal sinus aspergillosis appears to be less common than in adults [27, 29], primary cutaneous aspergillosis has been preferentially reported in the paediatric setting in association with lacerations by arm boards, tapes, and electrodes and at the insertion site of peripheral or central venous catheters [5, 27, 30]. With combined surgical and medical therapy, primary cutaneous aspergillosis has a comparatively more favourable prognosis [27].

Central nervous system (CNS) aspergillosis in infants and children predominantly presents as single or multiple brain abscesses and appears to have a better outcome compared to published adult data [31]: a systematic review of 90 cases of CNS aspergillosis in infants and children published up to 2005 found that leukaemia was the most frequent underlying disease and that the outcome has improved recently with published mortality as low as 40% [32]. CNS aspergillosis is discussed in more detail by Dr Schwartz in a separate chapter in this book.

The long-term outcome of IA in children with haematological malignancies is difficult to evaluate. In the aforementioned single-centre series, all 11 patients who were diagnosed and treated for a minimum of 10 days responded to either medical or combined medical and surgical treatment, and 7 were cured (64%). However, overall long-term survival was only 31% after a median of 5.7 years following diagnosis [26]. Whether IA ultimately was the cause or effect of ineffective cancer control remains undetermined.

3.3.3 Allogeneic Haematopoietic Stem Cell Transplantation (HSCT)

Irrespective of age, IA is an important infectious complication following allogeneic HSCT and associated with high mortality rates [4, 33, 34]. Whilst there are distinct phases of risk (pre-engraftment during granulocytopenia and mucositis vs. post-engraftment during management of acute or chronic GVHD), the clinical presentation and species distribution are essentially similar to those observed in cancer patients [4, 34].

In an interim analysis of an ongoing prospective, multicentre epidemiologic survey including 4,621 HSCT of all ages at 19 USA sites during 2001 through 2002 (TransNet Survey), the aggregate cumulative incidence of aspergillosis at 12 months was 2.3% after allogeneic HSCT from an HLA-matched related donor, 3.2% after transplantation from an HLA-mismatched related donor, and 3.9% after transplantation from an unrelated donor. The aggregate cumulative incidence at 12 months was similar following myeloablative or non-myeloablative conditioning (3.1 vs. 3.3%). Mortality at 3 months following diagnosis of aspergillosis reached up to 84.6% in unrelated-donor transplants [34].

Similar data have been reported for paediatric patients: based on discharge records of the USA 2000 Kids Inpatient Database, a total of 101 cases of IA were diagnosed amongst 2,219 paediatric allogeneic HSCT patients during the year 2000, accounting for an incidence rate of 4.5%. Overall in-hospital mortality was 45%, and attributable mortality was 34% [4] (Fig. 1). In a retrospective multicentre survey conducted at six major USA medical centres between 2000 and 2005, 51 of 139 cases of proven and probable paediatric IA occurred following allo-HSCT (27% of all cases). Patients with allo-HSCT were 6 times more likely to die than those with other diagnoses, and the overall case fatality rate of was 78% [3]. Epidemiological data from single-centre surveys are similar worldwide with incidence rates between 2.7 and 8% and case fatality rates exceeding 80% [35–37]. Whilst a predominant association with GVHD has been observed in single-centre surveys [35, 37], national [4] and multicentre data [3] do not support a current shift in incidence toward the post-engraftment graft versus host disease (GVHD) setting in paediatric patients.

3.3.4 Solid Organ Transplantation (SOT)

IA is an uncommon complication of SOT, but continues to be associated with a poor outcome. In the interim analysis of the prospective USA TransNet survey including 4,110 SOTs during 2001 and 2002, the aggregate cumulative incidence of proven or probable IA at 12 months was 2.4% after lung transplantation, 0.8% after heart transplantation, 0.3% after liver transplantation, and 0.1% after kidney transplantation. Mortality at 3 months after diagnosis of aspergillosis ranged from 20% for lung transplants to 66.7% for heart and kidney transplants [34].

Virtually similar data have been reported following paediatric SOT: Based on the USA 2000 Kids Inpatient Database, there were a total of 6 cases of IA amongst 1,593 paediatric SOT patients during the year 2000. Incidence rates were 5% after lung transplantation, 0.3% after heart transplantation, and 0.5% after liver transplantation; mortality was 33% [4]. Sixteen out of 139 cases of proven and probable paediatric IA diagnosed at six major USA medical centres between 2000 and 2005 occurred following SOT, accounting for 11.4% of all cases. The majority of cases occurred following lung and heart lung transplantation (10), followed by heart and liver transplantation (3 cases each) [3]. The case fatality rates in both analyses were 33 and 50% [3, 4].

3.3.5 HIV Infection

Children are recognized as one of the most rapidly expanding populations worldwide infected with the HIV. Without highly active antiretroviral therapy, mucosal and invasive fungal infections are major causes of morbidity and mortality in advanced stages of the disease [38, 39].

HIV-related impairment of phagocytosis by leukocytes [40] greatly contributes to the increased susceptibility of children with advanced HIV infection to IA [41]. IA was diagnosed in 7 (1.5%) of 473 HIV-infected children followed at the Pediatric Branch of the National Cancer Institute (NCI, USA) from 1987 to 1997. Invasive pulmonary aspergillosis occurred in five, and aspergillosis of the skin and adjacent soft tissues in two patients. All patients had low $CD4^+$ counts (median, 2 cells/μl; range, 0–338). Neutropenia (<500 cells/μl) lasting for >7 days or therapy with steroids were encountered in only two patients. Consistent with the experience in other immunocompromised children [27], patients with cutaneous aspergillosis were diagnosed during life and successfully treated, whereas diagnosis of pulmonary aspergillosis was made ante-mortem in only one patient [41].

3.3.6 Children with Severe Acute Illnesses or Trauma

Paediatric patients in critical condition are vulnerable to nosocomial invasive *Candida* infections, partly because of the severity of the underlying medical problem and partly because of the interventions and procedures of advanced life support. Unlike in adults [42], IA in critically ill non-immunocompromised children is extremely rare. However, similar to adults, the isolation of *Aspergillus* spp. in a patient admitted to an intensive care unit carries a particularly dismal prognosis [43].

Invasive infections in otherwise healthy children as a results of accidents have been reported in the form of invasive sinopulmonary aspergillosis following near-drowning [44], or as skin and soft tissue infections following mechanical trauma [45] or burns [46] and may cause life-threatening diseases.

3.3.7 Children with Chronic Airway Diseases

Fungal infections may also occur in children and adolescents with chronic sinopulmonary infection and lung destruction, as it may be associated with congenital B-cell defects, the hyper-IgE syndrome, and, most commonly, cystic fibrosis. Non-invasive fungal diseases associated with the colonisation of the respiratory tract by *Aspergillus* spp. and other moulds such as allergic bronchopulmonary aspergillosis (ABPA) and fungal ball (aspergilloma) formation clearly predominate in this setting. However, invasive pulmonary mould infections have been reported [21, 22, 47, 48].

4 Diagnostic Considerations

Effective management of IA requires an early and accurate microbiological diagnosis. Therefore, acquisition of an appropriate and sufficient diagnostic specimen should be attempted by all possible means. Microscopy and culture still are the

standard of mycological diagnosis. However, the diagnostic utility of conventional cultures is limited by long incubation times and often, negative results. Histology and microscopic examination are rapid techniques, but with approximately 50% sensitivity, the diagnostic yield is similarly unsatisfactory [17]. The use of molecular techniques on diagnostic aspirates or biopsies has demonstrated promising sensitivity and specificity in the research setting and is likely to play an important role in the near future [49, 50]. Drs Lass-Flörl and Freund provide us with a review on invasive interventions for the diagnosis of IA in another chapter of this book.

High-resolution computerized tomography (CT) constitutes a sensitive diagnostic screening method of pulmonary mould infections in adult haematological patients based on the detection of the halo- and air crescent signs [51]. The data accrued thus far indicate a lesser utility of these useful CT findings in the paediatric setting. In a multicentre retrospective survey including 139 cases of IA, the most frequent diagnostic radiological finding of IA were nodules (35%). Only 2.5% of children showed the air crescent sign, 11% demonstrated the halo sign, and cavitation was seen in 23% of patients [3] (Table 3). In another series of 20 patients, radiographic findings included segmental and lobar consolidation, peri-hilar infiltrates, multiple small nodules, peripheral nodular masses, and pleural effusion. Pulmonary findings were varied and often non-specific and no patient with IA exhibited a halo sign [52]. The exact reasons for these differences remain to be elucidated but may be due in differences in angioinvasiveness in non-haematological paediatric patients, differences in underlying disease as well as the particular age-dependent immune response of the host [17].

Early diagnosis of IA in adults has been improved with galactomannan ELISA assay (discussed in more detail in this book in the chapter by Drs Maertens, Theunissen, and Lagrou). A summary of published data revealed an overall sensitivity and specificity of >85% [53, 54], and there is evidence for its practical usefulness in the management of patients in conjunction with serial CT-imaging [51]. However, its use in paediatric patients and especially in young infants is problematic due to conflicting data on specificity or sensitivity [17]. In a prospective study that included 450 adult allogeneic HSCT patients (3,883 samples) as well as 347 children with haematological malignancies (2,376 samples), the rate of false-positive test results in adult patients was 2.5% as compared to 10.1% in children [55]. In another study, the false-positive rate in a group of neutropenic patients with 797 episodes of fever of unknown origin (including 48 paediatric patients) was 0.9% in adults and 44% in children, accounting for a positive predictive value of 92.1% for

Table 3 Type of radiographic findings in 82 paediatric patients with probable or proven invasive pulmonary aspergillosis (modified from reference [3])

Radiographic finding	Frequency (%)
Nodules	35
Cavity	23
Halo sign	11
Air crescent sign	2.5
Other infiltrates	35

adults and only 15.4% for children ($p < 0.0001$) [56]. The reasons for these differences may be similar to those observed with CT-imaging, and underline the need for separate paediatric validation. Antibiotics, primarily piperacillin-tazobactam, represent a source of extraneous galactomannan that may compromise the clinical specificity of the assay [57]. In a prospective study in paediatric HSCT patients including 826 serum samples from 64 patients, 11 patients received piperacillin-tazobactam therapy, and 4 of the 11 patients had a positive assay result coinciding with the dates of piperacillin-tazobactam administration. When samples from these patients were excluded, specificity by patient increased from 87.3 to 91.5% [58].

An important age-dependent limitation is the low specificity of the assay in neonates and young infants. Lipoteichoic acid, a constituent of bacterial wall of *Bifidobacterium bifidum* subspecies *pennsylvanicus* that of the intestinal microbiota of neonates may mimic the epitope recognized by the monoclonal antibody EB-A2 used in the ELISA galactomannan kit [59]. Further causes for false-positive assay results include galactomannan-positive infant formula, galactomannan-containing food and even water in conjunction with the immature or damaged intestinal epithelium of the neonate [17, 54]. Disease-specific differences may also exist. For instance, in patients with CGD or Job's syndrome and IA, galactomannan was detected in only 4 of 15 (27%) cases, as compared to 24 of 30 (80%) cases of all other immunocompromising conditions ($p = 0.0004$) [60]. The reasons of this difference may be due to more localized and less angioinvasive disease in CGD patients, to particular sites of infection, or to certain immune factors unique in CGD patients.

Beta-D glucan assay has been studied in adult patients with fungal infections [61] and in neutropenic patients treated for leukaemia [62, 63]. The importance of Beta-D glucan testing for the diagnosis of IA is discussed in the chapter by Dr Yoshida. Particularly in neonates, the assay is very promising for the diagnosis of invasive candidosis. However, since beta-D-glucan is a component of the cell wall of many opportunistic fungi, the potential usefulness of this test is likely more in the realm of preemptive treatment strategies. More detailed data in paediatric patients with IA are currently lacking.

Polymerase chain reaction (PCR) may be a powerful tool for early diagnosis of IA but has not been standardized for routine use yet. Drs Lewis and Barnes discuss the importance and limitations of PCR testing in another chapter of this book. Use of PCR in paediatric cancer patients has yielded varying performance data in the few series published thus far [64–66] without signal for differences as compared to adults.

5 Options for Treatment

The principles of treatment and the general pharmacology of antifungal agents have been comprehensively reviewed in other chapters of this textbook. The following sections focus on the available options for treatment in paediatric age groups with

brief reference to pharmacokinetics and efficacy as indicated. A detailed review of the paediatric pharmacology of antifungal agents can be found elsewhere [67].

Due to the still existing difficulties in achieving an exact microbiological diagnosis and the threat of clinical deterioration and death, treatment commonly needs to be started preemptively on the basis of risk profile, imaging studies and serological markers. The diagnostic criteria set forth by the EORTC/MSG [68] for clinical research purposes may conceptually be helpful for clinical practice also (more on this in the chapter by Dr Donnelly), but should not be misunderstood as guidelines for diagnosis and treatment. However, even if treatment is started preemptively, all efforts should be made to perform the necessary diagnostic procedures to obtain a diagnostic microbiological specimen for its potential consequences on the selection and duration of treatment.

Based on pivotal phase III trials with similar design performed in adult patients [69, 70] and the existence of approved paediatric dosages [71–74], options for primary treatment of IA in paediatric patients $\geq$2 years of age include voriconazole and liposomal amphotericin (L-AMB) (Table 4). As the dosage of voriconazole for children <2 years has yet not been defined, L-AMB is the only approved first-line option in this age group. Because of inferior responses and survival in the randomized comparative trial with voriconazole [70], there is little rationale for the use of amphotericin B deoxycholate in primary treatment of IA. It is important to note, however, that there is little systematic data available on the efficacy of both voriconazole and L-AMB in paediatric patients with IA [72, 75]. In particular, there is virtually no published treatment experience for the dosage of voriconazole in children aging 2–12 years, which has been preliminarily approved by the European Medicines Agency (EMEA) only and not yet by the Food and Drug Administration (FDA, USA).

Options for second-line therapy in paediatric patients $\geq$2 years of age not responding to or being intolerant of treatment with either voriconazole or L-AMB are similarly based the existence of an approved paediatric dosage, efficacy data generated in adults and limited published efficacy data in children and adolescents. An exception to this is amphotericin B lipid complex (ABLC), for which there is a large published phase II and IV experience in paediatric patients of all age groups [16, 76] (Table 4). Other options include amphotericin B colloidal dispersion (ABCD) [77] and caspofungin, which is approved for all pediatric age groups [78–80]. Non-approved options include itraconazole, for which there exists a paediatric dosage for the oral but not the intravenous solution [81, 82], and, based on limited pharmacokinetic and treatment data, posaconazole for adolescents $\geq$13 years of age [83, 84]. Alternatives for second-line therapy in children <2 years are limited to amphotericin B lipid complex [85] and caspofungin [67] (Table 4).

Certain clinical situations bear notice. Although not formally investigated in a clinical trial, when a change in antifungal chemotherapy is considered necessary for refractory disease, a switch in class is advised; this is likely also true for patients experiencing breakthrough infections whilst on antifungal prophylaxis. Whereas antifungal combination therapy is promising [86, 87] and widely used in clinical practice, its role, however, remains to be defined through randomized

Table 4 Paediatric dosages of systemic antifungal agents used for treatment of invasive aspergillosis (for detailed indications, please refer to the text)

	Daily dosage per age group			
Antifungal agent	13–18 years	2–12 years	1–24 months	Neonates
Amphotericin B deoxycholate	1–1.5 mg/kg IV	1–1.5 mg/kg IV	1–1.5 mg/kg IV	1–1.5 mg/kg IV
Liposomal amphotericin B (L-AMB)	3–5 mg/kg IV	3–5 mg/kg IV	3–5 mg/kg IV	3–5 mg/kg IV
Amphotericin B lipid complex (ABLC)	5 mg/kg IV	5 mg/kg IV	5 mg/kg IV	5 mg/kg IV
Amphotericin B Colloidal dispersion (ABCD)	3–4 mg/kg IV	3–4 mg/kg IV	3–4 mg/kg IV	NA
Itraconazole oral suspension	5 mg/kg PO (divided in 2 doses)	5 mg/kg PO (divided in 2 doses)	NA	NA
Voriconazole IV	6 mg/kg IV twice daily on D1, then 4 mg/kg IV 12 hourly	7 mg/kg IV 12 hourly (no need for a loading dose)	NA	NA
Voriconazole oral suspension/capsules	200 mg PO 12 hourly	200 mg PO 12 hourly	NA	NA
Posaconazole oral suspension	800 mg PO (divided in 2–4 doses)	NA	NA	NA
Caspofungin	50 mg IV (D1: 70 mg) (max: 70 mg)	50 mg IV (D1:70 mg) (max: 70 mg)	50 mg/m^2 IV (D1:70 mg/m^2)	NA

Legend: IV, intravenous; PO, oral; D1, day 1; NA, no or no sufficient data is available.

comparative studies. Until the generation of such data, antifungal combination therapy is presently only justified in fulminant or refractory disease [88, 89].

There are emerging data suggesting a rationale for therapeutic drug monitoring of antifungal triazoles to optimize outcome in IA [90–92]; however, more systematic investigation is required before its implementation into clinical practice. Similarly, little is known about site-directed antifungal efficacy. For invasive pulmonary aspergillosis, the most common entity, there is no pharmacodynamic data that would suggest differences between triazoles and amphotericin B. Whilst the same is true for most entities, non-comparative clinical data acquired in adult patients currently suggest that voriconazole may be the preferred agent for treatment of CNS-aspergillosis [31] with high-dose ($\geq$5 mg/kg/d) L-AMB being the next best alternative based on pharmacokinetic/pharmacodynamic animal data [93]. Because of its high solubility in water, voriconazole may also be a rationale option

for endophthalmitis, peritonitis, and joint infections [94]; in a small series in mostly adult patients with *Aspergillus* bone infections, the response rate appeared to be encouraging [95] (see specific chapters on extra-pulmonary aspergillosis throughout this book).

Beyond patient-specific and general pharmacological information, the availability of microbiological data has become essential in making treatment decision. Multi-azole resistant *Aspergillus* species have been reported [96, 97]. *A. terreus*, on the other hand, has a reduced susceptibility to amphotericin B [98, 99] and is best treated with voriconazole [100]. Zygomycetes are resistant to voriconazole and caspofungin, and non-*Aspergillus* hyalohyphomycetes and phaeohyphomycetes are considered resistant to caspofungin [101]. Identification to the species level is recommended, and in vitro susceptibility testing is desirable despite the absence of a firm in vitro-in vivo correlation [102].

Similar to adults, the duration of treatment for IA is defined by the complete disappearance of all signs and symptoms and resolution of the underlying deficiency in host defences [89, 94]. Patients who have responded to initial intravenous therapy and who are clinically stable may be consolidated with orally available agents [89, 94].

Recommended adjunctive treatments include the administration of colony-stimulating factors (G-CSF, GM-CSF) in granulocytopenic patients with severe *Aspergillus* infections and the discontinuation or at least dose-reduction of steroids. Granulocyte transfusions may be indicated in individual granulocytopenic patients, but their general usefulness is unclear [94, 103]. Surgery is considered essential for *Aspergillus* endocarditis and endophthalmitis, and should be strongly considered in progressive sinusitis, skin- and soft tissue infections, CNS-lesions and other focal processes amenable to a surgical procedure (see specific chapters about these conditions) [94, 104]. The exact role of surgery in the management of invasive pulmonary aspergillosis and the optimal time points for intervention have not been defined – several case series suggest that surgery can be safely and effectively performed in patients who have localized infections, even during periods of neutropenia [105, 106].

6 Approaches to Prevention

General measures for prevention of IA have been discussed in other chapters of this textbook and elsewhere [107]. Since there are no principal differences in this area between paediatric and adult patients, we will limit the discussion in this section to the current approaches to chemoprophylaxis in paediatric patients.

6.1 Primary Prophylaxis

The lack of effective tools for an early diagnostics, the poor prognosis of IA and the frequency of this condition in certain high risk populations provide the rationale for primary chemoprophylaxis. A variety of different approaches have been explored in the past – mostly in adult patients with haematologic malignancies and/or allogeneic

HSCT – without providing convincing preventative evidence for efficacy against IA [107–109]. Two recent large randomized clinical trials, however, have demonstrated that prophylactic posaconazole is effective in preventing IA in patients with acute myeloblastic leukaemia or myelodysplastic syndrome [110] and those with GVHD receiving augmented immunosuppressive therapy [111]; the first study also demonstrated a survival advantage in the posaconazole cohort. Whilst a few patients 13 years and older were included in these trials, data in children are currently lacking.

Institutional incidence rates of 10% and higher are commonly observed in paediatric patients treated for de novo and recurrent AML, recurrent ALL, bone marrow failure syndromes, and following allogeneic HSCT [25, 26, 37]. Based on the inclusion of a few paediatric subjects ≥13 years of age [110, 111], favourable paediatric safety data within the manufacturer's compassionate use program, and limited exposure data obtained in 12 paediatric subjects ≥8 years of age that suggest no fundamental differences in trough plasma concentrations in older children as compared to adults [84], at the Department of Pediatric Hematology/Oncology in Muenster (Germany), patients ≥13 years of age with de novo and recurrent AML, recurrent ALL, bone marrow failure syndromes, and those with augmented immunosuppression for GVHD receive prophylactic posaconazole 200 mg three times daily by mouth. Patients 2–12 years of age receive voriconazole 200 mg two times daily orally. Patients <2 years of age and those unable to take oral medication receive either L-AMB (at 1 mg/kg every other day for all age groups) or intravenous voriconazole (if ≥2 years of age), under careful consideration of contraindications and drug interactions (Table 5).

Table 5 Paediatric dosages of systemic antifungal agents used for prophylaxis of invasive aspergillosis (for detailed indications, please refer to the text)

	Daily dosage per age group			
Antifungal agent	13–18 years	2–12 years	1–24 months	Neonates
Liposomal Amphotericin B	1 mg/kg IV daily or every other day	1 mg/kg IV daily or every other day	1 mg/kg IV daily or every other day	1 mg/kg IV daily or every other day
Itraconazole oral suspension	2.5 mg/kg PO bid	2.5 mg/kg PO bid	NA	NA
Posaconazole oral suspension	200 mg PO tid	NA	NA	NA
Voriconazole IV	4 mg/kg IV bid	7 mg/kg IV bid	NA	NA
Voriconazole oral suspension/capsules	200 mg PO bid	200 mg PO bid	NA	NA
Micafungin	50 mg IV (1 mg/kg if <50 kg)	50 mg IV (1 mg/kg if <50 kg)	NA	NA

Legend: IV, intravenous; PO, oral; D1, day 1; NA, no or no sufficient data is available; bid, twice daily; tid, three times daily.

6.2 Secondary Prophylaxis

Patients with IA who undergo further intensive chemotherapy or allogeneic HSCT have a ∼30% likelihood for exacerbation or recurrence of the infection [112, 113]. Whilst the role of surgical resection of residual lesions, if feasible, is unclear, patients should have had at least a partial response before continuation of anticancer treatment and continue to receive effective antifungal therapy [94, 107]. In a recently published paediatric case series, patients with leukaemia and a history of invasive pulmonary aspergillosis undergoing allogeneic HSCT received L-AMB (1 mg/kg/d) from conditioning until engraftment, followed by oral voriconazole (200 mg 12 hourly) until the end of the at-risk period. Both regimens were acceptably well-tolerated. At +180 days post-transplant, 8 patients were alive, 6 with complete, and 2 each with near complete/ongoing resolution of pulmonary infiltrates. All but one were in haematological remission. Three patients had succumbed to recurrent leukaemia or refractory graft failure, two with secondary possible or probable invasive pulmonary aspergillosis. In the absence of chronic GVHD, breakthrough infection appeared to be associated with recurrent leukaemia/graft failure and shorter duration of post-engraftment prophylaxis [114].

7 Conclusions

Paediatric age groups display important differences in host biology, predisposing conditions, epidemiology and presentation of fungal infections relative to the adult population. Over the past decade, major advances have been made in the field of medical mycology, including the development of new diagnostic tools and new antifungal agents. Although the paediatric development of new antifungal drugs is moving forward at steady pace, the discussion of new diagnostic and interventional modalities clearly demonstrates the limited fund of paediatric data. Cognizant of past and present epidemiological trends, invasive fungal diseases will remain a frequent and important complication in children with severe underlying illnesses. Well-designed, carefully conducted, cooperative clinical investigations are needed more than ever to translate progress in medical mycology into paediatric medicine.

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Invasive Aspergillosis in the Intensive Care Unit: Beyond the Typical Haematological Patient

Wouter Meersseman

Abstract Data about incidence of invasive aspergillosis in intensive care units (ICU) are scarce and variable. Incidences ranging from 2 to 24% have been reported, which might reflect different autopsy policies amongst centres. Recent studies have shown that many patients with invasive aspergillosis do not have a haematological diagnosis. Instead, conditions such as chronic obstructive pulmonary disease and liver failure became recognized as important risk factors. The diagnosis remains difficult in these patients, since diagnostic tests have not been widely validated outside the haematological boundaries. Mechanical ventilation precludes the interpretation of clinical signs and radiological diagnosis is clouded by underlying lung pathology. Respiratory cultures lack sensitivity and specificity. At the moment, diagnosis is best made by testing for galactomannan in bronchoalveolar fluid samples (sensitivity and specificity of > 87%). Testing galactomannan in sera has limited sensitivity for the non-neutropenic. Modern diagnostic tests such as PCR and beta-glucan have never been validated in an ICU population. Due mostly to major delays in the diagnosis, mortality exceeds 50%. Although our therapeutic armamentarium against invasive aspergillosis has improved in recent years, data concerning safety and efficacy of new antifungal agents in the ICU setting are lacking.

Keywords Antifungals · Aspergillosis · COPD · Diagnosis · Galactomannan · ICU

Contents

W. Meersseman (✉)
Department of General Internal Medicine, Gasthuisberg University Hospital, Leuven, Belgium
e-mail: wouter.meersseman@uzleuven.be

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1 Is Invasive Aspergillosis (IA) a Problem in the ICU?

Autopsy studies show the emergence of *Aspergillus* as a major pathogen, as well as the expansion of the spectrum of patients at risk for IA. In a non-selected patient population, the prevalence of invasive fungal infections in an academic hospital rose from 2.2 to 5.1% over a 12-year period, largely due to an increase in *Aspergillus* infections [1]. However, estimates about the incidence of IA in critically ill patients are sparse and variable. For various reasons, figures about the true incidence are difficult to generate. First, in case of a positive culture for *Aspergillus* species, discriminating between colonisation and infection remains challenging. Second, very few institutions perform post-mortem examinations routinely, while in most cases, this is the only way for proving the definite nature of the diagnosis. Third, characteristic radiological signs of IA are usually absent in the non-neutropenic ICU patient. Finally, to date, the diagnostic utility of recently available non-culture based microbiological tools, including the detection of fungal antigens and the detection of *Aspergillus*-specific DNA through polymerase chain reaction (PCR) techniques, has not been properly validated in the non-haematology ICU population. In addition, typical ICU patients such as those with chronic obstructive pulmonary disease (COPD) or liver disorders were not considered amongst hosts at high risk for IA in the recently updated EORTC/MSG guidelines [2].

A summary of available studies in ICU patients is listed in Table 1 [3–9]. In a medical ICU, we have observed high incidences of IA in two separate retrospective autopsy-controlled studies. In the largest one, 127 of 1850 (6.9%) hospitalised patients had microbiological or histopathological evidence of aspergillosis during their ICU stay, including 89 cases (70%) without underlying haematological malignancy. The observed mortality of 80% was much higher than the predicted mortality as per SAPS II score (48%) [4]. An earlier study looked for unsuspected causes of death in the same medical ICU and showed that, out of 100 autopsies, there were 15 cases of IA, of which 5 were missed pre-mortem [3]. During a 6-year period, Cornillet et al. found a mean number of 15 patients per year diagnosed with IA; approximately half of them were in the ICU [9]. These inter-centre differences can be explained by differences in underlying patient characteristics, case mix and different autopsy policies.

In a recent study published by our group [10], patients with fever new lung infiltrates were screened for IA using galactomannan testing in bronchoalveolar (BAL) fluid. From a total of 1,109 patients admitted to the ICU, 110 patients fulfilled the entrance criteria and were evaluated. Most patients had non-haematological diseases (67%), including liver cirrhosis (21%), COPD (14%) and other systemic conditions (15%). The incidence of proven IA in this population was surprisingly high at 23.6%, which might have been associated with the high frequency of autopsy

Table 1 Relevant epidemiological studies of IA in the intensive care unit

References	Year	No. of patients	Duration study	Type of study	Aim of the study	Autopsy protocol[a]	Incidence of IA	Important findings
(1) Studies examining the incidence of IA widespread in hospital (not confined to ICU)								
Groll et al. [1]	1996	8,000	12 years	Retrospective single centre	Describing trends in post-mortem epidemiology of IFI	Yes	3.1%	Increasing trends in incidence of IA compared to invasive candidosis
Cornillet et al. [9]	2006	88	6 years	Combined retrospective and prospective cohort 47% ICU patients	Comparing features of IA in neutropenic and non-neutropenic patients	No	15 cases/year	Overall mortality 71% (non-neutropenic patients 89%)
(2) Studies specifically examining the incidence of IA in the ICU								
Bulpa et al. [15]	2001	23	4 years	Case series of COPD patients mixed ICU	Describing IA in patients with COPD admitted to the ICU	Yes	–	100% mortality in ventilated COPD patients
Meersseman et al. [4]	2004	127	3 years	Retrospective single centre, medical ICU	Determining incidence of IA in a medical ICU	Yes	5.8%	IA in increasingly recognized in patients without classical risk factors
Garnacho-Montero et al. [28]	2005	1,756	9 moths	Multicentre prospective, 73 mixed ICU	Describing characteristics of patients with positive sputum sample for *Aspergillus* in 73 ICU	No	1.1%	Mortality 50% in patients colonised with *Aspergillus* and 80% in patients considered to have IA
Vandewoude et al. [7]	2006	172	7 years	Retrospective single centre mixed ICU	Describing characteristics of patients with positive sputum samples for *Aspergillus*	No	0.33%	60% nonhaematological patients, mortality 77% in IA, 40% in colonisation

Table 1 (continued)

References	Year	No. of patients	Duration study	Type of study	Aim of the study	Autopsy protocol[a]	Incidence of IA	Important findings
Meersseman et al. [10]	2008	1,109	1.5 year	Prospective single centre study	To investigate the role of a diagnostic test for IA in the ICU	Yes (95%)		
(3) Other studies (more general autopsy studies/or studies examining the aetiology of pneumonia in the ICU								
Roosen et al. [3]	2000	100	1 year	Retrospective single centre study in a medical ICU	Comparison pre-mortem diagnosis and autopsy findings (all causes of death)	Yes	15%	IA is a more important missed diagnosis in a medical ICU than any other illness
Valles et al. [8]	2003	67	7 years	Prospective cohort study, in 2 mixed ICU's	Describing patients with hospital acquired pneumonia admitted to the ICU	No	19%	IA was the second most frequent cause of HAP requiring ICU admission. COPD was a significant risk factor
Dimopoulos et al. [5]	2004	222	1 year	Retrospective single centre mixed ICU	Comparison pre-mortem diagnosis and autopsy findings (all causes of death)	Yes	3.7%	In 6/14 cases with major missed diagnoses, IA was responsible
Kumar et al. [6]	2006	2,154	15 years	Retrospective multicentre cohort	Determining the impact of antimicrobial therapy in all patients with septic shock admitted to the ICU	No	0.7%	No data of proven cases, inclusion solely based on culture results

Legend: IA, invasive aspergillosis; ICU, intensive care unit; COPD, chronic obstructive pulmonary disease.
[a]Studies in which autopsy was performed in more than 50% of cases.

(95% of fatalities), as well as the use of a sensitive diagnostic tool (more comments about galactomannan testing are presented below). As this was a single-centre study, the presence of an outbreak is also a possibility.

2 Who is at Risk for Developing IA in the ICU?

Over the past two decades, IA has emerged as a life threatening fungal infection in patients with haematological diseases. Although many of these infected patients will eventually be admitted to the ICU for advanced supportive care, it seems that IA has also gained a foothold in less severely compromised ICU patients [7]. So, can a threshold of immunosuppression needed for the development of IA be defined? We grouped the risk factors for IA in the ICU are into 3 categories (high, intermediate, low) (Table 2).

Various factors adversely affect the defence systems of previously healthy individuals, including the prolonged use of antibiotics, the use of central venous

Table 2 General risk categories for invasive aspergillosis amongst patients admitted to the intensive care unit (medical, surgical or mixed)

High risk category
– Neutropenia (< 500 neutrophils/mm^3)
– Haematological malignancies (particularly acute leukaemia)
– Allogeneic haematopoietic stem cell transplantation
Intermediate risk category
– Prolonged treatment with steroids before admission to the ICU
– Autologous haematopoietic stem cell transplantation
– COPD
– Liver cirrhosis with ICU stay > 7 days
– Solid-organ cancer
– HIV
– Lung transplantation
– Systemic diseases requiring immunosuppressive therapy
Low risk category
– Severe burns
– Other solid-organ transplant recipients (e.g., heart, kidney, liver)
– Receipt of steroids for $\leq$ 7 days
– Prolonged stay in the ICU (> 21 days)
– Malnutrition
– Post-cardiac surgery

Legend: ICU, intensive care unit; COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus.
Note: environmental factors such as exposure to fungal high inoculums are not being considered in this Table. Other immune defects putting patients at risk for invasive aspergillosis are discussed in the chapter by Dr. Romani.

catheters and/or mechanical ventilation. Although these factors are present in most ICU patients, many of them do not develop IA. One of the intriguing hypotheses for immunosuppression in the apparently immunocompetent patient with multiple organ dysfunctions is related to the biphasic response to sepsis. The initial hyperinflammatory phase is followed by relative immunoparalysis [11]. This latter process is characterized by neutrophil deactivation and may put the patient at risk for developing opportunistic infections such as IA. Further epidemiological study is warranted to better delineate this phase of immunoparalysis. More detail on the interactions between *Aspergillus* and the immune system are presented in the chapter by Dr Romani.

Patients in the ICU (medical and surgical) are often treated with steroids. Recent work concluded that the mortality is reduced if septic shock patients with adrenal dysfunction receive hydrocortisone for a 7-day period [12]. In vitro, however, pharmacological concentrations of hydrocortisone accelerate the growth of *Aspergillus* spp. [13]. Clearly, high steroid intake diminishes both lines of cellular defence against IA (macrophages and neutrophils). Palmer reported that the threshold steroid level varies according to the type of patients and emphasized that underlying lung disease is a risk factor for IA even at low doses [14]. Further study is needed to investigate whether the 7 day course of hydrocortisone at 200-mg/day in patients with septic shock puts them at risk for IA, knowing that recognition of fungal infection may be delayed, since the anti-inflammatory properties of steroids blunt the signs of infection.

Two at-risk groups not included in the EORTC/MSG definitions stand out for IA, COPD and cirrhosis patients. Patients with COPD are an increasingly recognized group of patients at risk for developing IA and in some institutions outnumber cases in "classic" patients. Bulpa et al. analyzed a group of 16 COPD patients with proven or probable IA requiring ICU admission. All patients were on steroid treatment. The outcome was invariably poor [15]. This is in accordance with the report of Rello et al., who describes another 8 COPD patients with IA and universally fatal outcome [16]. Guinea et al. from Madrid recently presented results from a large series of IA cases in association with COPD ($n = 57$) [17]. Steroids were identified as a risk factor in 98% of patients, with 74% of patients having received total doses of > 700 mg. Most cases of IA in COPD patients had only lung involvement, but 2 patients also had probable brain involvement. Data from the same group also revealed that COPD became the leading underlying disease associated with IA (52.4% of cases), far more frequent than classical conditions such as haematological malignancies (15.2%) [18].

Hepatic failure is generally not recognized as a risk factor for IA. A literature review revealed that 5 of 14 previously reported cases of IA in seemingly immunocompetent hosts were associated with liver disease [19]. Our study revealed 3 fatal cases of IA [4]. Patients with cirrhosis have depressed phagocytosis, which may increase their risk for severe infections.

It is expected that new risk categories of IA will come up as new immunosuppressive agents are made available such as alemtuzumab and etanercept (TNF- α blocker) [20].

3 Do Patients Acquire IA in the ICU?

There are numerous sources of *Aspergillus* species for patients in the ICU [21, 22]. It is believed that the primary ecological niche is decomposing material. However, aerosolised spores may become a potential source of infection through improperly cleaned ventilation systems, water systems or even computer consoles. The use of High Efficiency Particulate Air (HEPA) filtration reduces the risk of IA but does not reduce it to zero, probably partly because patients may be colonised before admission to the ICU, partly because of breaks in airflow. Pittet described two patients who developed fatal IA in the ICU. In retrospect, high concentrations of airborne *Aspergillus* spores could be found, closely related to air filter change in the ICU [23]. Besides the airborne route, contaminated water has been implicated as a source of infection [24]. A study of ventilators as a source of infection has not been undertaken. Of note, the development of IA is depends on an interplay between the inoculating dose, the ability of the host to resist infection (which also depends on the lung architecture) and the virulence of the infecting organism.

The concept that increasing fungal burden due to specific ICU treatments for other diseases than IA (e.g. steroids for septic shock) parallels the progression from subclinical to clinical aspergillosis, needs to be explored with more sensitive markers (e.g. PCR). PCR in respiratory secretions as a modality for surveillance is an interesting topic for research.

4 Disease Manifestations in the ICU

Generally speaking, there are types of pulmonary interactions between *Aspergillus* species and humans. The most frequent interaction is colonisation of the airways. This can be present in patients with defective mucociliary clearance and structural changes in the bronchial wall. These changes are present in almost every mechanically ventilated patient, making them particularly susceptible to colonisation. IA will not develop in these patients unless a critical level of immunodeficiency has been reached. The second type of interaction is allergic in nature and is beyond the scope of this review (these are discussed in other chapter in this book). The most relevant form of interaction for ICU physicians is the invasive disease that develops in persons with impaired immunity. The aggressive angioinvasive form is frequently encountered in neutropenic patients, whereas cavitating infiltrates are observed most frequently in patients on steroids, patients with COPD, cirrhosis, and solid organ transplant recipients. Other more rare presentations include endocarditis, wound infections, mediastinitis (post-cardiac surgery), infection of vascular grafts, and osteomyelitis. These are occasionally a problem in immunocompromised patients and may occur as outbreaks. Infection of the central nervous system is frequently an ominous sign and may arise from haematogenous seeding (in which the lung is the most common primary site) or spread from the sinuses or following neurosurgery.

The pathogenesis of IA in steroid-immunosuppressed patients differs greatly from that in neutropenic patients. Data demonstrate that the pathological lesions are often widespread and that death is related to a high fungal burden in neutropenic animals, while the pathogenesis in non-neutropenic, steroid-treated animals is driven by an adverse inflammatory host response, frequently confined to the lungs, with a low fungal burden in the lung parenchyma and other organs [25, 26]. The reader is referred to the chapter by Drs Ben-Ami and Kontoyiannis for more detail on the pathogenesis of IA.

Clinical signs are usually non-specific and do not necessarily differ from other causes of nosocomial pneumonia. In addition, critically ill patients with prolonged stays in the ICU often develop pulmonary infiltrates, atelectasis and/or acute respiratory distress syndrome (ARDS), whereas patients with prior lung disease (e.g. COPD) may present with pre-existing cavities on conventional chest radiographs (Fig. 1).

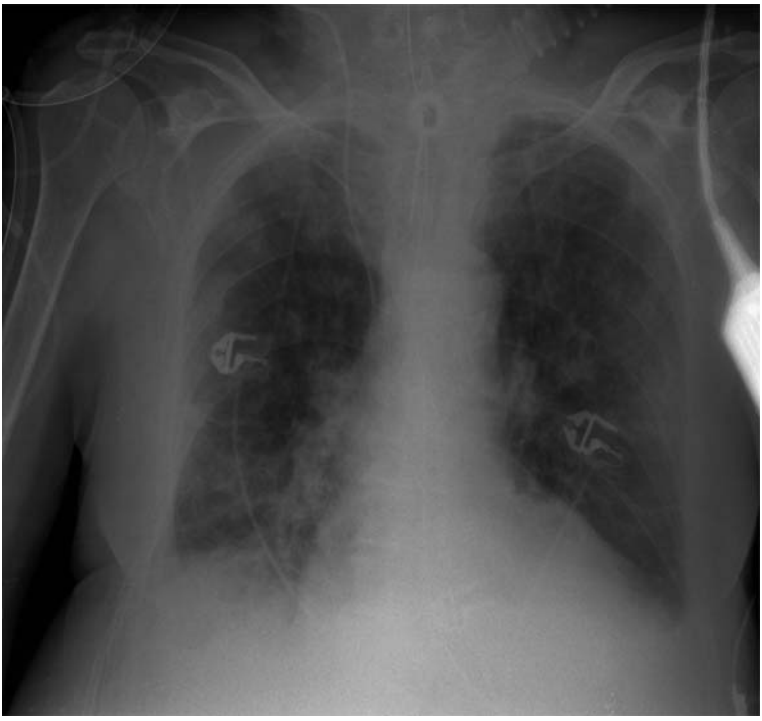

Fig. 1 Chest X-ray from a COPD patient on steroids, admitted to the ICU because of an exacerbation with respiratory failure. Patchy, hazy infiltrates with predominantly a peripheral localisation and a right sided pleural effusion were seen. BAL culture was positive for *Haemophilus influenzae* and negative for fungi. Serum galactomannan was negative but showed a value of 2.6 ng/ml in the BAL fluid. Despite treatment with caspofungin (patient was in renal failure), he died and autopsy showed invasive aspergillosis, confined to the lungs

5 Are the Available Diagnostic Tools Applicable to Patients in the ICU?

Making a timely diagnosis of IA in the ICU population is probably even more challenging than establishing an early diagnosis in patients with haematological disease, basically because the index of suspicion is lower since most patients do not belong to one of the well-established risk groups. Moreover, the diagnostic tools were mainly developed in haematological patients. In general, the diagnosis is based on a combination of compatible clinical findings, radiological abnormalities, and microbiological confirmation or on the histological proof of tissue invasion by the fungus. Table 3 gives an overview of the available diagnostic tools.

Over the past few years, lung computed tomography (CT) scan has become one of the most important tools for the diagnosis of IA [27]. Virtually diagnostic signs for angioinvasive pulmonary mycosis – not only due to aspergillosis but occasionally also due to zygomycosis as well as other vascular conditions – include single or multiple small nodules with a "halo" sign. It should be recognized that the utility of this sign has been evaluated almost exclusively in neutropenic patients. In other groups, including ICU patients, similar CT-findings are frequently absent and, if present, are far less specific [4]. Many ICU patients have non-specific interfering radiological abnormalities due to atelectasis, or ARDS (Figs. 2, 3, and 4).

A positive respiratory specimen by culture or by direct microscopic examination is present in only half of the patients with IA. The predictive value of a positive culture depends largely on the immunocompromised status of the patient and ranges from 20 to 80%. Given the ubiquitous nature of *Aspergillus* spores, differentiating colonisation from infection remains problematic. Two studies have examined the significance of isolation of *Aspergillus* spp. in ICU patients and confirmed the poor positive predictive values [7, 28]. Therefore, surveillance cultures in the ICU will add little to the diagnosis of IA.

Serological techniques based on the detection of circulating fungal cell wall components such as galactomannan (GM) or β-D-glucan and detection of circulating fungal DNA by PCR techniques hold promise in patients with haematological malignancy but limited data exist with the use of these tests in diagnosis of IA in the ICU [10]. Although very useful in the haematological patient [29], serum GM is not a sensitive marker for IA in the non-neutropenic individual, as demonstrated in lung and liver transplant recipients [30, 31]. Viable fungi could endure in the lung tissue (with encapsulation by an inflammatory process), while circulating markers remain undetectable because of clearance by circulating neutrophils. BAL fluid could be a better specimen for GM detection as recently was demonstrated in a prospective study performed in a medical ICU in a tertiary referral hospital [10]. This is reinforced by the data with the solid organ transplant population [32–34]. On the other hand, GM testing in BAL fluid samples seems very promising for the diagnosis of IA in non-neutropenic patients [10, 35]. Results from a single ICU showed that test sensitivity and specificity were 88 and 87%, respectively using a cut-off of 0.5 for BAL testing. In contrast, the sensitivity of serum GM was 42% only. A bit of caution, however, is required with this, since the best cut-off for GM testing in BAL

Table 3 Tools for diagnosis of invasive aspergillosis and applicability in the intensive care unit

Diagnostic tool	Characteristic finding	Relevant studies	No. of patients	Applicability for ICU	Comment
CT scanning	Halo sign	Caillot et al. [43]	25 proven cases	No, too early sign (5 days before the onset of disease) (see Figs. 1, 2, 3, and 4). Non-neutropenic patients usually do not manifest halo signs	Not specific for *Aspergillus* (also other moulds)
	Air crescent sign	Caillot et al. [43]	25 proven cases	Probably not – obscured by atelectasis, ARDS and/or pleural effusion (see Figs. 1, 2, 3, and 4)	CT scan often not feasible in a patient with high FiO_2
Histopathological evidence	Acutely branching (45°), septated hyphae in mainly lung tissue	Meersseman et al. [4][a] Roosen et al. [3][a]	129 (56 proven) 100 (15 proven)	Yes, global standard	Biopsies often not feasible (thrombocytopenia, high F_iO_2)
Culture	Growth on Sabouraud agar Isolation of the species takes several days	Vandewoude et al. [7][a] Garnacho-Montero et al. [28][a] Perfect et al. [44] Bouza et al. [45]	172 (17 proven) 36 (5 proven) 1209 (24 centres) 260 (31 proven)	Moderate applicability for both culture and microscopy, since poor sensitivity and specificity	50% of cases are missed based on culture and microscopy; discrimination colonisation vs invasive disease difficult, PPV increases with increased immunosuppression
Direct microscopy	PAS, Grocott stain, Calcofluor, etc.				

Table 3 (continued)

Diagnostic tool	Characteristic finding	Relevant studies	No. of patients	Applicability for ICU	Comment
Galactomannan assay	Polysaccharide released by the fungus in case of invasiveness	Meerseman et al. [10]	110 (26 proven cases)	GM detection in BAL: sensitivity 88%, specificity 87% (using a 0.5 cut-off). The sensitivity of serum GM was only 42%	In the non-neutropenic critically ill patient: better performance for BAL fluid testing than serum
Polymerase chain reaction (PCR)	DNA material of *Aspergillus fumigatus*	Tuon et al. [46]	–	Not tested in the ICU	In the nonneutropenic critically ill patient: BAL fluid may be more performant than blood
Beta, 1–3, D-glucan	Fungal cell wall component	Digby J, et al.[38]	61	Only one study	Not specific for *Aspergillus*, also present in yeasts and bacteria, may be useful as a negative predictor of fungal infection

Legend: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; CT, computed tomography; DNA, deoxyribonucleic acid; F_iO_2, fraction of inspired oxygen; GM, galactomannan; ICU, intensive care unit; PAS, periodic acid Schiff's stain.
[a]Studies confined to the ICU.

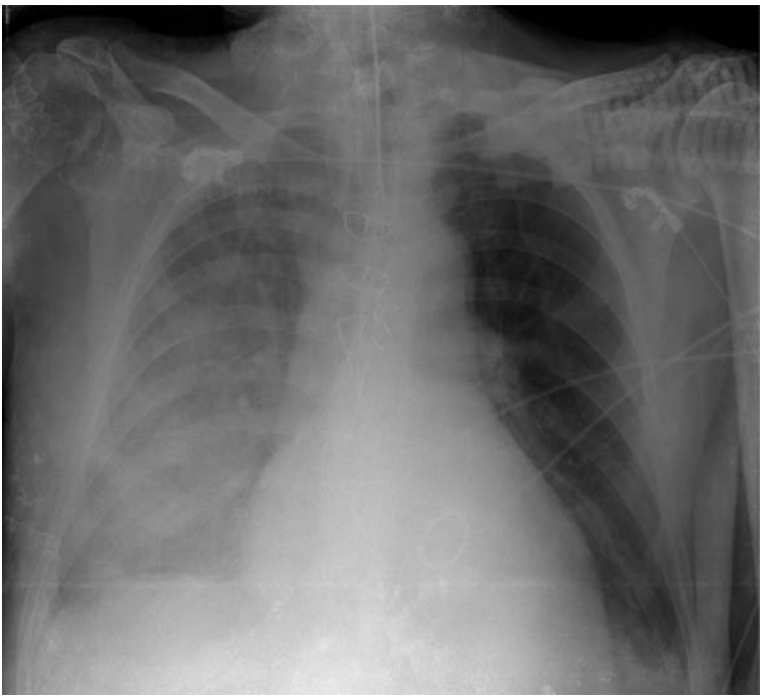

Fig. 2 Chest X-ray from a liver transplant recipient reveals predominantly right sided air-space disease. No nodular lesions are seen. Findings are compatible with the diagnosis of pneumonia. Chest CT scan was not feasible because of high FiO_2 requirements. BAL culture results were negative for bacteria and fungi (while on broad spectrum antibiotics). Galactomannan in serum was negative. Patient died and autopsy showed disseminated aspergillosis

fluid samples is still a matter of debate. It has been demonstrated experimentally that the dynamics of IA result in higher and earlier release of GM in the alveoli, in comparison to the endothelial compartment [36]. Accordingly, many studies have shown that testing GM in BAL fluid samples result in higher optical densities than testing sera [33, 37]. In a study with non-immunocompromised patients, all cases of IA were associated with GM optical densities of ≥ 1.18 in the BAL [34]. A higher cut-off value for BAL has also been suggested by other authors, in comparison to sera [32, 33]. False-positive results have been observed when BAL is tested for GM in patients colonised with *Aspergillus* species, particularly lung transplant recipients [32] (Dr. Pasqualotto, unpublished data).

Although attractive, other modern diagnostic tests have not been systematically evaluated for the diagnosis of IA in ICU patients. The use of β-D-glucan detection in ICU is hampered by false-positive readings (use of albumin, wound gauze, hemodialysis and bacterial infections) [38]. Galactomannan gives less false-positive results, although the presence of β-lactam antibiotics such as piperacillin-tazobactam may pose also a problem [39]. The impact of piperacillin-tazobactam is probably reduced if GM is tested in the BAL fluid instead of sera, since the epithelial

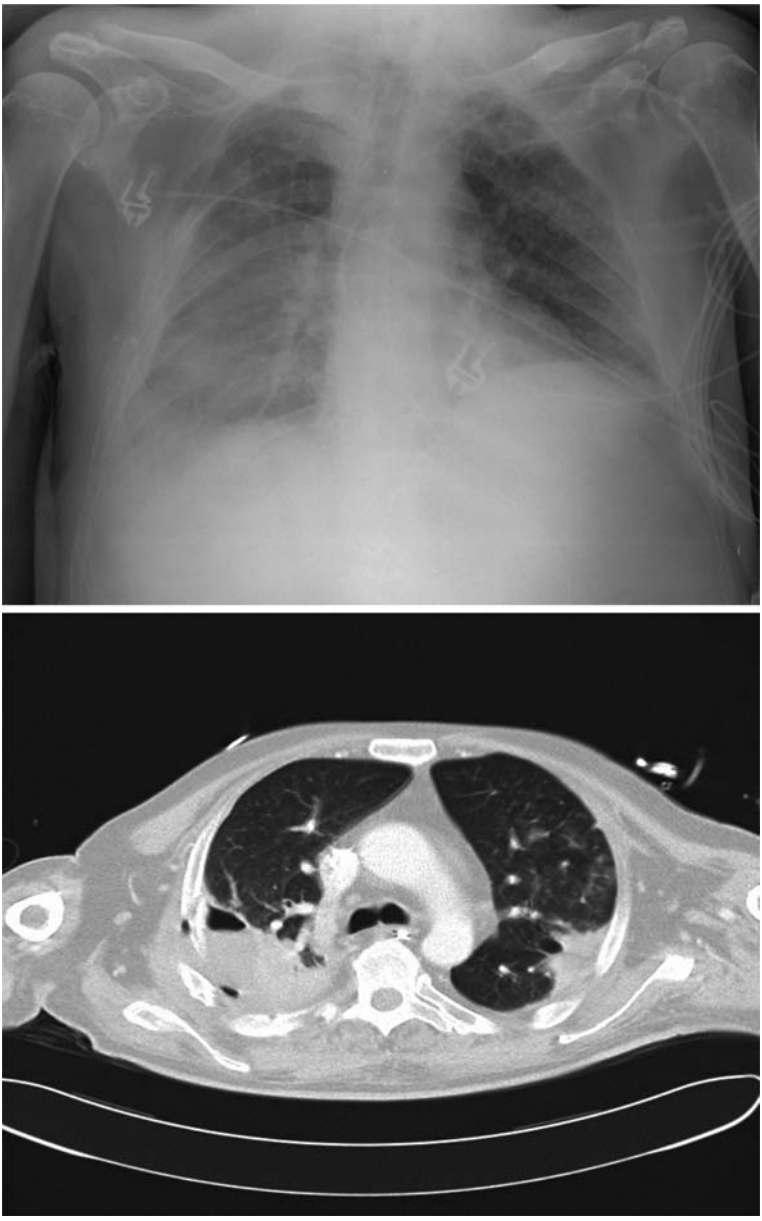

Fig. 3 Chest X-ray and CT scan from a patient on high dose steroids because of graft-versus-host disease 4 months after haematological stem cell transplantation for acute myeloid leukaemia. Chest X-ray reveals a right-sided pleural effusion and adjacent lung infiltrate. CT scan confirms a right sided complicated parapneumonic effusion, a mass filled partially with air between the 4th and 5th rib (with partial destruction of the bone) and a wedge-shaped infiltrate on the left side. In the culture specimen of the pleural fluid grew *Aspergillus fumigatus*. Findings are compatible with a bronchopleural fistula, secondary to rupture of a cavitating infiltrate and adjacent bone destruction

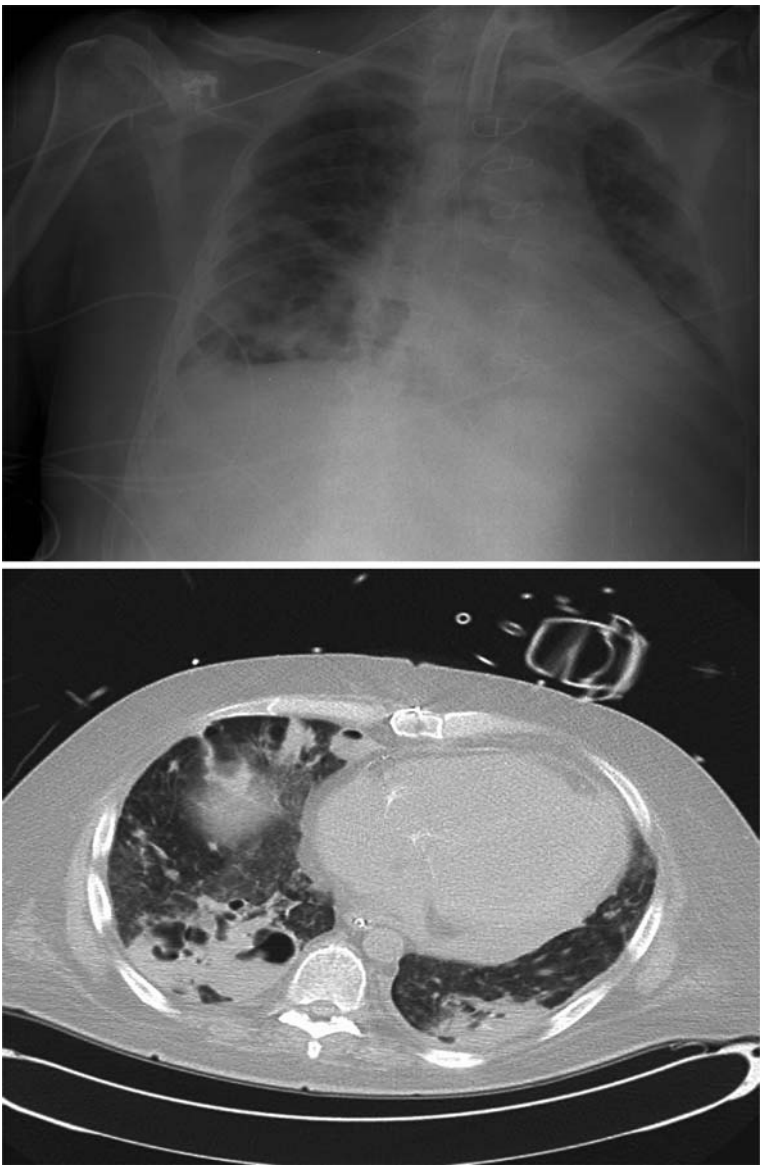

Fig. 4 Chest X-ray and CT scan 2 months post kidney transplantion for end stage diabetes. Bilateral lower lobe cavities with adjacent pleural effusion on the right side are seen. Transbronchial biopsy revealed *Aspergillus fumigatus*. Serum and BAL galactomannan was 0.1 and 5.7 ng/ml, respectively. Despite antifungal treatment, patient died of proven *Aspergillus* endocarditis of the tricuspid valve

lining fluid concentration of piperacillin is about half of the serum steady state concentrations [40]. Thus far, no prospective data on PCR detection are available in ICU patients, and the usefulness of combining different diagnostic test in these patients is also unknown.

6 Antifungals for the Treatment of IA in the ICU

Treatment options for IA are reviewed in the chapter by Dr Marr. In summary, amphotericin B deoxycholate was the mainstay for the treatment of IA for a long time. However, this formulation is infamous for the occurrence of serious side effects (e.g., nephrotoxicity, hypokalemia, and infusion-related reactions). These events often result in the use of suboptimal dosing regimens. Recently voriconazole, a derivative of fluconazole, has become the new standard of care for treating IA [41]. Caspofungin, micafungin, and anidulafungin belong to a new class of antifungal drugs, the echinocandins, which act by inhibiting the synthesis of ß-(1,3)-D-glucan in the fungal cell wall. Echinocandins display activity against *Aspergillus* species, as demonstrated in several salvage studies, but convincing first-line data are still lacking.

However, most patients recruited in these first- and second-line treatment studies suffered from an underlying haematological disorder or were transplant recipients. Patients with baseline characteristics that are commonly seen in ICU patients have usually been excluded from these studies, including those with liver function abnormalities, coagulation disorders, or renal dysfunction, and patients in need of advanced cardiovascular or pulmonary support including mechanical ventilation. Therefore, data on antifungal treatment in the ICU remain anecdotal.

In addition, many aspects of antifungal therapy that are relevant to the ICU population have not been sufficiently addressed in clinical studies, including the pharmacokinetic profile of antifungals in patients with underlying renal, hepatic and/or cardiac dysfunction; the dose-response relationship; and the best route of administration (oral, enteral, or parenteral). For instance, a recent study showed found nasogastric/gastric administration of voriconazole to be an independent predictor for undetectable voriconazole serum concentrations [42]. Other important questions that should be better addressed include the monitoring of drug-related toxicities, and especially drug interactions with frequently used “ICU-drugs”.

7 Future Directions

In an era of increased availability of new immunosuppressive drugs and better intensive care with prolonged survival, we can expect a continuing rise in the incidence of IA. Its occurrence in ICU usually entails a poor prognosis despite major recent improvements in the diagnosis and treatment of IA in patients with haematological diseases. Multicenter studies are warranted to explore the exact incidence of

IA in the ICU and to better delineate the difference between hospital-acquired, ICU-acquired and community-acquired aspergillosis. Evaluating the value of galactomannan, β-D-glucan and PCR in non-neutropenic critically ill patients in different sample types (and especially in respiratory samples) is urgently needed as well as a better delineation of the patient population at risk for IA in the broad group of critically ill patients. Finally, antifungal pharmacokinetics and pharmacodynamics and interactions with other drugs need to be explored more thoroughly. Meanwhile, all new diagnostic techniques and therapeutic measures must be validated against post-mortem findings, since only proven cases offer the most valuable information.

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Aspergillosis in Surgical Patients

Alessandro C. Pasqualotto

Abstract Most physicians will promptly consider the possibility of invasive aspergillosis in high-risk patients in the presence of an appropriate clinical syndrome. Aspergillosis will however only rarely be listed amongst the possible aetiologies of surgical site infections, although hundreds of cases have been published so far. This chapter reviews the current literature on post-operative aspergillosis. A few illustrative clinical cases are also shown. In most patients, the source was presumed to be airborne infection during the surgical procedure. Diagnosis relies on a high index of suspicion and treatment usually requires a combination of medical and surgical interventions. Prevention of post-operative aspergillosis requires special care with the ventilation system in the operating room.

Keywords Aortitis · Endocarditis · Neurosurgical infection · Osteomyelitis · Surgical site infection

Contents

A.C. Pasqualotto (✉)
Serviço de Controle de Infecção Hospitalar, Hospital Dom Vicente Scherer, Santa Casa-Complexo Hospitalar, Porto Alegre 90035-075, Brazil
e-mail: acpasqualotto@hotmail.com

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1 Introduction

Fungi belonging to the genus *Aspergillus* are ubiquitous and occur in soil, water and decaying vegetation [1, 2]. Like other filamentous fungi, *Aspergillus* species are primarily acquired from an inanimate reservoir, usually by the inhalation of small airborne spores [3]. Whilst nosocomial aspergillosis typically affects immunocompromised patients [4], post-operative aspergillosis occurs mainly in immunocompetent patients, some given steroids temporarily. Aspergillosis as a post-operative infection seems to have been first recognised in 1933, in a 40-year-old woman who was operated on for an abdominal tumour [5]. After 16 days the dressing was removed and an ulcer was observed. There were no systemic manifestations of the infection, and *A. niger* grew in the surgical dressings covered with a dark powder. The authors speculated that the contamination might have occurred in the operating room. They also commented about 2 previous similar cases in their medical centre, each of them exhibiting similar pustulation confined absolutely to the area of the gauze dressing and healing rapidly after local treatment with iodine and alcohol.

Post-operative aspergillosis seems to be an underappreciated problem. A literature review on the subject showed that >500 cases have been described to date [6]. Moreover, a recent study from Spain presented in abstract form [7] showed that post-operative aspergillosis represented 8.5% of all cases of aspergillosis occurring over a 10-year period. The purpose of this chapter is to review the main clinical presentations of aspergillosis affecting surgical patients. A few clinical cases are presented to better illustrate the challenges associated with these diseases. These cases were originated from Drs Pasqualotto and Denning personal knowledge.

2 Post-Operative *Aspergillus* Endocarditis, Aortitis and Vascular Prosthetic Infections

2.1 Illustrative Case (1)

Aortic valve replacement was recommended for a 70-year-old male patient with a history of coronary artery disease. The patient had a history of hypertension and hypercholesterolemia which were under treatment and congestive heart failure, New

York Heart Association class I. He had developed gout and he has been taking allopurinol. The patient underwent elective aortic valve replacement due to severe aortic stenosis with the use of a 22 mm aortic cadaveric homograft valve. His intra-operative course was smooth without any difficulty. His post-operative course was somewhat complicated with renal failure requiring hemodyalisis.

Four months after surgery he experienced fatigue and diminished physical endurance, which worsened in the following weeks. A transthoracic echocardiogram showed no remarkable findings. Seven months after surgery profuse diarrhoea started. Stool cultures were negative and viral diarrhoea was suspected. The patient was treated symptomatically. Minor alteration in vision prompted consultation with an ophthalmologist. The patient had previous been diagnosed with open angle glaucoma and had a history of peripheral retinal degeneration and a choroidal nevus in the left eye. Dilated funduscopic exam revealed a small flame haemorrhage just distal to an arteriovenous crossing inferior and slightly temporal to the optic nerve of the right eye. There was also a peripheral white centred haemorrhage in the nasal peripheral retina and some intra-retinal haemorrhaging in the far periphery in the right eye. A small dot haemorrhage was also observed in the temporal mid-peripheral retina on the left eye. The aetiology of the retinopathy was not elucidated.

One week later, the patient was hospitalised due to chills, low-grade fever, weakness, severe diarrhoea, and lethargy. His white count was $19{,}000 \times 10^6$/l and he appeared quite ill. A transthoracic echocardiogram revealed normal left ventricular size and function. There was mild aortic regurgitation and moderate mitral regurgitation and no vegetation was found. A transoesophageal echocardiogram however revealed a discrete focal mass/thickening on the ventricular surface of the non-coronary cusp of aortic valve measuring 0.8 cm, with associated moderate aortic insufficiency. Blood cultures were all negative which was attributable to prior use of antibiotics – he had received clindamycin 2 days prior to admission as endocarditis prophylaxis at the time of dental work, as he was allergic to penicillin. A working diagnosis of *Streptococcus viridans* endocarditis was made. Initial therapy in the hospital was vancomycin, gentamicin, and ceftriaxone. Vancomycin and gentamicin were suspended after blood cultures were negative. He was discharged on ceftriaxone and metronidazole (the patient was found to have *Clostridium difficile* toxin on his initial stool specimen).

Over the next week whilst on antibiotics he continued to deteriorate, and he was readmitted for congestive heart failure. At admission, he had $15{,}000 \times 10^6$ white cells and was febrile. He was very short of breath and unable to walk for more than a few steps. Antimicrobial therapy was changed to a combination of vancomycin, doxycycline, metronidazole, and ceftriaxone. On the 2nd hospital day, he developed dense right hemiparesis, dense aphasis, no gag reflex on the right, and was flaccid, with incontinence. He died three days later.

Autopsy showed massive cerebral haemorrhage and hemorrhagic infarction of the right cerebral hemisphere with a 10 cm cavitation containing blood clot. Examination of the thrombus taken from the right middle cerebral artery showed typical hyphae of *Aspergillus*. Sectioning of the endocarditis lesion also showed multiple hyphae characteristic of *Aspergillus*. The surface of the aortic valve homograft did

show some inflammatory cells and the vegetation was attached on the homograft. The vegetation had extensive inflammatory cells and hyphae. No other site of infection was found.

2.1.1 Key Messages for This Case

The difficulty in establishing the diagnosis of *Aspergillus* endocarditis is well known, since the fungus is infrequently isolated from blood cultures. Surgical exploration is usually required. The role of antibody testing and molecular tests on blood is not well studied. It is possible that the homograft was contaminated prior to the surgical procedure in this patient. This case illustrates that a low suspicion index in association with a difficult diagnosis are key components for a poor outcome. The patient received no antifungal therapy and the diagnosis of *Aspergillus* endocarditis was performed post-mortem only.

2.2 *Literature Review on* Aspergillus *Endocarditis Following Heart Surgery*

A recent literature review [6] found that ~190 cases of *Aspergillus* endocarditis and *Aspergillus* aortitis have been reported following surgical procedures. In addition, more than 20 cases of *Aspergillus* infections have followed vascular prosthetic surgeries. Additional notable features for these cases are: (i) absence of immunosuppression in almost all patients; (ii) a post-operative course consistent with culture-negative endocarditis; (iii) no evidence of bronchopulmonary aspergillosis; (iv) extensive local necrosis and invasiveness of fungi with propensity to late embolisation; (v) a very low rate of ante-mortem diagnosis (43.1%); and (vi) an extremely high mortality. Male gender was markedly predominant (~70%), which may be related to the higher prevalence of coronary and aortic valve surgery in males than in females. The aortic vale is involved in 60.5% of cases, followed in frequency by the mitral valve (30.6%). The incubation period is variable, typically from 1 to 6 months (median 2.7 months). Overall mortality was 92.7% [6]. Importantly, *Aspergillus* species are rarely isolated from blood cultures [8–12] and pre-autopsy diagnosis is usually associated with the examination of the valve or emboli to large peripheral arteries. Serology was positive in 2.4% of patients only – since most of these individuals are not immunosuppressed, this might be underestimated. Many of these studies were autopsy series. Mortality was also extremely high despite an ante-mortem diagnosis (83.0%) or receipt of surgical treatment (80.9%).

Most cases of *Aspergillus* endocarditis/aortitis in the literature were caused by *A. fumigatus* (58.7%). Other involved *Aspergillus* species include *A. terreus* (12.5%), *A. flavus* (11.2%), *A. niger* (11.2%), *A. glaucus* (2.5%), *A. clavatus* (1.2%), *A. ustus* (1.2%), and *A. sydowi* (1.2%) [6]. Most of the reports have associated these infections with contaminated grafts, contaminated sutures or intra-operative dispersion of spores. In one investigation, pigeon excreta and moss in the immediate vicinity of the ventilator intake port were found to harbour large numbers of *Aspergillus* spores [8]. Similarly, *Aspergillus* spp. were isolated from all areas of the surgical

theatre in another study [9], except in the room fitted with laminar air flow. Air conditioner cooling coils and pigeon droppings on the ledges outside the suite were found to harbour *Aspergillus* spores in large amounts.

Aspergillus graft infections usually occur on the suture line of a previous aortotomy [13]. Almost all cases were reported in immunocompetent males [6]. Definitive diagnostic procedures for these patients are generally culture of the excised aortic graft or the peripheral embolus and biopsy of the contiguously affected vertebral disk space [14–18]. Similar to endocarditis, almost all cases affect immunocompetent males [14–25]. The median time from the placement of the graft to diagnosis is 8 months, a longer period when compared to *Candida* graft infections (usually <6 weeks) [26]. Fever is absent in about one-half of the cases. Graft removal seems to be required for cure. In one report, in situ replacements or no excision of the infected graft was associated with recurrence and death in all cases, whereas all patients who underwent extra-anatomic bypass through a clean field survived [20]. No difference seems to exist in the clinical presentation of fungal and bacterial vascular prosthesis infections, particularly when *Staphylococcus epidermidis* is considered [27].

Diaz-Guerra et al. [28] reported the simultaneous isolation of one *A. flavus* isolate from the aortic prosthesis of a heart surgery patient and another 2 isolates recovered from a dual-reservoir cooler-heater used in the operating room where this patient was operated on. Genetic typing of these isolates revealed identical genotypes, indicating the nosocomial origin of the strain. Similar results were reported in another investigation [29].

3 Aspergillosis Following Neurosurgery

3.1 Illustrative Case (2)

A 16-year-old girl was submitted to neurosurgery for Chiari I malformation with placement of a bovine pericardium dural graft, through a posterior approach. Her post-operative course was marked by persistent headache, nausea, photophobia, and neck stiffness. When discharged from the hospital she was placed on a regimen of 2 mg dexamethasone four times daily.

One week after surgery she presented to the Emergency Room with aseptic meningitis and her steroid course was extended (3 mg two times daily). However, as a second steroid taper was attempted 2 weeks later, she had return of her symptoms and was hospitalised. A lumbar puncture revealed an opening pressure of 180 mm of water. Vancomycin and cefotaxime were started, and her dexamethasone dose was increased to 4 mg four times daily. Cerebrospinal fluid (CSF) cultures grew a few colonies of *A. fumigatus* but this was felt to be a culture contaminant. She continued to have severe headaches, and a wound exploration revealed that sutures had dehisced. Cultures again revealed *A. fumigatus*, and amphotericin B deoxycholate was started.

Two days later severe lethargy occurred and an emergent craniotomy was performed for evacuation of a wound haematoma, and establishment of external ventricular drainage for hydrocephalus. Cultures revealed again *A. fumigatus*. She continued on amphotericin B. Because of persistence of her symptoms, a third re-exploration of the wound was undertaken to remove the dural graft. *Aspergillus* was demonstrated in the surgical specimens and in culture. She died 2 months after the first surgical procedure. Autopsy studies revealed abundant hyphae in the origin of the basilar artery and bilateral vertebral arteries, with multifocal transmural destruction of arterial walls. No other focus of aspergillosis was found.

3.1.1 Key Messages for This Case

This tragic case illustrates the challenge inherent in the diagnosis of post-operative aspergillosis and the invasiveness of these fungi. Despite medical and surgical treatment, *A. fumigatus* involved arteries of the posterior circulation, with multifocal destruction of the artery walls. Continued therapy with steroids probably contributed to the patient demise. The importance of not considering positive cultures for *Aspergillus* species in this setting as contaminants should be emphasised. The hospital did an exhaustive investigation of the operating room and found no *Aspergillus* source of contamination, and there was no history of such contamination before this procedure. These findings suggested contamination of dural graft previous to the surgical procedure.

3.2 Illustrative Case (3)

A 43-year-old woman underwent laparoscopic cholecystectomy. She had a history of diabetes mellitus, alcoholic liver disease and haemochromatosis. There was no overt liver cirrhosis. Ten days after surgery, she became confused, dysphasic and eventually had tonic-clonic generalised seizures. CSF fluid analysis revealed 35 cells/mm^3 (90% lymphocytes), glucose 2.5 mg/dl and proteins 4 mg/dl. CSF culture was negative for bacteria. A CSF polymerase chain reaction (PCR) test was negative for tuberculosis, and computed tomography scan showed non-communicating hydrocephalus with ventriculitis. Since the diagnosis was unclear she received anti-tuberculosis therapy, meropenem, vancomycin, and dexamethasone, given for all or part of a 3 month period. Little clinical improvement occurred and an external ventricular drain was inserted in the left frontal lobe and in the parietal horn. Undertreated bacterial ventriculitis was suspected and the patient was treated with high dose meropenem. CSF cultures revealed coagulase-negative staphylococci (twice) and *Diphtheroid* spp. (on three occasions). CSF culture was negative for tuberculosis. She improved significantly and discharged back home.

One month later, she had generalised seizures. Oedema adjacent to fluid density area in left parietal lobe with mass effect and midline shift were seen at scan. She was taken to theatre for left temporal craniotomy and marsupialisation of trapped left temporal horn and biopsy, with antibiotic and dexamethasone cover. At a depth

of about 4 cm the trapped left temporal horn was opened to release clear CSF. The wall of the ventricule appeared necrotic and was biopsed before being sent for both histology and microbiology. No acid-fast bacilli were seen and there was no growth on the culture. Histological examination merely showed lymphocytic perivascular cuffing and polymorphs, the appearances in keeping with an active chronic meningoencephalitis. There was no evidence of fungi and the inflammatory infiltrate was not typical of tuberculosis. There was also no evidence of any neoplasm.

The drainage of this temporal horn was followed by a truly remarkable clinical improvement so that she became alert, responsive, independently mobile and broadly orientated. A few days later, she became less responsive and drowsy. A new scan revealed ring enhancing lesion in the left temporoparietal lobe again with considerable oedema and mid-line shift (Fig. 1). She was submitted to left pterion craniotomy, and heavy growth of *A. fumigatus* was retrieved from the abscess. Amphotericin B deoxycholate and 5-flucytosine were started. Apart from the fungus no other organisms were retrieved from the abscess. Antifungal therapy was changed voriconazole due to intolerance to amphotericin B (severe chills) and worsening disorientation. Voriconazole dosing (which varied from 300 mg to 100 mg twice daily) was guided by plasma concentrations as induction with rifampicin and carbamazepine, and alcoholic liver disease complicated her treatment. She had a good (if prolonged) recovery. Steroids were gradually reduced during the following months. The patients completed 9 months of therapy for tuberculosis and 9 months of voriconazole with no evidence of relapse over 6 months of follow-up.

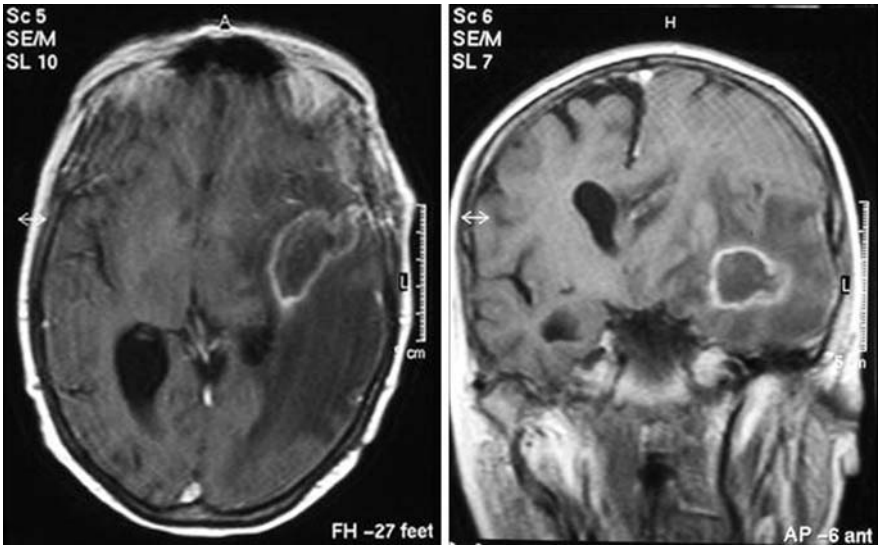

Fig. 1 (**A**) and (**B**). *Aspergillus* cerebral abscess in a patient with post-operative neurosurgical infection (T1 weighted, gadolinium enhanced magnetic resonance imaging)

3.2.1 Key Messages for This Case

Despite air filtration in the operating rooms, this patient apparently acquired an intra-operative infection at the time of the first neurosurgical intervention. This was probably accelerated in presentation by concurrent dexamethasone. Rapid diagnosis and optimisation of voriconazole dosing lead to a good outcome.

3.3 Literature Review on Aspergillosis After Neurosurgery

At least 25 cases of aspergillosis following neurosurgery have been published [30–48]. Previous treatment with steroids was mentioned in more than 50% of the studies, including patients receiving regimens of pulse therapy, high doses regimens during weeks to months, and even physiologic steroid replacement therapy [6]. All proven cases were caused by *A. fumigatus*. Interestingly some cases were described following trans-sphenoid surgery [32, 36, 40, 48], a situation in which previous colonisation of paranasal sinuses may have played a role. Different clinical presentations were described, including meningitis, CNS abscess, mycotic aneurisms and infarction. Median time from surgery to diagnosis was 3 months (ranging from <1 to >12 months).

In contrast to *Aspergillus* endocarditis, males were not over-represented in neurosurgical patients (44.0%). In addition, a higher ante-mortem diagnosis and a lower mortality rate are seen with the latter. Ante-mortem diagnosis was performed in 64.0% of patients, usually by abscess examination (36.0%) but also in some cases by CSF culture (20.0%). Mortality rate was 68.0%, which is "surprisingly low" when compared with cerebral aspergillosis occurring as a disseminated infection in the neutropenic patient – in which a mortality rate of ≥90% is usually observed. This may reflect three main factors: (i) imaging of the brain facilitates an earlier diagnosis; (ii) if diagnosed, immunosuppression with dexamethasone is stopped unlike in those with more permanent immunosuppression; and (iii) neurosurgical intervention is likely to have been more prompt.

4 *Aspergillus* Wound Infections and Mediastinitis

In a review of 33 cases of post-operative wound aspergillosis, the median interval between surgery and infection was only 17 days (range, <7 to 180 days) [4, 12, 49–67]. Moreover, many of these patients were immunosuppressed, particularly affecting patients submitted to liver and kidney transplantation and heart surgery. As this condition can progress to profound or disseminated infection, all patients need to be aggressively treated with combined medical therapy and surgical debridement. *A. flavus* is more common than *A. fumigatus* in many of these manifestations [68].

Chronic lung disease was the only independent risk factor for *A. fumigatus* sternal wound infection after open-heart surgery in a case-control study [69]. *A. fumigatus* grew at the same time from the bronchial washing of one patient, suggesting that colonised patients may be at increased risk for these infections.

In another study, a cluster of four cases of surgical and burn wound aspergillosis was associated with the outside packages of dressing supplies, which had become contaminated during hospital construction [70]. This resulted in patients with large exposed surface areas being inoculated directly with *Aspergillus* spores. When outbreaks of cutaneous aspergillosis occur wound dressings and tapes should be cultured [71, 72]. Cases of *A. flavus* sternal wound infection coinciding with hospital renovation activities were also reported [73].

Environmental studies conducted during an outbreak of *A. flavus* sternal wound infection [60] revealed massive fungal contamination in some areas of the surgical ward [74]. When strains from patients and from the environment were typed the same genotype was observed, proving the clonal single-source of the environmental contamination and the intra-operative acquisition of *A. flavus*. The importance of considering this condition in the differential diagnosis of slowly progressive but destructive wound infections and culture-negative mediastinitis after cardiac surgery is reinforced.

Aspergillus mediastinitis in surgical patients have been described in association with deep sternal wound infection [4, 60, 75], heart transplantation [76–78], aortitis [4, 10], and patch infection after repaired tetralogy of Fallot [79]. Two immunocompetent patients were also affected during an outbreak of aspergillosis related to contaminated air-handling system [4].

5 Aspergillosis Following Ophthalmological Surgeries

Ocular aspergillosis usually presents as keratitis and rarely as endophthalmitis [80–84]. Dr Phillip Thomas discusses these in detail elsewhere in this book. Post-operative ophthalmologic infections have been described following penetrating keratoplasty [85–87], radial keratotomy [88–91], excimer laser photorefractive keratectomy [92], laser-assisted in situ keratomileusis [93–96], pterygium excision [97, 98], hydroxyapatite orbital implant surgery [99], cataract surgery [81, 82, 100–109], scleral buckling procedures [110, 111], sutureless surgery [112, 113] and trabeculectomy [101, 114]. Sampling at the site of infection provides the best chance of obtaining a positive culture [93, 115–117]. Whilst some cases of *Aspergillus* endophthalmitis and keratitis have been associated with hospital construction [106, 118], others have been linked to contaminated irrigating fluids used during surgery [105]. The efficacy of the antifungal therapy depends to a great extent on the ability of the compound to penetrate the eye [116, 119].

6 Aspergillosis and Surgical Dental Procedures

A number of studies have reported a connection between endodontic treatment and non-invasive sinus aspergillosis, mainly in the presence of obturating pastes within the maxillary antrum [120–130]. Treatment is surgical mainly and consists in removal of all material and promotion of sinus aeration. Antifungal drugs should be used only if hyphal invasion is diagnosed.

7 Other (Rare) Clinical Presentations

Bronchial stump aspergillosis, i.e., *Aspergillus* infection of bronchial granulation tissue surrounding endobronchial sutures after pulmonary resection, is a rare clinical presentation of *Aspergillus* infection [131–137]. This condition is more common when silk thread is used as the suture material instead of stainless steel [138, 139], and should be considered in the differential diagnosis of haemoptysis, coughing, and dyspnoea occurring up to several years after lung operation, although asymptomatic presentation is possible [132–134, 139]. *Aspergillus* suture granuloma may simulate recurrent neoplastic disease in patients submitted to resection for pulmonary cancer [140]. Although no comparative studies exist, removal of the silk suture probably constitutes the key therapy for this condition.

Fungal infections of the soft tissue have been reported in association with prosthetic breast implants [141–144]. A 39-year-old healthy woman with bilateral inflatable silicone breast implants was colonised by *A. niger* as a possible consequence of massive operative contamination [142]. *A. flavus* was also cultured from a saline-filled implant removed from a healthy young patient who developed unilateral breast inflammation 4 years after surgery [143]. It has been shown that fungi can grow and reproduce in a saline-only environment, and their survival periods differ amongst the species [145–147]. Colonisation of a tissue expander with *A. niger* resulting in mechanical obstruction of the device has also been described [148].

A 64-year-old man had undergone hip replacement and was admitted after 9 years because of progressive hip pain [149]. *A. niger* grew from aspirated hip fluid, and he was treated with amphotericin B and prosthesis replacement. Two cases of aspergillosis following laminectomy were also reported in this paper. In another report, *A. fumigatus* spondylodiscitis occurred 2.5 months after lumbar disc surgery, and the patient was treated with itraconazole in combination with surgical debridement [150]. Other cases of post-operative aspergillosis following lumbar surgery have also been reported [151–153].

In a prospective study of 658 consecutive total hip arthroplasty procedures in which cultures were taken at surgery, *Aspergillus* was recovered in 4.1% of cases. *Aspergillus* was cultivated from the air in the operation room in this study as well [154]. In another paper, a 22-year-old man developed *Aspergillus* osteomyelitis after a closed fracture of the femur and subsequent operation [155]. The first case of aspergillosis complicating total knee arthroplasty was described in an 80-year-old man receiving chronic corticotherapy [156]. This patient presented with a popliteal cyst 27 months after surgery and was cured with a 12-week course of amphotericin B in combination with surgical debridement and prosthesis removal. Other similar cases have also been published [157, 158].

Multiple abdominal visceral infection by *A. fumigatus* occurred in a 37-year-old woman after laparostomy for Crohn's disease [159]. She was successfully treated with a 4-week course of amphotericin B. Sampling of air from the patient's environment yielded one isolate of *A. fumigatus* that matched the patient's isolates, suggesting that this patient acquired her infection from air in the intensive care unit. Peritoneal aspergillosis has also been reported following laparotomy in

non-transplant patients [4, 160], as well as *Aspergillus* cholangitis complicating portoenterostomy for a biliary atresia [161]. One case of *A. flavus* renal pelvis infection following a pyelolithotomy was described, with *Aspergillus* around suture material [162]. Post-operative cases of wound infection [12, 62, 63, 65–67, 163], peritonitis [163, 164], and renal allograft infection [165] have also been described following solid-organ transplantation. Although not always apparent, it is possible that some of these patients had occult foci from which the *Aspergillus* may have disseminated [6].

8 Diagnosis

Due to the low frequency at which post-operative *Aspergillus* infections occur, diagnostic strategies have not been properly evaluated for these patients. Accordingly, post-operative aspergillosis was not considered neither in the updated EORTC/MSG criteria for the diagnosis of invasive fungal diseases [166] nor in the latest IDSA guidelines for the treatment of aspergillosis [167]. Diagnosis relies on a proper clinical suspicion. This includes culture-negative cases of endocarditis, particularly in the presence of large vegetation. Physicians should not expect patients to manifest concomitant pulmonary aspergillosis. All biopsied samples should be submitted to both histopathology and microscopy/culture, and *Aspergillus* species should not be disregarded as laboratory contaminants. Since most patients with *Aspergillus* surgical infections are immunocompetent, *Aspergillus* IgG testing may have a role in the diagnosis. Galactomannan testing has also not been adequately evaluated in this context. In a review of 7 cases of post-operative aspergillosis [7], galactomannan was found to be positive in only 2 cases (endotipsitis with endocarditis and mediastinitis). A better diagnostic test is ultimately required, in order to allow for an early diagnosis of the diseases.

9 Treatment

The optimal treatment of *Aspergillus* surgical infections has also not been specifically studied. Survival seems to depend on the excision of the infected tissue; in cases involving prosthesis, the new prosthesis should be placed in a non-infected field. Concomitant use of a systemic antifungal agent is also crucial. Although there are no data favouring any one antifungal drug, the response rates of voriconazole in immunocompromised patients were superior to amphotericin B deoxycholate [168]. Caspofungin and other echinocandins may also be useful. Longer-term oral extension with itraconazole or voriconazole is appropriate for some patients. The duration of therapy is unknown and is likely to differ between patients, but once as much infected tissue as possible has been removed, any immunocompromising factors such as steroid therapy minimised, and treatment for 3 months beyond the last evidence of active disease may be appropriate [6].

10 Prevention

As discussed along this chapter the main sources for post-operative *Aspergillus* infections are contaminated grafts, contaminated sutures and intra-operative dispersion of *Aspergillus* conidia, even though only a few studies have undoubtedly linked the acquisition of aspergillosis with the surgical operation. Preventing post-operative aspergillosis would therefore require proper disinfection and storage of surgical devices and grafts. Another important step would be reducing *Aspergillus* conidia concentration in the surgical theatre. Care should also be taken in order to minimise wound exposure to *Aspergillus* conidia. Patients colonised with *Aspergillus* species may be at higher risk for a post-surgical *Aspergillus* infection, so a higher suspicion index is required for these.

Operating room air may contain microorganisms, dust, aerosol, lint, skin squamous epithelial cells, and respiratory droplets. The microbial level in operating room air is directly proportional to the number of people moving in the room [169]. Therefore, efforts should be made to minimise personnel traffic during operations. In addition, operating rooms should be maintained at positive pressure with respect to corridors and adjacent areas [170].

Most modern operating theatres have conventional plenum ventilation with filtered air, using filters with an efficiency of 80–95% to remove airborne particles of $\geq$5 μm [315]. However, these filters are not sufficient to contain *Aspergillus* spores (2.5–3.0 μm). Laminar airflow is designed to move particle-free air over the aseptic operating field at a uniform velocity (0.3–0.5 m/s), sweeping away particles in its path. This airflow can be directed vertically or horizontally, and recirculated air is passed through a high efficiency particulate air (HEPA) filter. Laminar airflow systems with HEPA filter which remove airborne particles of $\geq$0.3 μm with 99.97% efficiency. These systems are generally used for orthopaedic and other implant surgery [171], as well as in protective environments for high-risk patients [172–177]. In surgical site infections, it has been suggested that laminar airflow and ultraviolet (UV) germicidal irradiation as adjunct measures may reduce infection risk for certain operations. Neither laminar airflow nor UV light, however, has been conclusively shown to decrease overall risk for these infections [176]. Although theoretically attractive, the use of these systems cannot, at this moment, be recommended for the prophylaxis of post-operative aspergillosis. Since no study has been systematically designed to evaluate this subject, it is not known if the universal use of these technologies will decrease the incidence of these infrequent infections. Furthermore, these strategies are limited by the expense of both constructing and maintaining these systems and by the lack of consensus about the level of airborne conidia at which the risk can be numerically defined for aspergillosis [177]. Moreover, data demonstrating a survival benefit for patients submitted to these systems are lacking [178].

Since outbreaks of post-operative aspergillosis have occasionally occurred in units with HEPA filters [4], periodic maintenance of ventilation systems seems to be warranted [9, 176, 179]. These incidents were due to contamination of insulation in variable airflow volume units that had deteriorated after becoming wet. For

optimal performance, filters require monitoring and replacement in accordance with the manufacturer's recommendations and standard preventive maintenance practices. Since fungi can inhabit water distribution systems and may cause nosocomial infection, tap water should not be used on surgical wounds. Larger controlled studies, however, are needed to determine the role of water in the transmission of aspergillosis [2, 3].

Finally, microbiological sampling has its place in the investigation of epidemics, validation of changes in products and procedures in the maintenance of operating theatres (cleaning, disinfection, and ventilation) and education [178].

11 Conclusions

Post-operative aspergillosis is relatively common compared with many other manifestations of invasive aspergillosis and rarely affects the lungs. Mortality is high in non-cutaneous infections. Several different organs and surgical procedures may be involved, depending on the surgery performed. These infections are usually indolent, in some cases occurring several months after surgery. In most patients, the source of the conidia seems to have originated from the air during the surgical procedure; contamination from paranasal sinuses, bronchopulmonary lesions, haematogenous dissemination, and contaminated grafts is also possible. Infected patients should be treated aggressively with combined medical and surgical therapy. Prevention of these uncommon infections should include special care with the ventilation system in the operating room.

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Invasive Aspergillosis in Chronic Granulomatous Disease

Brahm H. Segal

Abstract Chronic granulomatous disease (CGD) is a rare inherited disorder of the NADPH oxidase complex in which phagocytes are defective in generating superoxide anion. NADPH oxidase activation leads to release of sequestered neutrophil granular proteases, which are likely the principal antimicrobial effectors. CGD is characterized by recurrent life-threatening bacterial and fungal infections and by inflammatory complications such as wound dehiscence, obstructive granulomata of the genitourinary tract, and inflammatory bowel disease. Despite routine use of interferon-gamma prophylaxis, fungal infections have remained a persistent problem with an incidence of 0.1 fungal infections per patient year. Invasive pulmonary aspergillosis in CGD manifests with neutrophils and lymphohistiocytic inflammation; hyphal angioinvasion is not observed, indicating that NADPH oxidase-independent pathways appear to be adequate to protect against vascular invasive disease. Chronic prophylaxis with a mould-active agent is standard of care in CGD. "Mulch pneumonitis" is characterized by rapid onset life- pulmonary inflammation following mould exposure, and treated with systemic antifungals and prolonged corticosteroids. Haematopoietic stem cell transplantation is curative in CGD, but transplant-related mortality from GVHD and infectious complications is substantial, particularly in cases of donor/recipient HLA-antigen disparity. Since CGD is a disorder of myeloid stem cells, gene therapy is an attractive option, but maintaining stable population of myeloid gene-corrected cells has been a persistent challenge. The strategy of non-myeloablative conditioning followed by gene therapy facilitates the expansion of gene-corrected cells. Studies of experimental aspergillosis in CGD point to defective tryptophan metabolism as a contributing factor to impaired host defence and increased lung inflammation in CGD.

Keywords Aspergillosis · CGD · Chronic granulomatous disease · NADPH

B.H. Segal (✉)
Division of Infectious Diseases, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY, USA
e-mail: brahm.segal@roswellpark.org

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Contents

1 Clinical Manifestations, Genetics, and Diagnosis of Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is an inherited disorder of the NADPH oxidase complex in which phagocytes are defective in generating the microbicidal reactive oxidant superoxide anion and its metabolites, hydrogen peroxide, hydroxyl anion, and hypohalous acid. As a result of the defect in this key host defence pathway, CGD patients suffer from recurrent life-threatening bacterial and fungal infections by a subset of catalase producing pathogens [1–3]. A national database describing the spectrum of infections in 368 patients with CGD has been created [4]. The most common pathogens include: *Aspergillus* species (pneumonia, osteomyelitis), *Staphylococcus aureus* (suppurative adenitis, subcutaneous infections, and liver abscess), *Serratia marcescens* (osteomyelitis, pneumonia), *Nocardia* species (pneumonia), and *Burkholderia cepacia* (pneumonia and sepsis). CGD is also characterized by abnormally exuberant inflammatory responses leading to granuloma formation, such as granulomatous enteritis, genitourinary obstruction, poor wound healing, and dehiscence. CGD affects approximately 1 in 200,000 persons.

Chronic granulomatous disease results from mutations in genes encoding gp91^{phox}, p22^{phox}, p47^{phox}, or p67^{phox}. X-linked CGD results from defects in gp91^{phox}, and accounts for ∼65% of cases of CGD. Female X-CGD carriers usually have a dual population of circulating neutrophils based on random X-chromosome inactivation (lyonization), in which one population has normal NADPH oxidase activity and the second is defective. X-CGD carriers usually have no increased susceptibility to infection, with the exception of cases of extreme skewing in favor of NADPH oxidase-defective cells, such that the proportion of normal circulating neutrophils is below 10% [5, 6]. The p22^{phox} (5%), p47^{phox} (25%), and p67^{phox} (5%) forms of CGD are inherited in an autosomal recessive pattern.

Laboratory methods used to diagnose CGD rely on demonstration of defective NADPH oxidase function. In the nitroblue tetrazolium test (NBT), superoxide anion reduces the tetrazolium compound to a formozan precipitate, which is detected microscopically. NBT tests may yield false normal results in cases of $p47^{phox-/-}$ and variant X-CGD, in which low levels of reactive oxidants are produced [7, 8]. We use fluorescent cytometry employing the dihydrorhodamine 123 probe, an ultrasensitive method for detecting intracellular H_2O_2 production [9].

2 NADPH Oxidase

The phagocyte NADPH oxidase functions to rapidly generate superoxide anion by transferring electrons from NADPH to molecular O_2 in response to physiologic stimuli such as phagocytosis. The cytochrome, composed of $gp91^{phox}$ (phox, *ph*agocyte *ox*idase) and $p22^{phox}$, is embedded in membranes (Fig. 1). In neutrophils, approximately 85% of the cytochrome is in the membranes of specific granules or gelatinase-containing granules, and the remainder is present in the plasma membrane and in secretory granules [10, 11]. The NADPH binding site is located on the cytoplasmic side of membranes. Upon activation of the oxidase, the cytoplasmic subunits $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ appear to translocate en-bloc to the membrane-bound cytochrome. Activation of *rac*, a member of the low molecular weight GTP-binding proteins, and translocation of *rac* to the membrane-bound cytochrome are also critical for NADPH oxidase activation [12, 13]. NADPH is oxidized to $NADP^+$, and electrons are transported down a reducing potential gradient to FAD and then possibly to 2 non-identical heme groups. On the vacuolar or extracellular side of the membrane, the final step in the electron transport chain occurs when oxygen accepts an electron and is converted to superoxide anion. The net equation involves the reduction of 2 molecules of O_2 to 2 molecules of superoxide anion (O^-_2) at the expense of 1 molecule of NADPH.

Superoxide, a relatively weak microbicidal oxidant, is metabolised to the more toxic hydrogen peroxide by superoxide dismutase. Hydrogen peroxide can in turn be converted to hypohalous acid by myeloperoxidase, and to hydroxyl anion. Possible molecular targets of these species include genomic DNA, electron transport, and sulfhydryl groups of proteins and nonproteins.

In the past, neutrophil host defence pathways were divided into NADPH oxidase and granular proteins, without the knowledge that these two pathways were linked. Reeves et al. [14] showed that activation of the NADPH oxidase in neutrophils leads to an influx of reactive oxidant species into the endocytic vacuole, resulting in an accumulation of anionic charge. To maintain electrogenic neutrality, K+ ions cross the membrane in a pH-dependent manner. The rise in ionic strength leads to the release of cationic granule proteins, including elastase and cathepsin G, which are bound to the anionic proteoglycan matrix in the inactivated state within primary (azurophilic) granules. Knockout mice deficient in the cationic granule proteins

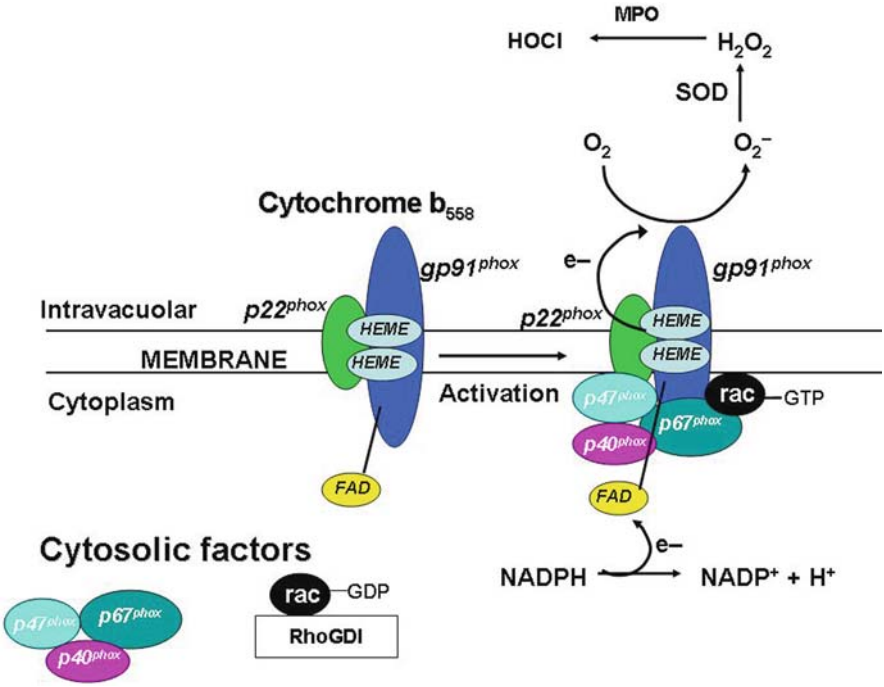

Fig. 1 Schematic of NADPH oxidase activation. NADPH oxidase is composed of a membrane-bound flavocytochrome and cytosolic constituents. Activation requires translocation of the cytosolic phox constituents and rac to the membrane; this is accompanied by phosporylation events and conformational changes. Following oxidation of NADPH, electrons are transported via flavine adenine dinucleotide (FAD) and heme proteins, across the membrane, where molecular oxygen is converted to superoxide anion and downstream reactive oxidants. In neutrophils, NADPH oxidase activation is accompanied by activation of antimicrobial proteases sequestered in primary granules. MPO, myeloperoxidase; SOD, superoxide dismutase

recapitulate some of the features of the CGD phenotype regarding susceptibility to experimental bacterial and fungal infections [15]. Elastase and cathepsin G had non-redundant roles in protecting against systemic aspergillosis [15]. Taken together, these studies point to activation of preformed granular proteases as an important mechanism for NADPH oxidase-mediated antimicrobial host defence.

Fungal cell wall constituents activate NADPH oxidase through ligation of pathogen recognition receptors. Dectin-1, a receptor and immunomodulator of beta-glucans, is likely to be important for NADPH oxidase activation following exposure to zymosan (comprised principally of particulate Saccharomyces-derived cell wall β-glucans) [16]. The requirement for NADPH oxidase in host defence against *A. fumigatus* appears to be stage-specific. NADPH oxidase is dispensable regarding neutrophil-mediated [17] and macrophage-mediated [18] conidiocidal activity. However, NADPH oxidase is required for neutrophil-mediated damage against *Aspergillus* hyphae [17, 19].

3 Invasive Aspergillosis in CGD

Invasive aspergillosis is the most important cause of mortality in CGD [20, 21]. CGD patients are susceptible to a broad spectrum of opportunistic filamentous fungi, but *Aspergillus* infection is by far the most common. Despite the routine use of interferon-gamma prophylaxis (see below), fungal infections have remained a persistent problem with an incidence of 0.1 fungal infections per patient year [4]. Patients with X-linked CGD appear to be at increased risk for invasive aspergillosis compared with the autosomal recessive forms [22, 23]. Invasive aspergillosis may be the first manifestation of CGD. Infection usually manifests within the first 2 decades of life [22]. Invasive aspergillosis in CGD patients during the neonatal period is occasionally observed [24, 25]. Very rarely, invasive aspergillosis may occur in a female carrier of X-linked CGD in whom the random process of lyonization (X-chromosome inactivation) has led to a skewing of the circulating normal neutrophil population to less than 10% oxidant competent [5].

A. fumigatus and *A. nidulans* are the most common *Aspergillus* species in CGD. *A. nidulans* is a rare pathogen in most patient populations with quantitative or qualitative neutrophil defects. We reviewed all cases in which *A. nidulans* was isolated from patients at the National Institutes of Health (Bethesda, USA) between 1976 and 1997 [22]. *A. nidulans* infection occurred in 6 patients with CGD, but was not a pathogen in any other patient group. *A. fumigatus* was a more common pathogen in CGD (n=17 cases), but *A. nidulans* was more virulent. *A. nidulans* was significantly more likely to result in death compared with *A. fumigatus* (3 of 6 versus 1 of 17 cases, respectively), to involve adjacent bone, and to cause disseminated disease. Patients with *A. nidulans* received longer courses of amphotericin B therapy than patients with *A. fumigatus*, and were treated with surgery more often. In contrast to *A. fumigatus*, *A. nidulans* was generally refractory to intensive antifungal therapy, suggesting that early surgery may be important. However, the need for early resection of pulmonary lesions will need to be reevaluated with the availability of 2nd generation antifungal triazoles (see below).

CGD patients often do not have typical symptoms and signs of infection [1]. Even in the setting of life threatening infections, CGD patients may be asymptomatic or have mild non-specific symptoms. Fever and leukocytosis may be absent, and an elevated sedimentation rate may be the only abnormal laboratory test [1]. In a review of aspergillosis in CGD patients at the NIH, one third of patients were asymptomatic at diagnosis and only ~20% were febrile [22]. In many of these patients, a pulmonary infiltrate on routine screening chest x-ray or computed tomography (CT) scan was the first indication of an infection. The white blood cell count was $\leq$10,000/μl in 13/23 cases and the sedimentation rate was $\leq$40 mm/h in 9/20 cases.

Patients with CGD may have concurrent bacterial and fungal infections [26]. It is therefore essential to establish a culture diagnosis when feasible prior to initiating antimicrobial therapy. Biopsy material should be submitted for pathology as well as bacterial and fungal culture, including *Nocardia*. In addition, recrudescent fungal infection can occur after initial control of infection had been achieved – a feature that is common with *A. nidulans* [22]. Thus, a high level of vigilance in searching for

infection is necessary in caring for CGD patients. Frequent radiographic evaluation (e.g., chest radiographs during routine clinic visits and CT scans in patients with fever or focal signs) is critical to making early diagnoses.

Pulmonary infection is the most common site of invasive aspergillosis in CGD. The radiographic appearance is usually an infiltrate, mass, or nodule, and multiple lesions may be present. In contrast to patients with chemotherapy-induced neutropenia, hyphal angioinvasion is not a feature of CGD (Fig. 2). Thus, a halo sign (reflecting angioinvasion with surrounding haemorrhage), cavitated lesions, and pulmonary infarction are not typical features of CGD. Serum galactomannan is not elevated in experimental pulmonary aspergillosis in CGD mice [27] and appears to be an insensitive diagnostic marker of aspergillosis in CGD patients [28].

Invasive pulmonary aspergillosis can cause a chronic infection in CGD. The terms, "chronic septic granulomatosis" and "microgranulomatous aspergillosis" have been used to describe these lesions [29]. A chronic diffuse nodular pneumonia may also occur [30]. Lesions typically contain a mixed inflammatory cell response consisting of neutrophils, histiocytes, and lymphocytes. Hyphae may be observed within these lesions. "Mulch pneumonitis" is a recently described life-threatening complication in CGD characterized by rapid onset life-threatening pulmonary inflammation following mould exposure, and treated with systemic antifungals and prolonged systemic corticosteroids [31].

Knockout mouse models further shed light on the pathogenesis of aspergillosis in CGD. Even low virulent mutant strains of *A. nidulans* caused mortality in pulmonary aspergillosis in CGD mice due to excessive inflammation [32]. In addition, intratracheal administration of heat-killed hyphae elicited mild self-limited inflammation in wild-type mice, but robust and persistent inflammation in CGD mice [33]. Intratracheal zymosan was also capable of eliciting progressive pyogranulomata in

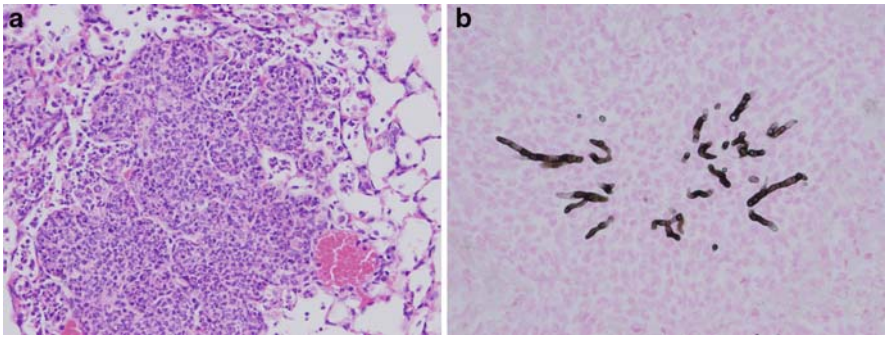

Fig. 2 Invasive aspergillosis in the p47$^{phox-/-}$ mouse model of CGD. (**a**) Robust inflammatory responses characterized by a central neutrophilic infiltrate surrounded by histiocytes and lymphocytes (Haemotoxylin and Eosin, 200×). (**b**) Vascular invasion was not observed (note the intact blood vessel). Invasive hyphae are evident on silver staining (400×). The lack of hyphal angioinvasion in CGD suggests that while NADPH oxidase is required to defend against parenchymal invasive aspergillosis in the lung, NADPH oxidase-independent pathways may be adequate to defend against vascular invasive disease. These photomicrographs were previously published (Fig. 4 in reference [27])

CGD mice that resembled histologically the finding in experimental aspergillosis (unpublished data). These findings emphasize the unique pathophysiologic features of aspergillosis in CGD mice that must be considered in studies of immunotherapy.

4 Therapy for Invasive Aspergillosis

The Infectious Diseases Society of America (IDSA) guidelines appropriately recommend voriconazole as first-line therapy for invasive aspergillosis [34]. This recommendation is based on the landmark study by Herbrecht et al. [35]. showing superior outcomes and improved overall survival in voriconazole compared with amphotericin B recipients in a randomized trial of primary therapy for invasive aspergillosis. This study principally enrolled patients with haematological malignancies and allogeneic stem cell transplant recipients. Since it is not feasible to conduct randomised trials of therapy for invasive aspergillosis in CGD patients or other rare disorders of host defence, it is reasonable to extrapolate the results of this pivotal randomised study to all patients with invasive aspergillosis, regardless of the underlying host defence deficit. The data on voriconazole therapy in invasive mould infections in paediatric patients with CGD is limited, but the frequency of successful outcomes appears to be at least as good as patients with iatrogenic immunosuppression [36].

The data on salvage therapy for refractory aspergillosis in CGD is limited. The IDSA guidelines suggest a number of options for refractory aspergillosis (not specific for CGD patients) that include use of posaconazole or switch in class to a lipid formulation of amphotericin B or echinocandin [34]. Our limited experience showed that posaconazole was effective as salvage therapy for invasive mould diseases in CGD patients, including voriconazole failures [37]. Granulocyte transfusions are discussed below.

5 Prophylaxis Against Fungal Infections

In a European prospective open label study of itraconazole prophylaxis, the rate of *Aspergillus* infections was reduced compared with historical controls, and the drug was well tolerated [21]. A randomised, double-blind, placebo-controlled cross over study of itraconazole prophylaxis has recently been completed at the NIH [38]. Thirty-nine patients over 5 years of age were enrolled. One serious fungal infection occurred in itraconazole recipients versus 7 cases in the placebo group. No significant toxicity was observed. This study supports the routine use of itraconazole prophylaxis in CGD.

This study employed itraconazole capsules, which have erratic bioavailability. In approximately half of the plasma samples drawn during itraconazole therapy, the level was in the range (<0.3 μg/ml) that is considered sub-therapeutic. The cyclodextrin (oral suspension) formulation of itraconazole has enhanced and more reliable bioavailability. Regardless of which oral formulation is used, monitoring of

plasma levels is advised (trough level of at least 0.25 μg/ml is advised) based on the experience using itraconazole prophylaxis in patients with haematologic malignancies and haematopoietic transplantation in which therapeutic levels of itraconazole were protective [39, 40].

6 Interferon-γ

Interferon (IFN)- γ is a macrophage activating factor critical in host defence against intracellular infections such as *Leishmania* and *Mycobacteria* species in humans and mice [41]. In clinical trials in patients with cancer [42] and with lepromatous leprosy [43], IFN-γ increased H_2O_2 generation in circulating monocytes, providing a rationale for evaluating IFN-γ in CGD. In preliminary studies, addition of recombinant IFN-γ in vitro to phagocytes from some, but not all, CGD patients augmented superoxide production [44, 45]. Augmentation of superoxide production was observed in phagocytes from cytochrome positive X-linked and the majority of autosomal recessive CGD patients, as well as in ∼30% of X-linked cytochrome negative patients. In a study of patients with cytochrome positive X-linked CGD, two consecutive subcutaneous injections of IFN-γ resulted in a 5- to 10-fold increase in superoxide production from granulocytes and monocytes [46]. The effect lasted for more than 2 weeks and was associated with an increase in bactericidal activity. Rex et al. [47]. showed that administration of IFN-γ to CGD patients augmented the in vitro ability of CGD neutrophils to damage *Aspergillus* hyphae.

Given these promising results, a multicenter, randomised, double blinded, placebo-controlled study of prophylactic recombinant IFN-γ (50 μg/m^2 subcutaneously thrice weekly) was conducted [48]. One hundred and twenty eight CGD patients (67% X-linked and 33% autosomal recessive) were enrolled. Prophylactic IFN-γ reduced the number of serious infections by over 70%, and was beneficial in both the X-linked and autosomal recessive forms of CGD. In contrast to earlier studies, no significant differences occurred between the IFN-γ and placebo groups with regard to reactive oxidant generation, cytochrome B expression, or in vitro bacterial killing. Subsequent studies in CGD patients [49, 50] and CGD mice [51] confirmed that IFN-γ did not improve NADPH oxidase function or increase levels of its constituent proteins.

The benefit of IFN-γ prophylaxis in CGD likely occurs through augmentation of oxidant-independent antimicrobial pathways. For example IFN-γ increases TNF-α; production, tryptophan metabolism, expression of Fc-γR1 receptors on phagocytes, B_2-integrin expression on monocytes, granule protein synthesis, and MHC-expression [41, 52].

7 Granulocyte Transfusions

Granulocyte transfusion in CGD is supported by the principle that a small number of normal neutrophils may be able to complement the oxidative defect in a large number of CGD phagocytes. In vitro, a small proportion of normal neutrophils mixed

with CGD neutrophils kill *A. fumigatus* hyphae as well as a population consisting entirely of normal neutrophils [53]. The likely explanation is that H_2O_2 generated by the minority of normal neutrophils can diffuse into CGD neutrophils and provide the necessary reagent to generate hypohalous acid and hydroxyl anion [53, 54]. Transfused granulocytes retain respiratory burst activity and appear to traffic normally based on their recovery from sites of infection (e.g. wound drainage sites from liver abscesses), from mouth rinse preparations, from bronchoalveolar lavage, and from neutrophil exudate in experimental skin windows [55–57].

Granulocyte transfusions have been used in life threatening infections and in infections refractory to antimicrobial and surgical treatment in CGD patients. Granulocyte transfusions are generally well tolerated, but adverse effects include fevers, development of leukoagglutinins leading to rapid loss of transfused granulocytes, and rarely, pulmonary leukostasis. The likelihood of pulmonary leukostasis may be increased if amphotericin B and granulocytes are administered concomitantly [58]; therefore granulocyte transfusions and amphotericin B should be administered several hours apart. One concern regarding granulocyte transfusions is alloimmunization, which is of theoretical concern for patients under consideration for bone marrow transplantation.

8 Haematological Stem Cell Transplantation

Because CGD results from a defect in haematopoietic stem cells, stem cell transplantation is a rational option to establish a stable population of normal myeloid progenitors. Horwitz et al. [59] evaluated peripheral stem cell transplantation using a non-myeloablative conditioning regimen in 10 patients with CGD. A matched related T-cell depleted allograft was used to reduce the risk of severe graft versus host disease (GVHD). After a median follow-up of 17 months the proportion of donor neutrophils in the circulation in 8 of the 10 patients was 33–100%, a level that can be expected to be protective. Significant GVHD occurred in three of four adults with engraftment, but in none of the five children. Three of 10 patients died. Given the risks of this procedure, this non-myeloablative transplant regimen should probably be reserved outside of research protocols to CGD patients who have had recurrent serious infections despite antibiotic, antifungal, and IFN-g prophylaxis and who have HLA-matched donor siblings.

Haematopoietic stem cell transplantation has been successfully employed as a therapeutic measure in a few published cases of CGD patients with active refractory *Aspergillus* infection [60, 61]. The rationale is that a stable population of circulating neutrophils achieved through transplantation may cure the fungal infection. This option should only be considered as a last resort given that life-threatening fungal infections may progress during neutropenia and in the setting of GVHD following transplantation. In general, transplantation should only be performed in the absence of active infection.

9 Gene Therapy

CGD is an ideal candidate disease for haematopoietic stem cell gene therapy for the following reasons [62]: (i) Engraftment of a stable population of normal myeloid stem cells is curative in CGD, as illustrated by successful bone marrow transplantation; (ii) The genes encoding the subunits of NADPH oxidase have been identified, cloned, and sequenced; (iii) Transfer of the relevant gene corrected the NADPH oxidase defect in myeloid and EBV-transformed B cell lines [63–68] and in primary monocytes [69] from CGD patients, confirming that expression of the missing protein was sufficient to restore oxidase function in intact cells; (iv) Reconstitution of phagocyte NADPH oxidase activity has been achieved in the X-linked and autosomal recessive forms of CGD by transduction of bone marrow [70, 71] and peripheral blood stem cell progenitors [72–74] with retroviral vectors containing the relevant gene; (v) In the X-linked [75] and p47$^{phox-/-}$ [76] mouse models of CGD, genetic correction of NADPH oxidase has been achieved in vivo with stem cell gene therapy and gene-corrected mice have increased resistance to experimental infection; (vi) Based on the experience in X-linked CGD carriers, only a small proportion of normal phagocytes are required for normal host defence.

In X-linked CGD mice, correction of the host defect either by bone marrow transplantation or retroviral-mediated gene transfer led to protection against challenge by *Aspergillus* and bacterial pathogens [75, 77]. Host defence was improved even with a limited proportion of gene-corrected neutrophils. Therefore, maintenance of a chimeric state in which there is a small population of circulating gene-corrected neutrophils may be of benefit in invasive aspergillosis in CGD patients.

The goal of gene therapy in CGD is to achieve a stable population of gene corrected myeloid precursors, which give rise to a biologically significant number of peripheral phagocytes. Gene therapy in patients with p47$^{phox-/-}$ CGD led to detectable, albeit miniscule, circulating oxidant producing neutrophils for as long as 6 months [78]. Successful gene therapy in CGD and in other haematopoietic disorders is limited by the transduction efficiency of myeloid precursors *ex vivo* and by the ability of corrected cells to replace uncorrected cells in vivo. The strategy of non-myeloablative conditioning followed by gene therapy facilitated the expansion of gene-corrected cells [79] and may be a promising approach.

10 Chronic Granulomatous Disease: A Disorder of Tryptophan Metabolism

Superoxide is a co-factor of indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan degradation along the kynurenine pathway. Tryptophan metabolism regulates both antimicrobial resistance and protective tolerance to proinflammatory microbial motifs. IDO and the other kynurenine pathway enzymes represent not only effector host defence pathways, but also, paradoxically, a means

of generating regulatory T-cells (Tregs) with anti-inflammatory, tolerogenic activity. Tregs and IL-17-producing T-cells mediate opposing responses. IL-17 stimulates production of G-CSF, GM-CSF, TNF-α, and chemokines that regulate myelopoiesis and neutrophil recruitment to inflammatory sites.

Tregs are recruited early in the inflammatory response to *Aspergillus* in wild type mice, and are capable of suppressing inflammation [80]. IL-17 augmented inflammation, but, paradoxically, impaired neutrophil antifungal host defence in mice [81]. Romani et al. recently demonstrated that IDO-mediated tryptophan metabolism along the kynurenine pathway is defective in CGD mice [82]. When challenged with intratracheal *Aspergillus fumigatus*, unrestrained γδ T cell and αβ Th17 expansion, defective regulatory Treg activity, and acute inflammatory lung injury leading to mortality occurred in CGD mice. The combination of recombinant IFN-γ and administration of a natural kynurenine distal to the IDO blockade protected CGD mice from experimental aspergillosis, restored production of downstream immunoactive metabolites and enabled the emergence of regulatory T cell responses. These results demonstrate a central role of NADPH oxidase in regulating the kynurenine pathway and the balance between Th17 and Treg cell development that can, in part, account for the impaired host defence and excessive inflammation in CGD. Natural kynurenines and kynurenine pathway enzymes are therefore highly promising and novel therapeutic targets in CGD.

There are important limitations to this study regarding application of mouse data to patients. First Th-17 development is under different regulatory control in mice and humans [83]. In addition, recent data suggest that superoxide is not a requirement for IDO activation in human cells [84]. Therefore, it will be important to correlate data from mouse models using human samples.

11 Conclusions

NADPH oxidase is a critical mediator of anti-fungal host defence. The host defence function of NADPH oxidase is mediated by the direct antimicrobial effect of reactive oxidant species and activation of sequestered proteases in neutrophil primary granules. NADPH oxidase is required for neutrophil-mediated damage of hyphae, but appears to be dispensable for conidiocidal activity. NADPH oxidase is required to protect against pulmonary aspergillosis, but NADPH oxidase-independent pathways appear to be sufficient to defend against hyphal vascular invasion and infarction. In addition to its host defence function, NADPH oxidase appears to have a role in counterbalancing inflammation induced by microbial products to limit tissue injury. Seen in this light, invasive aspergillosis in CGD is both a disease of impaired host defence and excessive inflammation, as illustrated by the recently described “mulch pneumonitis”. Knowledge gained about NADPH oxidase is important to develop novel strategies for CGD, but is also broadly relevant to our understanding of innate immunity.

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Aspergillosis in Drug Addicts

Juan-Carles Trullas, Josep Bisbe, and Jose M. Miro

Abstract Infections might seriously harm individuals who are addicted to illicit drugs, with systemic fungal infections being occasionally implicated. Drug users can acquire different forms of aspergillosis by inhalation of fungal spores during marijuana smoking or by direct inoculation from a contaminated drug supply or injection material. Marijuana may sometimes be contaminated with *Aspergillus*, and that is a potential hazard for individuals predisposed to aspergillosis. Marijuana smoking may subject immunosuppressed patients such as transplant recipients or those receiving chemotherapy for haematological malignancies to develop invasive pulmonary or disseminated aspergillosis (7 cases have been described so far). On the other hand, when the fungal infection is acquired by intravenous drug consumption, direct inoculation with fungi can cause haematogenous spread and local clinical manifestations, including endocarditis (5 cases), endophthalmitis (17 cases) and central nervous system (5 cases) or osteoarticular (7 cases) involvement. Disseminated aspergillosis has not been described in intravenous drug users. This chapter reviews different forms of aspergillosis occurring in patients using illicit drugs.

Keywords Aspergillus endocarditis · Aspergillus endophthalmitis · Cannabis · Marijuana abuse · Pulmonary aspergillosis · Substance abuse

Contents

J.M. Miro (✉)
Infectious Diseases Service, Hospital Clinic – IDIBAPS, University of Barcelona, Villarroel 170, 08036 Barcelona, Spain
e-mail: jmmiro@ub.edu

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1 Introduction

Infections are amongst the most serious complications of drug users. The Spanish Working Group for the Study of Infections in Drug Users performed a multicentre study (1977–1991) including 15,592 episodes of infection in intravenous drug users. Viral hepatitis, HIV infection, bacterial infections (particularly infectious endocarditis), and disseminated candidosis were the most frequent infections that occurred in this patient population [1].

The bacteria responsible for infections in drug users are acquired either from the normal microbiota of the drug user or externally as a result of organisms contaminating the drugs, drug adulterants, or paraphernalia. The most frequent bacterial infections in drug users [2] involve the skin and soft tissues (abscesses), musculoskeletal system (septic arthritis and osteomyelitis), endovascular system (endocarditis, septic thrombophlebitis and mycotic aneurysm), and the respiratory tract.

Systemic fungal infections in intravenous drug users are less common. The fungi most commonly encountered are *Candida* and *Aspergillus* species. Candidosis may be disseminated, with lesions in the superficial structures, the eye, and the skeletal system, or limited to the eye, the heart (as endocarditis), or the central nervous system. A syndrome of candidal infection consisting of cutaneous, ocular, and osteoarticular involvement (mainly costochondral) began to appear after 1980 in heroin addicts. Outbreaks were also described in different European countries and Australia during the 1980s. Persons at higher risk for the acquisition of these infections were intravenous drug addicts who used lemon juice to dissolve brown heroin and the causative agent was always *C. albicans* [3, 4].

Aspergillosis in drug addicts usually presents as endophthalmitis or as central nervous system infection. Zygomycosis is also occasionally seen, and various other fungi may cause endophthalmitis or endocarditis [5]. The purpose of this chapter is to provide a review on aspergillosis occurring in drug users.

2 Invasive Pulmonary Aspergillosis and Other Forms of Aspergillosis in Cannabis/Marijuana Smokers

Some studies have evaluated the potential of a cannabis substrate for the growth of *Aspergillus* species and other fungi. The spectrum of fungi in marijuana includes *A. fumigatus*, *A. niger*, *A. flavus*, *Mucor*, *Penicillium* species, *Thermoactinomyces*

candidus and *Thermoactinomyces vulgaris* [6–10]. When inhaled, these organisms are known to cause a variety of lung disorders, ranging from asthma, allergic bronchopulmonary mycoses, and hypersensitivity pneumonitis to invasive fungal diseases in immunocompromised hosts. Fungal spores present in marijuana can be inhaled during smoking (spores do not appear to be inactivated during the combustion of marijuana) and a prolonged inhalation of heavily contaminated air may result in hypersensitivity pneumonitis. Some of the respiratory complaints of marijuana smokers are likely due to an allergic reaction caused by the inhalation of *Aspergillus* spores. Marijuana smokers show a higher prevalence of precipitin antibodies against fungi, particularly *Aspergillus* species [7–9]. Individuals with underlying pulmonary or systemic diseases may develop invasive pulmonary or generalized forms of aspergillosis. Smoking marijuana contaminated with *Aspergillus* species has been identified as the route of infection in the seven cases of aspergillosis shown in Table 1 [11–17]. In 6 cases marijuana smoking occurred a few weeks before the onset of symptoms. A less evident temporal association occurred in case 3, in which the authors concluded that it could be the result of chronic marijuana smoking and perhaps the inhalation of a contaminant fungus with the marijuana smoke. In all cases but case 3 "cultures" fungi were cultured from patient's marijuana and/or pipe. As shown in Table 1, invasive pulmonary aspergillosis and disseminated aspergillosis occurred in immunocompromised patients. One patient with asthma had allergic bronchopulmoary aspergillosis. No patient had HIV infection. The species of *Aspergillus* most frequently identified was *A. fumigatus*. Four patients recovered with antifungal therapy and two patients died. These cases show that marijuana may sometimes be contaminated with *Aspergillus*, and that it is a potential hazard for individuals predisposed to *Aspergillus* infection. Marijuana smoking may subject immunosuppressed patients to potentially serious opportunistic fungal infections. Physicians caring for such patients should be aware of this potentially lethal complication, especially since patients may smoke marijuana to relieve the nausea brought on by chemotherapy [18]. For immunocompetent individuals, such exposure is probably of little practical significance (except for some rare cases of allergic bronchopulmonary aspergillosis).

3 Invasive Pulmonary Aspergillosis in Intravenous Drug Users

We performed a MEDLINE/PubMed search using the following MeSH terminology: "Aspergillosis" or "Aspergillus" and "Substance Abuse, Intravenous" or "Heroin" or "Cocaine". No cases of pulmonary aspergillosis directly related to intravenous drug use were found. Invasive pulmonary aspergillosis has been considered to be relatively infrequent in patients with HIV infection. Although formerly an AIDS-defining condition, it was dropped because of its very low prevalence. Reported series of HIV-infected patients with invasive aspergillosis include former intravenous drug users, but an association between drug use and aspergillosis was not straightforward. Invasive pulmonary aspergillosis in the HIV-infected population usually occurs amongst patients with <50 CD4 cells/mm^3 and major

Table 1 Reported cases of aspergillosis in marijuana smokers

Case	Authors	Age/Gender	Underlying disease	Clinical presentation	*Aspergillus* species	Treatment	Outcome
1	Chusid et al. [11]	17/M	Chronic granulomatous disease	IPA	*A. fumigatus*	*AMB IV*	Cured
2	Hamadeh et al. [12]	34/M	Chronic myelogenous leukaemia and HSCT	Disseminated[a]	*A. fumigatus*	*AMB IV and DHPG*	Fatal
3	Cunnington et al. [13]	23/F	None	Necrotizing Pulmonary Granulomata[b]	Fungal element seen in open lung biopsy (not identified)	None	Unknown[c]
4	Llamas et al. [14]	27/M	Atopy Asthma	ABPA	*A. fumigatus*	Steroids	Cured
5	Szyper-Kravitz et al. [15]	46/M	Acute myeloid leukaemia	IPA	*Aspergillus sp*	AMB IV	Cured
6	Marks et al. [16]	48/M	Renal transplantation	IPA	*Aspergillus sp*	AMB IV	Cured
7	Sutton et al. [17]	60/M	Small cell lung cancer	IPA	*Aspergillus sp*	Systemic antifungal therapy (not specified)	Fatal

[a] Autopsy revealed aspergillosis involving lung, endocardium and brain.
[b] Probably related with hypersensitivity to inhaled fungi (not definitely demonstrated in this case).
[c] Patient discharged herself from hospital prior to any treatment.
Legend: AMB, amphotericin B. ABPA, allergic bronchopulmonary aspergillosis; DHPG, experimental drug (dihydroxy-propoxymethyl guanine); F, female; IPA, Invasive pulmonary aspergillosis; HSCT, allogeneic bone marrow transplant; IV, intravenous; M, male.

predisposing conditions are the same as for the HIV-negative population, including neutropenia and steroid treatment [19–22] (see the chapter by Dr. Libanore and colleagues on aspergillosis in patients with HIV infection).

4 Other Forms of Aspergillosis in Intravenous Drug Users

4.1 Aspergillus Endophthalmitis

To date, 17 patients with *Aspergillus* endophthalmitis and intravenous drug use have been reported (Table 2). This topic is discussed elsewhere in this book by Dr Thomas. *Aspergillus* species has been cultured from street drugs [23] and the mechanism involved is probably haematogenous spread from direct inoculation of viable spores intravenously from a contaminated drug supply or injection material (diluting with tap water and cotton or cigarette filters used as filters) [24]. Extraocular involvement is rare (only one case reporting metastatis to the ribs [25]), which differs from endophthalmitis cases caused by *Candida* species, that are frequently complicated by cutaneous and chondrocostal involvement. Acute or subacute unilateral visual loss and ocular pain are the main symptoms in *Aspergillus* endophthalmitis. Clinical findings include conjunctival injection, anterior uveitis (hypopyon), vitreitis, macular chorioretinitis and fluffy vitreous infiltrates. Blood cultures are not useful for diagnosis and culture of vitrectomy material or vitreous needle aspirates are usually positive in untreated patients. *Aspergillus* species reported are *A. flavus*, *A. fumigatus*, *A. terreus*, and *A. glaucus*. In some cases, the diagnosis was by histopathologically confirmed only after enucleation. Pars plana vitrectomy, intravitreal and subconjunctival amphotericin B, systemic amphotericin B with/without 5-flucytosine and ketoconazole, fluconazole and itraconazole have all been used for treatment. Some degree of visual loss is the rule [24–32].

4.2 Aspergillus Endocarditis

Endocarditis and pericarditis will be reviewed in another chapter in this book so we have focused only on cases related to intravenous drug use. Our literature review show two cases of mitral *Aspergillus* endocarditis (one in a patient with both intravenous drug use and HIV infection) [33, 34], two cases of aortic endocarditis (one in a patient with both intravenous drug use and HIV infection), and one case of right (tricuspid valve) endocarditis in an intravenous drug user with HIV infection have been published [35] (Table 3). Vegetations were typically large, probably reflecting a late diagnosis. Three patients had systemic emboli, and the patient with right endocarditis had multiple septic lung infarcts. In two cases, *Aspergillus* species was recovered from the embolus material. All patients died. Blood cultures were negative in all cases. *A. fumigatus* ($n = 2$), *A. flavus* ($n = 1$), and *A. niger* ($n = 1$) were the species identified; in the fifth case, the diagnosis was by histopathology.

Table 2 *Aspergillus* endophthalmitis in intravenous drug users

Author [reference]	Age/Gender	HIV	Drug	Diagnosis	Species	Location	Treatment	Outcome
Sugar et al. [26]	22/M	NR	Amphetamines, lysergic acid, heroin and marijuana	Histologic	NR	Left eye	Enucleation	Blindness
Elliot et al. [27]	47/M	NR	Amphetamines	Vitreal culture	*A. flavus*	Left eye	Topical 5-FC	Partial visual loss
Doft et al. [28]	35/F	NR	NR	Vitreal culture	*A. flavus*	Unilateral	core vitrectomy + AMB intravitreal	Blindness
	21/F	NR	Cocaine	Vitreal culture	*A. flavus*	Left eye	PPV + AMB IV + AMB intravitreal + AMB PO + 5-FC	Partial visual loss
Michelson et al. [25][a]	31/M	NR	Cocaine	Vitreal culture	*A. flavus*	Right eye	Vitrectomy + AMB IB + AMB intravitreal + AMB PO + 5-FC	Partial visual loss
Wilmarth et al. [24].	27/M	NR	Methamphetamine	Vitreal culture	*A. fumigatus*	Right eye	Vitrectomy + AMB IV + AMB intravitreal. Enucleation	Blindness

Table 2 (continued)

Author [reference]	Age/Gender	HIV	Drug	Diagnosis	*Species*	Location	Treatment	Outcome
Roney et al. [29]	27/M	NR	Narcotics	Vitreal culture	*A. flavus*	Right eye	Vitrectomy + AMB PO + AMB IV + AMB intravitreal.+ 5-FC	Normal visual acuity
Lance et al. [30]	35/F	NR	Heroin	Vitreal culture	*A. flavus*	Right eye	Vitrectomy + AMB IV + AMB intravitreal	Partial visual loss
Barr et al. [31]	40/M	Neg	Cocaine, pentazocine and tripelennamine	Vitreal culture	*A. flavus*	Unilateral	Vitrectomy + AMB + 5-FC	Partial visual loss
	32/M	Neg	Cocaine, pentazocine and tripelennamine	Vitreal culture	*A. flavus*	Unilateral	Vitrectomy + AMB + 5-FC	Partial visual loss
	24/F	Neg	Cocaine, pentazocine and tripelennamine	Vitreal culture	*A. flavus*	Unilateral	Vitrectomy + AB + 5-FC	Partial visual loss
Weishaar et al. [32]	31/F	Neg	1 NR	Vitreal culture	*A. fumigatus*	Left eye	PPV + AMB IV + AMB intravitreal + miconazole PO + systemic fluconazole and itraconazole	Enucleation/ blindness

Table 2 (continued)

Author [reference]	Age/Gender	HIV	Drug	Diagnosis	*Species*	Location	Treatment	Outcome
	39/M	Neg	2 NR	Vitreal culture	*A. glaucus*	Right eye	PPV + AMB intravitreal + fluconazole + ketoconazole	Partial visual loss
	40/M	Neg	3 NR	Vitreal culture	*A. flavus*	Left eye	PPV + AB IV + AB intravitreal + AB PO + FC	Partial visual loss
	32/M	Neg	4 NR	Vitreal culture	*A. flavus*	Left eye	PPV+ FC + AB IV + AB intravitreal + AB PO + AMB topical + miconazole topical	Partial visual loss
	24/F	Neg	5: NR	Vitreal culture	*A. flavus*	Left eye	PPV + IV AMB + AMB intravitreal + AMB topical + miconazole PO + FC	Partial visual loss
	32/M	Neg	6: NR	Vitreal culture	*A. terreus*	Left eye	PPV + AMB intravitreal + AMB PO	Partial visual loss

[a]This patient had also metastatic rib osteomyelitis.

Legend: iv, intravenous. M, male; F, female; Pos, positive; Neg, negative; NR, not reported; PPV, pars plana vitrectomy; AMB, amphotericin B; 5-FC, 5-flucytosine; PO, periocular.

Table 3 *Aspergillus* endocarditis in intravenous drug users

Authors	Age/Gender	HIV	Drug	Diagnosis	Species	Embolus	Location	Treatment	Outcome
Henochowicz et al. [33]	32/M	Pos	NR	Echocardiogram. Necropsy. Negative blood cultures	*A. fumigatus*	Brain	Mitral	Any antifungal treatment	Fatal
Ligth et al. [34]	55/M	Neg	Heroin and cocaine	Echocardiogram. Positive embolus culture. Negative blood cultures	*A. flavus*	Distal aortic, femoral, brain, kidneys and spleen	Mitral	IV AMB	Fatal (stroke)
Petrosilo et al. [35]	35/M	Pos	NR	Echocardiogram, culture of valve vegetation, necropsy. Negative blood cultures.	*A. fumigatus*	Lung	Tricuspid	Any antifungal treatment	Fatal (cardiogenic shock)
	32/M	Neg	NR	Echocardiogram. Positive embolus culture. Negative blood cultures.	*A. niger*	Common iliac arteries	Aortic	IV AMB lipid	Fatal (stroke)
	39/M	Pos	NR	Echocardiogram. Negative blood cultures. Microscopic study of endocardial vegetation	*NR*	No	Aortic	IV Fluconazole	Fatal (cardiogenic shock)

Legend: AMB, amphotericin B; F, female; IV, intravenous; M, male; Neg, negative; NR, not reported; Pos, positive.

Table 4 *Aspergillus* osteomyelitis in intravenous drug users

Authors	Age/Gender	HIV	Drug	Diagnosis	Species	Location	Treatment	Outcome
Seligsohn et al. [36]	42/F	NR	Heroin	Biopsy culture	***A. terreus***	L1–L2	AMB	Cured
Michelson et al. [25]	31/M	NR	Cocaine	Bone histology + vitreal culture	*A. flavus*	Rib	Surgery + AMB	Cured
Brown et al. [37]	34/M	Neg	Heroin	Biopsy culture	*A. fumigatus*	L1–L2	Surgery + AMB	Cured
	30/M	Neg	Heroin	Biopsy culture	***A. terreus***	T6–T7	Surgery + AMB	Cured
Walker et al. [39]	34/M	Neg	NR	Surgical culture	*Aspergillus* species	Sternum	Surgery	Cured
	32/M	Neg	NR	Surgical culture	*A. flavus*	Sternum	Surgery + AMB	Cured
Salloum [38]	48/M	Neg	Heroin	Aspiration material culture	*A. fumigatus*	T7 + 9th and 10th ribs	Surgery + itraconazole	Cured

Legend: M, male; F, female; NR, not reported; Neg, negative; AMB, Amphotericin B.

Table 5 Central nervous system aspergillosis in intravenous drug users

Authors	Age /Gender	Drug/s	Diagnosis	CSF	Species	Symptoms	Treatment	Outcome
Burston J et al. [40]	37/M	Cocaine + Heroin	Necropsy: reactive basilar meningitis and multiple brain fungal abscesses	*NR*	*NR*	Hemiplegia	None	Fatal
Kaufman et al. [42]	31/F	Heroin	CSF culture	Pleocytosis + glucose 20 mg/dl	*A. fumigatus*	Headache for 9 months + blurred vision + diplopia + tinnitus + hearing loss	Surgery + AMB IV	Fatal
Gordon et al. [41]	34/F	Cocaine + Heroin	CSF culture	Pleocytosis + glucose 16 mg/dl	*A. oryzae*	Headache and neck stiffness (clinical meningitis)	5-FC + AMB IV	Cured
Bryan et al. [43]	26/M	NR	CSF culture	Pleocytosis + glucose 6 mg/dl	*A. flavus*	Hydrocephalus + lumbar arachnoiditis	Surgery + AMB IV	Cured
Morrow et al. [44]	36/M	Heroin	Necropsy: ventriculitis	Pleocytosis + glucose 29 mg/dl	NR	Seizures and fever	None	Fatal

Legend: AMB, amphotericin B; CSF, cerebrospinal fluid; F, female; 5-FC, flucytosine; IV, intravenous M, male; NR, not reported.
5-FC, Flucytosine.

4.3 Aspergillus Osteomyelitis

Three cases of vertebral osteomyelitis [36, 37], one of vertebral and rib osteomyelitis [38], two of sternal osteomyelitis [39], and one case of endophthalmitis and rib osteomyelitis [25] have been reported. *A. fumigatus, A. terreus*, and *A. flavus* (two cases each), and other of non-*fumigatus Aspergillus* species were involved. Patients were treated with surgical debridement and antifungal agents (systemic amphotericin B in five cases and itraconazole in one) and all recovered (Table 4).

4.4 Central Nervous System Aspergillosis

Since the first report of brain aspergillosis from the necropsy of an intravenous heroin and cocaine addict by J. Burston and W. Blackwood in 1963 [40], a further four cases of central nervous system aspergillosis have been reported in intravenous drug users [41–44]. All five cases were before 1980 so HIV status of patients is obviously unknown. Unlike brain aspergillosis in immunocompromised patients where parenchymal abscesses are the rule and cerebrospinal fluid (CSF) analysis shows little or no pleocytosis with normal glucose and negative culture, in drug users meningeal and or ventricular involvement is frequent (4/5 cases), with frank pleocytosis, low CSF glucose and isolation of *Aspergillus* species from CSF culture (3/4 cases). The outcome is usually fatal with only two patients surviving this diagnosis (Table 5).

4.5 Renal Aspergillosis

One case of renal aspergillosis [45] with excretion of multiple mucoid fungus balls has been reported. *A. fumigatus* was isolated from urine and the mucoid material excreted. The patient recovered after treatment with intravenous amphotericin B and urethral instillation of amphotericin.

5 Conclusions

Aspergillus infections in drug users are uncommon and most reported cases are from the 1980s and 1990s. When an immunocompromised host is exposed to contaminated and "non-controlled" street marijuana, invasive pulmonary aspergillosis can occur. On the other hand, when the fungal infection is acquired by intravenous drug consumption, direct inoculation of the fungi (from the contaminated drug or injection material) causes haematogenous spread that may produce local manifestations involving the heart (endocarditis), the eyes (endophthalmitis), the central nervous system, or the osteoarticular system. To our knowledge, disseminated forms of aspergillosis have not been described in intravenous drug users.

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Invasive Aspergillosis and HIV Infection

Marco Libanore, L. Sighinolfi, and F. Ghinelli

Abstract Invasive aspergillosis was previously considered an AIDS-defining disease. However, this infection is seen only occasionally in HIV-infected patients, usually affecting patients with advanced disease. The most commonly implicated factors for invasive aspergillosis are neutropenia, granulocyte dysfunction, exposure to broad-spectrum antibacterial therapy and a long term steroid use. The diagnosis of invasive pulmonary aspergillosis is hampered by the absence of typical clinic-radiological findings. New diagnostic tests such as galactomannan testing are of limited utility for AIDS patients. Cerebral aspergillosis seems to be more prevalent in the HIV population than in other groups. Treatment of invasive aspergillosis in the context of HIV infection is similar to treatment in patients not infected with the HIV, though special attention is required for drug-drug interactions.

Keywords AIDS · Aspergillosis · HIV · Invasive aspergillosis · SIDA

Contents

M. Libanore (✉)
Department Infectious Diseases, St. Anna Hospital and University, Ferrara, Italy
e-mail: m.libanore@ospfe.it

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1 Introduction, incidence and epidemiology

Highly active antiretroviral therapy (HAART) has determined a profound biological impact on the clinical manifestations of human immunodeficiency virus (HIV) infection. It has dramatically decreased the frequency of opportunistic infections independently of the use of specific prophylaxis [1]. The incidence of HIV related invasive aspergillosis (IA) is poorly known because the infection was included in the 1982 Center for Disease Control and Prevention surveillance case definition for acquired immunodeficiency syndrome (AIDS) but it was removed from the list of AIDS-defining conditions in 1984 because, at that time, information suggested that aspergillosis was not associated with cellular immune deficiency [2]. Nonetheless, aspergillosis does occur in HIV infected people, with many cases have be reported in the scientific literature since 1984 but only a few epidemiologic studies [3]. The most commonly implicated factors for invasive disease are neutropenia, granulocyte dysfunction, exposure to broad-spectrum antibacterial therapy and a long term steroid use. Invasive pulmonary aspergillosis (IPA) is a life-threatening opportunistic infection in the advanced stage of HIV infection. The disease normally takes a lethal course when antifungal therapy is not started in time. Compared with other types of immunosuppression it is however rare in AIDS. An analysis of the United States of America (USA) HIV archives revealed an average risk of IPA of 0.4% in HIV-infected patients. The risk increases to 1.0% when CD4 count decline <50/mm^3 and 3.0% in neutropenic patients [4]. Numerous studies have demonstrated that the frequency of invasive fungal diseases is underestimated in life if invasive procedures are not performed. In pre HAART era in the USA, an uncontrolled study reported an incidence of 0.3–5% in HIV positive subjects [5]. In eleven autopsy series involving a total of 927 HIV infected patients, 42 (4.5%) cases of aspergillosis were identified [3, 6–13]. The incidence in these studies ranged from 0.3 to 12.5%. There were significant differences in the distribution of the infection all over the world: the incidence was higher in the USA and Africa and lower in the United Kingdom (UK) [14–16]. In Italy the incidence was nearly the mean value of Europe [11, 17]. In HAART era the incidence of aspergillosis among HIV-infected people is 3.5 cases per 1,000 person-years. The incidence was higher among people aged >35 years, among individuals with CD4 count of 50–99 cells/mm^3 or CD4 count of <50 cells/mm^3, subjects with more than one AIDS-defining opportunistic illness, and patients who were prescribed at least one medication associated with neutropenia (prolonged use of steroids, exposure to broad spectrum antibacterial therapy and antiblastic treatment) [18]. IA affects almost exclusively patients with advanced AIDS with serious deficiencies of T-cell function. Characteristically, it develops in patients with neutropenia or granulocyte dysfunction with a history of other AIDS-defining opportunistic infections and malignancies who are not receiving HAART. A variety of defects in phagocytic function have been demonstrated in HIV-infected patients abnormalities of neutrophil chemotaxis, phagocytosis, and killing have been reported. Impaired monocyte chemotaxis, phagocytosis and intracellular killing have been noted too [3, 19]. Another factor for the development of IA in HIV-infected patients includes immunosuppression due to high

Table 1 Predisposing factors associated with aspergillosis in patients with AIDS [17]

Possible predisposing factors*	n	%
Intravenous drug users	42	77.8
Receipt of steroids	22	40.7
Leukopenia	16	29.6
Anti-neoplastic agents	5	9.2
Other	3	5.6

*Ten patients with more predisposing factors.

doses of steroids. Opportunistic infections, especially those involving the lungs, such as pneumocystosis and cytomegalovirus infection, may also predispose to the development of IPA due to impairment of pulmonary macrophage function. Intravenous drug use has also been noted as a risk factor of *Aspergillus* infection [18, 20] (Table 1). IA occurring in patients using illicit intravenous drugs is discussed elsewhere in this chapter. In the pre-HAART era the increased incidence of the infection among HIV-infected patients was attributed to the large use of steroids for the treatment of pneumocystosis and of other conditions such as mycobacteria other than tuberculosis and lymphomas [17, 18]. A comparison of the characteristic of aspergillosis in patients with AIDS *versus* other underlying conditions (i.e., haematological and transplant patients) showed many similarities, however in the first concomitant pulmonary infections were frequent and than later diagnosis [21].

2 Clinical Manifestations

Two major syndromes have been described among patients with AIDS: respiratory tract disease (semi-invasive pseudomembranous tracheitis or invasive pneumonitis) and, central nervous system infection occurring as a febrile diffuse meningoencephalitis syndrome with vascular infarction as a central feature, based on the predilection of *Aspergillus* organisms to invade blood vessel walls [14, 17, 20, 22, 2–5]. Semi-invasive pseudomembranous tracheitis is associated with fever, cough, dyspnoea, stridor or wheezing caused by airway constriction, culminating in airway obstruction if untreated. Bronchoscopi examination may demonstrate a confluent, exudative pseudomembrane adherent to the tracheal wall. Invasive pneumonitis occurs with fever, cough, dyspnoea, chest pain, haemoptysis, and hypoxemia (Table 2). Chest radiograph may show either a diffuse interstitial pneumonitis or a localized wedge-shaped dense infiltrate representing pulmonary infarction, which is related to the predilection of the organisms for invasion of vascular endothelium. Initially unspecific infiltrates are the radiographic findings most commonly reported. The development of consolidations with cavities has been also observed in numerous cases (Table 3). The air crescent sign – which is noted in up to 93% among certain groups of immunocompromised patients with IA – is less frequently among HIV-infected patients [21, 23]. A proposed explanation is that this sign is associated with the rapid recovery from severe immunosuppression, a phenomenon that is move slow usual among AIDS patients. Extra-pulmonary involvement has been often reported in studies where post-mortem examination was performed. Central nervous

Table 2 Clinical findings of patients with HIV infection/AIDS and aspergillosis

	Libanore et al. [17]		Mylonakis et al. [5]	
Symptoms and signs	n	%	n	%
Fever	41	75.9	48	86
Cough	25	46.3	39	70
Chest pain	16	29.6	15	27
Dyspnoea	17	31.5	37	66
Haemoptysis	4	7.4	3	5
Central nervous system symptoms	10	18.5	6	11
Generalized malaise/weight loss	15	27.7	7	13

Table 3 Pulmonary radiographic appearance of patients with HIV infection/AIDS and aspergillosis

	Libanore et al. [17]		Mylonakis et al. [5]	
Chest x-ray	N	%	n	%
Consolidations/infiltrates	30	55.5	32	73
Single focal dense lesion	15	27.8	6	14
Cavitary lesions	9	16.7	15	34
Pleural effusions	3	5.6	3	7

Table 4 Sites of aspergillosis in patients with HIV infection/AIDS

	Libanore et al. [17] (n = 54)		Holding et al. [4] (n = 144)	
Sites	n	%	n	%
Lung	48	88.9	114	79.2
As sole site	37	68.5	NR	
With extra-pulmonary disease	11	20.4	NR	
Brain	9	16.7	0	–
Kidneys	9	16.7	0	
Liver	2	3.7	0	–
Lymph nodes	2	3.7	0	–
Thyroid	2	3.7	0	–
Paranasal sinuses	2	3.7	7	4,8
Skin and subcutaneous tissues	1	1.8	5	3,5
Prostate	1	1.8	0	–
Tongue	1	1.8	0	–
Stomach	1	1.8	0	–
Ear	0	–	5	3,5
Vagina	0	–	1	0,7
Bowel	0	–	1	0,7
Bone	0	–	1	0,7
Eye	0	–	1	0,7
Blood	0	–	8	6,2
Multiple sites	12	22.2	1	0,7
Extra-pulmonary only	6	11.1	NR	

Legend: NR, Not reported.

system involvement appears to be more frequent in AIDS patients than in others conditions. Other localizations include kidneys, liver, lymph nodes, thyroid, paranasal sinuses, ears, skin, subcutaneous tissues, bones, prostates, tongue and stomach. Other sites recently reported are the eyes and heart valves [8, 11, 24] (Table 4).

3 Diagnosis

Blood cultures are of limited utility, because the results are often not positive even in the context of a disseminated infection. A definitive diagnosis requires the presence of relevant clinical signs and symptoms and the histopathologic demonstration of organisms in biopsy specimens obtained from involved sites or from a site that is expected to be sterile (e.g., liver or brain). IA is defined of the presence of characteristic closely septate hyphae with repeated acute angle branching in the biopsy materials or in the percutaneous tissue aspirates other than lung. Hyphae are identified using hematoxylin-eosin and methenamine silver stain. Nevertheless patients with advanced AIDS and concomitant pulmonary opportunistic infections present often nonspecific symptoms of IA therefore an aggressive diagnostic approach is extremely important [3, 5, 17, 18]. Biopsy with histopathologic demonstration is often difficult to perform for seriously ill patients with AIDS and may be non-diagnostic. IA is further divided into pulmonary and extra-pulmonary disease. A presumptive diagnosis of respiratory tract disease can be made with the absence of a tissue biopsy if *Aspergillus* spp. are cultured from a respiratory samples, and compatible lesions or syndromes are present, and no alternative caused process are identified. The positivity of sputum cultures is not always correlated with IA, because airway colonisation with *Aspergillus* species is not uncommon in patients with AIDS. The diagnosis of IA cannot be based only on cultures, however, in the clinical setting of HIV infection, and in particularly among patients with history of neutropenia and low CD4 count, biopsy is encouraged [5, 17]. The occurrence of a new cavity or infiltrate in a previously normal lung in a patient with AIDS in association with recovered of *Aspergillus* species in broncoscopy cultures warrant antifungal treatment pending definitive diagnosis [5]. Autopsy studies have shown that the ante mortem diagnosis of AIDS-associated IA is often underestimated. In vivo diagnosis is misunderstood since nonspecific symptoms often mask multiple infections [23] (Table 5).

Table 5 Mode of definitive diagnosis of patients with HIV infection/AIDS and aspergillosis

Diagnostic test	Libanore et al. [17] (%)	Mylonakis et al. [5] (%)
Autopsy	75.9	70
Biopsy	16.6	27
Bronchoalveolar lavage	3.8	43
Blood culture	1.9	<1
Cerebrospinal fluid culture	1.9	0

EIA for galactomannan detection has been validated in animal models and in patients as a surrogate marker for the detection of IA [25]. However, the sensitivity in non-neutropenic patients may be lower, may be because of a lower residual fungal burden or anti-*Aspergillus* antibodies. False-positive results have been reported in several contexts, including patients who on beta-lactam certain antibiotic (particularly piperacillin-tazobactam and amoxicillin-clavulanate) or diagnosed with other invasive mycoses. These include penicilliosis, cryptococcosis, histoplasmosis [26] – all important infections in the HIV setting. For this reasons galactomannan testing does not seem to help in patients infected with the HIV. Other potential circulating markers for the detection of aspergillosis include β-D-glucans detected by the Tachypleus or Limulus assay but the experience is limited and more researches are required. Polymerase chain reaction (PCR)-based diagnosis, which amplified *Aspergillus* specific fungal genes, has shown to be considerable very promising for IA. However, these systems have not been standardised, are not commercially available, and remain investigational [27].

4 Therapy

The following Food and Drug Administration (FDA)-approved compounds have in vitro, in vivo and clinical activity against *Aspergillus* species and are licensed for treatment of IA: deoxycholate amphotericin B (d-AMB) and its lipid formulations (AMB lipid complex, liposomal AMB, and AMB colloidal dispersion), itraconazole, voriconazole, posaconazole, and caspofungin. Based on the latest consensus by the Infectious Disease Society of America (IDSA, USA), voriconazole is considered first-line therapy for IA [27]. Alternatives include liposomal AMB, AMB lipid complex, caspofungin, micafungin, posaconazole and itraconazole. Anidulafungin has both in vitro, in vivo, and clinical activity against aspergillosis but are not yet licensed for this indication. The recommended treatment for HIV-related IA is voriconazole, in the same dosage as for other patients with IA: loading dose of 6 mg/kg IV twice daily for two doses followed by 4 mg/kg every 12 hours. The weight of evidence supports voriconazole as the primary recommendation for systemic antifungal therapy of cerebral aspergillosis [28] – surgery has also an important role for these patients. Treatment of IA is discussed elsewhere in this book. Special attention should be given to drug–drug interactions in HIV-infected patients, particularly for drugs such as efavirenz, carbamazepine and the rifamycins. d-AMB is no longer considered an option for the treatment of IA.

The overall outcome of AIDS-associated IA is poor among patients with advanced immunosuppression and the absence of effective HAART. Treatment failure is generally defined as failure to respond to initial therapy or progression of clinical signs and symptoms despite appropriate therapy. It is reported a mean post-diagnosis survival of 6 months. Early diagnosis and aggressive treatment improved the outcome among AIDS patients with IA. Surgical interventions are helpful in certain patients for the treatment of localized infection and should be considered for the treatment of AIDS patients with IA. However, no data are available to guide

recommendations for the management of treatment failures. If d-AMB was used initially, substitution with voriconazole might be considered; the alternative approach would be rational for those who began therapy with voriconazole. IA, even if the patient initially responds to the treatment, is a long-term challenge and requires close monitoring and a prolonged and aggressive course of antifungal treatment [1, 4, 8, 18]. In this population no data are available to base a recommendation for or against chronic maintenance or suppressive therapy among those who have successfully completed an initial course of treatment.

IPA has been also observed in association with a rapid immune reconstitution in AIDS patients. Since the introduction of HAART in 1996, new clinical observations of florid inflammatory responses to infectious agents have been noted, which have come to be known as immune reconstitution syndrome. This exacerbation of a wide variety of latent infections, with the expression of complex clinical pictures and paradoxical reactions, coincides with improvements in CD4+ lymphocytes counts and decreases in plasma viral load and is probably related to an antigen specific T-cell response. In contrast to clinical manifestation of opportunistic infections during immunosuppression, which are characterized by high microbial tissue burden and systemic dissemination, immune reconstitution syndrome is characterised by a local reaction with sparse isolation of the complicating pathogen. It is a diagnosis of exclusion [24].

Finally, aspergillosis should be considered in the differential diagnosis of fever of unknown origin in patients with AIDS, especially if there is evidence of pulmonary involvement [5, 17].

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Invasive Pulmonary Aspergillosis in Solid Organ Transplant Recipients

Fernanda P. Silveira and Shahid Husain

Abstract Invasive aspergillosis (IA) continues to be a significant cause of morbidity and mortality in solid organ transplant recipients. The highest incidence of IA is noted in lung transplant recipients, while the lowest incidence is in kidney transplant recipients. Common risk factors for the development of invasive aspergillosis include environmental exposures and net state of immunosuppression. Unique risk factors to lung transplants include airway colonization, and receipt of single lung transplant. Liver transplant recipients receiving renal replacement therapy on the other hand have the highest risk of IA. Liver transplant recipients also have the highest incidence of disseminated disease while tracheobronchitis is the most common manifestation of IA in lung transplant recipients. Prophylaxis with antifungal agents is recommended in lung and liver transplant recipients. The drug of choice for the treatment of IA remains to be voriconazole. The role of combination therapy for the treatment of IA in solid organ transplant recipients remains undetermined.

Keywords Aspergillosis · Liver transplant recipients · Lung transplant recipients · Solid organ transplantation · Tracheobronchitis

Contents

S. Husain (✉)
Transplant Infectious Diseases, Division of Infectious Diseases and Multi-organ Transplantation, University Health Network/University of Toronto, Toronto, ON, Canada M5G 2C4
e-mail: shahid.husain@uhn.on.ca

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1 Epidemiology and Risk Factors

The incidence of invasive pulmonary aspergillosis (IA) amongst solid organ transplant (SOT) recipients varies according to the type of transplanted organ [1, 2]. The highest incidence is observed in lung transplant recipients, ranging from 6 to 16% [3–9]. Liver and heart transplant recipients have a moderate risk of developing IA, with incidence rates ranging from 1–8% and 1–14% [10–12], respectively. The lowest risk of IA is observed amongst kidney/pancreas recipients, ranging from 0.4 to 5% [12, 13]. In a prospective, multicentre surveillance program which included 4,110 SOT recipients in 19 centres in the United States of America the cumulative incidence of IA at 12 months following SOT was 2.4% after lung transplantation, 0.8% after heart transplantation, 0.3% after liver transplantation, and 0.1% after kidney transplantation [14]. In this same study, mortality at 3 months following diagnosis of IA ranged from 20% for lung transplant recipients to 66.7% for heart and kidney recipients.

Some risk factors for IA are common to all SOT recipients. These include environmental exposure and the net state of immunosuppression, which is crudely equated with the use of high-dose steroids, use of lymphocyte depleting agents and infections caused by immunomodulatory viruses, particularly cytomegalovirus (CMV) [1, 2, 15–18].

However, there are some risk factors that are unique to the transplanted organ. In lung transplant recipients, for example, the direct exposure of the allograft to the environment, along with impaired defenses due to decreased mucociliary clearance and blunted cough reflex, make this patient group particularly vulnerable to IA [19]. Other risk factors contributing to an increased risk of IA in lung transplant recipients, include the relative ischemia at the anastomosis [20], receipt of single lung transplant [8], development of bronchiolitis obliterans, hypogammaglobulinemia [21], placement of a bronchial stent [9, 22, 23] and pre and post-colonization of the airways with *Aspergillus* [3, 5, 24, 25]. In a study, post-transplant *Aspergillus* colonization was associated with a relative risk of 11 for the development of IA [3]. Cystic fibrosis patients with pre-transplant *Aspergillus* airway colonisation have a higher risk of post-transplant *Aspergillus* tracheobronchitis [26]. Recently, there has been preliminary evidence that the lower CD4+ T cell function measured by ATP synthesis stimulated with phytohemagglutination may predict the development of invasive fungal disease [27].

Table 1 Risk factors for pulmonary aspergillosis in solid organ transplant recipients

All organs	Lung	Liver
Environmental exposure	Allograft exposure to the environment	Pre-transplant fulminant hepatic failure
Net state of immunosuppression	Airway ischemia	Retransplantation
CMV disease	Decreased mucociliary clearance and cough reflex	Requirement of renal replacement therapy
	Single lung transplantation	Primary allograft failure or severe dysfunction
	Bronchiolitis obliterans	High transfusion requirement
	Hypogammaglobulinemia	
	Bronchial stent	
	Pre and post-transplant *Aspergillus* colonisation	

Legend: CMV, cytomegalovirus.

Some unique risk factors for the development of IA are observed in liver transplant recipients. These include: pre-transplant fulminant hepatic failure, primary allograft failure or severe dysfunction, retransplantation, high transfusion requirement and dialysis requirement following transplantation [28–30].

Table 1 lists the main risk factors for IA in SOT recipients.

2 Clinical Presentation

Pulmonary IA in SOT recipients usually presents with a dry cough and dyspnoea. Low grade fever and haemoptysis may also occur. The sensitivity of chest radiograph is low, and computed tomography of the chest is recommended whenever pulmonary aspergillosis is suspected. Pulmonary aspergillosis in SOT recipients, in contrast to what is observed in stem cell transplant recipients, lacks a distinguishing radiographic finding [31, 32]. Common radiographic findings include nodular lesions with or without cavitation and focal areas of consolidation or infiltrate. The halo sign has very low sensitivity in SOT recipients and is often absent.

There are some features that are particular to each type of allograft, and they are discussed below. These features are summarised in Table 2.

2.1 Lung Transplant Recipients

In lung transplant recipients, *Aspergillus* infection commonly has three types of presentation. These include *Aspergillus* airway colonisation, *Aspergillus* tracheobronchitis and invasive pulmonary aspergillosis. Amongst lung transplant recipients with IA, approximately 58% have tracheobronchitis, 22% have invasive pulmonary infection and 22% have disseminated infection [9]. Recent data however would suggest mare cases of invasive pulmonary infection.

Table 2 Differences in clinical presentation of invasive aspergillosis according to type of allograft

	Lung	Liver	Heart	Kidney
Time to onset	1st 3 months for tracheobronchitis, late for invasive pulmonary	Early, median time to onset 17 days[a]	Within 3 months	Late
Clinical presentation	Airway colonization Tracheobronchitis Invasive pulmonary	Disseminated disease in 50–60%	Invasive pulmonary	Invasive pulmonary
Mortality	23–29% for tracheobronchitis, 67–82% for invasive pulmonary	60–92%	53–78%	75–80%

[a]Recently a greater number of cases has been observed after 90 days post-transplantation.

Aspergillus airway colonisation occurs in 25–58% of lung transplant recipients [3, 6, 33], and may occur pre and post-transplant. In a study of patients with cystic fibrosis, 53% were found to have pre-transplant *Aspergillus* colonisation. Of these, 24% developed *Aspergillus* tracheobronchitis [33]. Post-transplant *Aspergillus* airway colonisation occurs in as many as 46% of lung transplant recipients [3]. Colonisation in the first 6 months following lung transplantation was shown to be associated with a relative risk of 11 for the development of IA [3]. The use of voriconazole prophylaxis for 4 months following lung transplantation decreased the rate of IA, but did not decrease the rate of *Aspergillus* colonisation at one year after transplantation [34], when compared to a cohort managed with targeted antifungal prophylaxis.

Aspergillus tracheobronchitis is a common presentation of IA following lung transplantation [9] and is found almost exclusively amongst lung transplant recipients, at the anastomotic site. It usually occurs within the first 3 months following transplantation and is characterised by the presence of necrosis, ulceration and pseudomembrane formation at the anastomotic site [35]. Diagnosis is made by bronchoscopy, and if not diagnosed early enough, infection may extend to the pulmonary parenchyma. Fever is generally absent (Fig. 1).

Invasive pulmonary aspergillosis is more common in patients with single lung transplants than double lung transplants and tends to occur later in the post-transplant period [9]. Fever is observed only in about 15% of patients and the most common radiographic findings are focal areas of patchy consolidation. Nodular infiltrates are seen in 27–30% of patients [9].

The mortality rate of IA in lung transplant recipients varies according to the clinical presentation, ranging from 23 to 29% in patients with tracheobronchitis to as high as 67–82% in patients with invasive pulmonary disease [2].

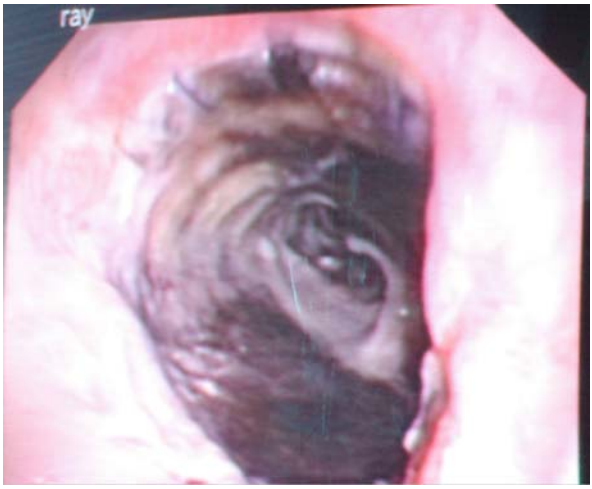

Fig. 1 *Aspergillus* tracheobronchitis in a lung transplant recipient

2.2 Liver Transplant Recipients

Amongst liver transplant recipients, *Aspergillus* infection typically occurs in the early post-transplant period, with a median time to onset of 17 days after transplantation [36], however a recent change in epidemiology has been observed, with an increase in the number of cases of IA occurring after 90 days post-transplantation [37]. Reasons for this shift are not completely clear, but may reflect improved survival in the early post-operative period, increasing the number of patients at risk for later development of IA, and also the fact that the use of CMV prophylaxis has delayed the onset CMV disease, a risk factor for development of IA [37].

Dissemination of infection beyond the lungs occurs in 50–60% of liver transplant recipients [32, 36]. More recently, a decline in the incidence of disseminated disease and central nervous system (CNS) infections has been observed and is thought to be secondary to the use of calcineurin inhibitors as maintenance immunosuppressive agents [37]. These agents have been shown to have anti-*Aspergillus* [38–40] activity and to enhance the activities of antifungal agents in vitro. Despite the antifungal activities of these agents, their immunosuppressive effect outweighs these effects, and disseminated disease was still observed in 30% of patients on these agents [37].

The mortality rate of liver transplant recipients with IA has ranges from 60 to 92%, with the lower rate observed in a recent cohort from 1998 to 2002, largely due to a lower incidence of disseminated disease [28, 32, 36, 37].

2.3 Heart Transplant Recipients

Approximately 75% of cases of IA in heart transplant recipients occur within 3 months of transplantation [10]. The isolation of *Aspergillus* from the respiratory

tract of a heart transplant recipient, particularly *A. fumigatus*, was found to be highly predictive of IA in these patients [41].

One case of *Aspergillus* tracheobronchitis of the native lung has been reported in a heart transplant recipient [42].

The mortality rate of heart transplant recipients with invasive pulmonary aspergillosis has ranged from 53 to 78% [10].

2.4 Kidney Transplant Recipients

Despite the fact that kidney transplant recipients have the lowest incidence of IA compared to other organ transplant recipients, IA is still a devastating complication in this patient group, with associated high morbidity and mortality. The mortality rate in kidney transplant recipients with IA has ranged from 75 to 80% [2].

3 Diagnosis of Invasive Aspergillosis in Solid Organ Transplant Recipients

Failure to establish an early diagnosis and therefore initiate appropriate therapy is a major impediment to successful outcomes in the management of IA in SOT recipients. Respiratory tract cultures often lack sensitivity, and because *Aspergillus* species are ubiquitous in the air, culture results need to be interpreted with caution. Amongst SOT recipients, the predictive value of a positive respiratory tract culture for *Aspergillus* ranges from 17 to 72% [43–45]. A study with liver and kidney transplant recipients revealed that cultures with more than two colonies of *Aspergillus*, particularly if the species were *A. fumigatus* or *A. flavus*, or more than one site of infection were predictive of significant infection. In the contrary, two or fewer colonies from a single site likely represented contamination [46].

In recent years great interest and efforts have been devoted to identifying non-invasive and non-culture based methods for accurate and early diagnosis of IA. So far, results amongst SOT recipients have been disappointing. Very few studies have evaluated the performance of the serum galactomannan assay in the diagnosis of IA in SOT recipients. These studies have shown sensitivities ranging from 30 to 55.6% and specificities ranging from 87 to 95% [4, 47, 48]. Two of the studies used a cut-off of 0.5 and one used a cut-off value of 1.0. A cut-off value of 0.66, at least in lung transplant recipients, was associated with an increase in specificity and positive likelihood ratio, without a decrease in sensitivity [4]. In a meta-analysis, the overall sensitivity of the galactomannan assay for SOT recipients was 22% and the specificity was 84% [49].

Recently, interest has been directed towards the utility of detecting the galactomannan antigen in the bronchoalveolar lavage (BAL) of SOT recipients for diagnosis of IA. In a study of lung transplant recipients with an incidence of IA of 5.2% (6/116 patients), the sensitivity was 60% and the specificity was 98% when an index cut-off value of ≥ 1.0 was used. The positive and negative likelihood ratios were 28

and 0.40, respectively [50]. Based on this study, the detection of galactomannan in the BAL of a lung transplant recipient with a clinical picture compatible with IA is highly suggestive of IA, when a cut-off of $\geq$ 1.0 is used. In another prospective study evaluating the performance of galactomannan testing in 117 BAL samples from 60 lung transplant recipients, the best cut-off was determined as 1.5. This was associated with a 90.9% sensitivity, 90.6% specificity, and positive and negative predictive values of 48 and 99.1%, respectively (Dr. Pasqualotto, submitted data). The incidence of proven/probable IA in this study was 9.4% (11/117).

Another study looked at the utility of galactomannan assay in the BAL of 81 SOT recipients. There were 24 heart, 22 kidney, 19 liver and 16 lung transplant recipients. The incidence of proven or probable IA was 6.2% (5/81). For an index cut-off value of $\geq$ 1.0, the sensitivity, specificity, and positive and negative predictive values were 100, 90.8, 41.7 and 100%, respectively. All patients with proven or probable IA had a positive BAL galactomannan with values $\geq$2.1. A positive BAL galactomannan diagnosed IA several days to 4 weeks earlier than other diagnostic tests in three patients. A high frequency of false-positive results was observed amongst lung transplant recipients when a cut-off of $\geq$0.5 was used [51].

The utility of (1$\rightarrow$3)-ß-D-glucan is limited by its broad-spectrum of reactivity [52]. It detects not only *Aspergillus* but also *Fusarium*, *Trichosporum*, *Acremonium*, *Candida* and even *Pneumocystis jirovecii* [53]. Recently a study looked at its utility for diagnosis of invasive fungal infections amongst 180 living donor liver transplant recipients who were prospectively followed for one year after transplantation. Five patients developed aspergillosis; all considered proven by EORTC/MSG criteria [54]. All patients with IA had (1$\rightarrow$3)-ß-D-glucan level >40 pg/ml. At this cut-off, the sensitivity and specificity of (1$\rightarrow$3)-ß-D-glucan for overall fungal infection was 58 and 83%, respectively, and the positive and negative predictive values were 35 and 93%, respectively [55].

Polymerase chain reaction (PCR)-based methods for detection of *Aspergillus* DNA have the potential of becoming very useful; however they are largely unstandardised and not yet utilised in clinical practice.

The definitive diagnosis of IA in SOT recipients still relies on demonstration of hyphal invasion in tissue with growth of *Aspergillus* in culture of the same tissue.

4 Therapy of Invasive Aspergillosis in Solid Organ Transplant Recipients

The principles of therapy of IA in SOT recipients do not differ from other immunocompromised patients. Early institution of adequate therapy, as well as reduction of immunosuppression is crucial for a favourable outcome. Therapy should be continued for a total of 12 weeks or for at least 4–6 weeks after complete resolution of clinical and radiographic findings, whichever is longer [56].

Since voriconazole was shown to have superior efficacy and survival benefit when compared to amphotericin B deoxycholate in a randomised trial, it became the drug of choice for the treatment of IA [57]. Voriconazole interacts significantly with

tacrolimus and cyclosporine and close follow-up of its levels and dose adjustments are necessary. Co-administration with sirolimus is considered contra-indicated by the manufacturer; however a study with stem cell transplant recipients showed that voriconazole and sirolimus can be co-administered as long as the sirolimus dose is decreased by 90% and sirolimus trough levels are consistently monitored [58].

Monitoring of voriconazole drug levels seems reasonable to improve the efficacy and safety of therapy. It can be used to evaluate reasons for therapeutic failure due to sub-therapeutic drug levels, when combined to clinical response and also to assess for toxicity associated with excessive drug levels. A large variability in voriconazole trough levels has been observed [59]. A study showed a relationship between disease progression and drug concentration, with favourable responses in patients with voriconazole concentrations >2.05 μg/ml [60]. Plasma voriconazole concentrations >6 μg/ml have been shown to be associated with increased visual and hepatotoxicity [61]. The issue of therapeutic drug monitoring is discussed elsewhere in this book.

Posaconazole has been used in the therapy of IA refractory to conventional therapy in SOT recipients. Enough data for safety and efficacy is missing at this point in SOT recipients. Posaconazole interacts with tacrolimus and cyclosporine and immunosuppressant drug levels need to be closely monitored and doses adjusted. The chapter on antifungal azoles presents the most prevalent drug interactions with these drugs in the clinical practice.

Although the echinocandins have very low potential for drug interactions, tacrolimus levels need to be monitored, as it can be lowered.

Aerosolised amphotericin B can be used for prophylaxis as well as adjunctive therapy of *Aspergillus* tracheobronchitis in lung transplant recipients [62]. The safety of aerosolised amphotericin B lipid complex and liposomal amphotericin B has been compared to aerosolised amphotericin B deoxycholate in lung transplant recipients and all three formulations were found to be safe and well tolerated; however patients receiving amphotericin B lipid complex had fewer side effects [63–65].

In a prospective, multicentre, observational study of 87 SOT recipients, the use of combination therapy with caspofungin and voriconazole as primary therapy for IA was independently associated with an improved 90-day survival in patients with *A. fumigatus* infection and renal failure when compared to a control group which received a lipid formulation of amphotericin B [66]. However, at the present time, not enough evidence exists to support the use of combination antifungal therapy for IA.

5 Prophylaxis of Invasive Aspergillosis in Solid Organ Transplant Recipients

The optimal approach to prophylaxis of IA in SOT recipients remains undetermined. Several factors, including the risk of infection, efficacy of different strategies, drug interactions and adverse reactions need to be taken into consideration. At the present time, evidence exists to support the use of prophylaxis only in liver and lung transplant recipients, but even in these groups of patients, a wide variation in

Table 3 Recommended antifungal prophylaxis for invasive aspergillosis in organ transplant recipients

Organ	Antifungal agent	Duration
Lung	Aerosolized amphotericin B 6–30 mg/day Or Itraconazole /Voriconazole 200 mg twice a day	2 weeks to lifelong
Liver[a]	Lipid formulation of amphotericin 2.5–5 mg/kg/day Or Voriconazole 200 mg twice a day Echinocandins?	4 weeks or until resolution of risk factors

[a]Prophylaxis is targeted towards patients with at least one of the following risk factors: pre-transplant fulminant hepatic failure, primary allograft failure, retransplantation, requirement for renal replacement therapy, high transfusion requirements during surgery, use of monoclonal antibodies.

practice exists [12, 67–69]. Table 3 summarizes the recommendations for IA prophylaxis in liver and lung transplant recipients.

Low-doses of amphotericin B deoxycholate (0.1–0.5 mg/kg/day) and of lipid formulations of amphotericin (1 mg/kg/day) failed to provide adequate prophylaxis in liver transplant recipients [2, 70, 71]. Lipid formulations of amphotericin B at doses ranging from 1 to 5 mg/kg/day have been shown to be efficacious in the prophylaxis of IA in liver transplant recipients at high risk for development of IA. The efficacy of a lipid preparation of amphotericin B as prophylaxis for invasive fungal infections was evaluated in a cohort of liver transplant recipients requiring renal replacement therapy. In this study, prophylaxis with a lipid preparation of amphotericin B decreased significantly the rate of invasive fungal infections, including invasive aspergillosis, however, there was no improvement in patient survival [72]. In another study, patients who had 4 or more risk factors for invasive fungal infection were given a cumulative dose of 1–1.5 g of a lipid formulation of amphotericin B, with a reduction in the risk of IA from 23 to 5% [73]. The magnitude of the effect of prophylaxis was even more pronounced in patients undergoing dialysis, from 32 to 0%. In this study, the use of prophylaxis also did not show an impact in patient survival.

Amongst lung transplant recipients, there are two strategies employed for antifungal prophylaxis against IA: targeted prophylaxis directed to patients with a positive culture for *Aspergillus* spp. on surveillance bronchoscopy or universal prophylaxis, where an antifungal agent is given to all patients for a specified period of time in the early post-operative period. The later strategy is more commonly utilised [62, 64, 74]. Since in this patient population the majority of infections are tracheobronchitis or pulmonary, the use of aerosolised amphotericin is an attractive option, and this is actually the most common form of prophylaxis utilised [68, 69]. Aerosolised amphotericin allows the direct administration of the drug into the

transplanted lung, avoiding systemic side effects and drug–drug interactions. Its use is limited by tolerability. Common side effects include cough, bronchospasm and nausea. Amphotericin B deoxycholate and the lipid formulations (lipid complex and liposomal) have been shown to be safe and tolerated [64, 65], however aerosolised amphotericin B lipid complex was associated with fewer side effects [63]. One disadvantage of aerosolised amphotericin B is the fact that distribution in single lung transplant recipients occurs preferentially in the allograft, with unreliable distribution in the native lung, which could remain as a source of infection [63].

Voriconazole is also used for IA prophylaxis in lung transplant recipients. It has been shown to decrease the rate of IA in lung transplant recipients to 1.5%, however, a significant proportion of patients developed liver enzyme abnormalities [34], which need to be monitored on a regular basis. Due to interactions with calcineurin inhibitors, levels need to be measured and doses adjusted routinely.

Posaconazole is the other potential candidate for prophylaxis. The fact that it is available in an oral formulation and its activity against Zygomycete make it attractive, however, sufficient data is lacking at this time and its use in the SOT setting is not currently recommended.

6 Conclusions

In conclusion the picture of invasive aspergillosis in SOT recipients continues to evolve. Use of newer immunosppresive and administration of prophylactic strategies has resulted in the decrease incidence of *Aspergillus* infection in these patients. The appropriate prophylactic strategy and as well as role of combination therapy in these recipients remains to be determined.

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PART III
CHRONIC CAVITARY PULMONARY ASPERGILLOSIS

Chronic Cavitary Pulmonary Aspergillosis and Fungal Balls

Luciana Silva Guazzelli, Melissa Orzechowski Xavier, Flávio de Mattos Oliveira and Luiz Carlos Severo

Abstract Chronic cavitary pulmonary aspergillosis (CCPA) is an *Aspergillus*-related disease that results in considerable morbidity. Most affected patients develop CCPA after lung damage caused by conditions such as tuberculosis. Fatigue and chronic cough are common symptoms and some patients may present with life-threatening haemoptysis. The diagnosis of CCPA relies on the presence of chronic respiratory symptoms in association with a positive serology/sputum to *Aspergillus* species and elevated serum inflammatory markers. Chest imaging is essential to evaluate the extent of the disease, to monitor disease progression and to evaluate for the presence of fungal balls. Since the majority of patients are not good surgical candidates due to limited lung function or extensive pleural involvement, long-term treatment with antifungal drugs are mandatory. This is however limited by the few available options for oral therapy and by cross-resistance between the azoles.

Keywords Aspergilloma · CCPA · Chronic cavitary pulmonary aspergillosis · Chronic necrotising aspergillosis · Chronic pulmonary aspergillosis · Fungal balls

Contents

L.C. Severo (✉)
Laboratório de Micologia, Hosiptal Santa Rita, Santa Casa-Complexo Hospitalar.
A.v Independência 75, 90035-150 Porto Alegre, Brazil
e-mail: severo@santacasa.tche.br

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1 Introduction

The terminology and classification of chronic cavitary pulmonary aspergillosis (CCPA) is complex and rather confused. CCPA actually represents an umbrella term that embraces several subcategories, which are distinguished mainly on the basis of their dominant clinical and radiological manifestations. Previous nomenclatures included "semi-invasive aspergillosis", "chronic invasive pulmonary aspergillosis", "symptomatic pulmonary aspergilloma", "progressive chronic pulmonary aspergillosis", "simple/complex aspergilloma", and "*Aspergillus* pseudotuberculosis". Cavities may or not contain visible fungal balls (aspergillomas). "Simple aspergilloma" is characterised by a single cavity containing a fungal mass, which is relatively stable over time and affects non-immunocompromised patients. In contrast, a "complex aspergilloma" is characterised by chronic and progressive formation and expansion of lung cavities affecting not obviously immunocompromised individuals. Some authors would reserve the term CCPA for patients with complex aspergillomas [1].

Denning et al. [1] have proposed that both simple aspergillomas and CCPA cases should be grouped under the more general term, "chronic pulmonary aspergillosis" (CPA). However, that might be a bit tricky since "chronic pulmonary aspergillosis" and "chronic cavitary pulmonary aspergillosis" are very similar names. Moreover, the marked existing overlap in the *Aspergillus*-related conditions makes disease classification rather tough. For instance, conditions such as allergic bronchopulmonary aspergillosis (ABPA) are also theoretically chronic infections caused by *Aspergillus* species affecting the lungs (therefore CPA), though these cases result from an allergic-type reaction. It might seem logical to separate an infectious process (CPA) from an allergic condition (ABPA), but many CCPA patients also present

with eosinophilia and demonstrate elevated serum IgE against *Aspergillus* species. Conversely, inflammation, lung cavities and fibrosis might also occur with ABPA (the author is referred to the chapter by Dr. Soubani on "*Aspergillus* overlap syndromes"). When one is dealing with aspergillosis, there is no clear-cut separation between allergic and infectious processes, so "disease" is preferred to "infection". In addition, simple and stable pulmonary fungal balls are also by definition chronic cavitary forms of aspergillosis affecting the lungs – therefore, CCPA. Why some patients present with one or another disease phenotype depends largely on immune factors, and many aspects of these have been revealed in recent years.

Based on the arguments above, we make no distinction in this chapter between CPA and CCPA. Therefore all chronic cavitary conditions caused by *Aspergillus* species affecting the lungs were named CCPA. That also applied to patients with a single fungal ball showing little or no evidence of radiological progression over time. The term CPA was abandoned in this chapter.

Some other previous definitions also deserve to be considered. "Intra-cavitary *Aspergillus* colonisation" and "saprophytic aspergillosis" have both been widely used to describe CCPA. One may argue that it is inappropriate to use "colonisation" in this context, since most patients show some evidence of inflammation, based on elevated serum inflammatory markers. Moreover, many patients are symptomatic. Regarding "saprophytic aspergillosis", it should be noted that the Greek suffix "phytic" refers to "plant", and fungi no longer belong to the Plant Kingdom. The expression "mycetoma" is also inappropriate, and it should be reserved for chronic granulomatous infections usually of subcutaneous tissue and caused by traumatic inoculation in tropical and subtropical regions.

In this chapter we also demonstrate that many non-*Aspergillus* fungi have been involved in CCPA cases. Since the aetiology of the intra-cavitary content cannot be ensured on clinical or radiological bases only, the term "aspergilloma" was therefore avoided along this book. Instead, terms like "fungal ball" or "fungus ball" were preferred.

2 Aetiology

The commonest cause of a fungal ball (rounded mass located inside a preformed and poorly drained pulmonary cavity) is *A. fumigatus*. Occasionally *A. flavus* and *A. niger* are the causative agents [2]. For this reason, a positive precipitin reaction directed against all these *Aspergillus* species should be performed [3–6].

Soil and decomposing organic material are common sources of the conidia that become airborne easily [7]. The penetration of the *A. fumigatus* conidia into the lung is facilitated by their small diameter (3–5 μm). Conversely, the bigger size of *A. flavus* and *A. niger* conidia (5–6 μm and 6–7 μm, respectively) create difficulties for these to reach the alveoli [6, 8–10]. Although the term "aspergilloma" is widely used in the literature, it is worth reinforcing that fungal balls have been produced by many other fungi, especially *Scedosporium apiospermum* (teleomorph stage, *Pseudallescheria boydii*) [6, 11, 12].

3 Epidemiology

The most common and best-recognised form of aspergillosis worldwide is fungal ball that is an important complication of healed tuberculosis [13]. In the mid 1960s the British Tuberculosis Association reviewed 544 patients with healed pulmonary tuberculosis who had residual cavities of ≥2.5 cm in diameter. These patients were re-evaluated after a period of 3–4 years with chest radiographs and precipitating antibodies to *A. fumigatus* in serum. The prevalence of fungal balls was 11%, which was elevated on the next 2–3 years of study follow-up to 17% [14–16]. Although previous tuberculosis is by far the most frequent predisposing factor for CCPA, many other pulmonary conditions resulting in bulla, cyst or cavity formation have been implicated, as discussed below.

4 Pathogenesis

As already mentioned the empty residual cavity left after tuberculosis treatment is the main predisposing factor for *Aspergillus* intra-cavitary colonisation [6, 12, 14, 17]. Other associated illnesses for this syndrome include bronchiectasis [18], emphysema, bullae or lung cysts, pulmonary fibrosis, healed abscess cavities, previous *Pneumocystis jirovecii* pnemonia, cavitated bronchogenic carcinoma [19], pulmonary infarction, sarcoidosis [20–24], lung abscess, infection caused by *Mycobacteria* other than tuberculosis, and radiation fibrosis [25–27]. A fungal ball may also develop in pre-existing lung spaces caused by other fungal disease, such as paracoccidioidomycosis [28], coccidioidomycosis, cryptococcosis, blastomycosis [29], histoplasmosis [30–32], and in a damaged central bronchus of allergic bronchopulmonary aspergillosis. A similar process may occur in chronically obstructed paranasal sinuses [29, 30].

Although fungal balls are usually seen as a secondary condition to patients with pre-existing lung cavities, the co-existence of CCPA with active tuberculosis has rarely been reported [33]. CCPA may also occur following allergic conditions that affect the lungs. In those cases, *Aspergillus* antigens stimulate antibody production causing allergic bronchitis and pneumonitis, which results in central bronchiectasias. Bronchial stenosis and fungal mass in the central bronchus naturally accelerates tissue destruction of the bronchial wall and surrounding lung parenchyma and causes cavitation. The fungal ball cavity originated in the central bronchus with progression to new lesions in peripheral lung tissue may be a phenomenon specific to aspergillosis [34]. Since the fungal ball stands anatomically midway between the large bronchi and the periphery, this type of aspergillosis occasionally presents clinical symptoms of type IV allergic reaction rather than those of types I and III [26, 34].

Although haemoptysis is a frequent complication of fungal balls, the pathogenesis of haemoptysis remains unclear. Usually, the bleeding originates from an ulceration of the epithelial layer in a region of the cavity wall characterised by granulation tissue with dense proliferation of capillaries and small vessels (Fig. 1). Rarely the

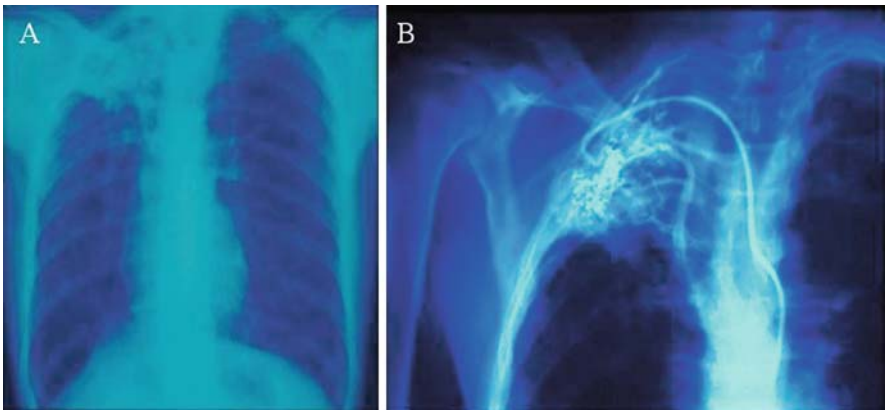

Fig. 1 (**a**) Chest radiograph showing a cavity with air crescent in the right lung; (**b**) A catheter inserted via subclavian artery demonstrating that the cavity is supplied by blood from the systemic circulation. This explains the severity of haemoptysis as occurs in many of these patients. Figure 1B, reprinted from reference [46] with permission from the publisher

bleeding originates from a bigger vessel. The triggering factor is unknown. Some believe that is traumatic due to mechanical friction by movement of the fungal ball within the cavity traumatising the highly vascular granulation tissue, whilst others suggest an enzimatic mechanism following release of by-products of the *Aspergilli* with a fibrinolytic activity, as well as *Aspergillus* endotoxins with haemolytic and anticoagulant properties [26, 34]. An immunogenic mechanism seems more plausible: symptoms, especially haemoptysis, could follow a type III hypersensitivity reaction (Arthus), leading to antigen-antibody complex formation with complement activation within the wall of the cavity. The levels of antibody, the histopathological findings, and the response to the steroid all support this idea [35–38].

In contrast to invasive aspergillosis, CCPA is thought to affect primarily considered to affect immunomocompetent patients with previous damage to the lung architecture. However, a growing number of studies in recent years have shown that subtle immune defects may put patients are higher risk of acquiring CCPA. These include a deficiency of mannose-binding lectin (which binds *Aspergillus* species avidly in vitro) [39], genetic polymorphisms in the collagen region of surfactant proteins A2 (SP-A2) [40] and cytokine abnormalities [41]. Although differences in natural history and pathogenesis between patients with simple aspergillomas and CCPA may be genetically determined, this assumption remains speculative. This topic is discussed in detail elsewhere in this book.

5 Clinical Manifestations and Natural History

CCPA resembles many chronic pulmonary diseases, and may be indistinguishable clinically from pulmonary tuberculosis. The appearance of haemoptysis in old tuberculosis when repeated attempts to find bacilli in the sputum are negative should

make one think of fungal ball. The frequency of haemoptysis in these patients has varied between 50 and 85% [42]. Haemoptysis may cause fatal asphyxiation in about 26% of patients [37].

Prominent symptoms include chronic productive cough, dyspnoea (results from the thickening of the pleura and the cavity wall), and develop systemic symptoms of malaise, fatigue, fever and weight loss [17, 26, 34, 43–45]. These are actually very common clinical symptoms (reviewed in details in reference [1]). CCPA patients frequently have a chronically ill appearance, reporting weight loss, low-grade fevers and low energy levels, and frequently over a period of several months [1, 6, 46].

After initially colonising the pre-existing lung cavity, *Aspergillus* species thrive in the poorly drained air space to grow in the cavity walls. Marked inflammation is usually observed in the surrounding parenchyma, without tissue invasion or fungal dissemination via the bloodstream. It would be incorrect however to assume that CCPA is by definition a non-invasive disease, since some degree of tissue invasion by hyphae may be observed in patients who are more immunosuppressed by condition such as diabetes mellitus, liver cirrhosis, AIDS or treatment with steroids. This clinical presentation of aspergillosis was initially described as semi-invasive pulmonary aspergillosis and chronic necrotising pulmonary aspergillosis (CNPA) [47–49].

The presence of a fungal ball makes phagocytosis of conidia by macrophages difficult. *Aspergillus* species can grow in the cavity producing hyphae that are not readily eliminated by polymorphonuclear leukocytes. The cavity generally communicates with the bronchial tree. Inside the cavity a dense mass of tangled, branched, septate hyphae is found, in association with fibrin, amorphous debris, and a few inflammatory cells. *Aspergillus* conidiophores are occasionally seen. The natural history of fungal ball is variable. Disease progression usually results in expansion of previous cavities and the formation of new ones. In late stages, the pleura may also be involved, and marked pleural thickening may occur, as well as *Aspergillus* empyema [6, 46]. Chronic fibrosing pulmonary aspergillosis (CFPA) refers to the final stage of untreated CCPA, characterised by the presence of marked lung fibrosis [1].

Fungi continuously grow and die within the cavity, which is partially regulated by conditions existing in the cavity. Devitalisation of the fungus is accompanied by changes in the mycelium and was eliminated with the sputum as small fragments of degenerating mycelium (Fig. 2). Rarely, spontaneous regression of fungal balls occurs, in about 10% of cases [26, 50]. Occasionally the lysis is associated to a pyogenic infection. The course of patient is more related to the underlying disease than to the infection itself. This dynamic process can be divided into six stages: (i) initial stage, based on the finding of mycelium in a thin layer covering part of its inner wall of the cavity; (ii) abortive form, representing a frustrate attempt of the fungus to develop and grow within the cavity; (iii) fully developed fungal ball, in which the fungus is both growing and dying showing zonation of hyphal grow and deliquescence of central hyphal strands; (iv) fungal ball containing dead fungus; (v) calcified fungal ball, with dead fungi remaining within a cavity that was calcified. This calcification may remain focal or involve the greater part

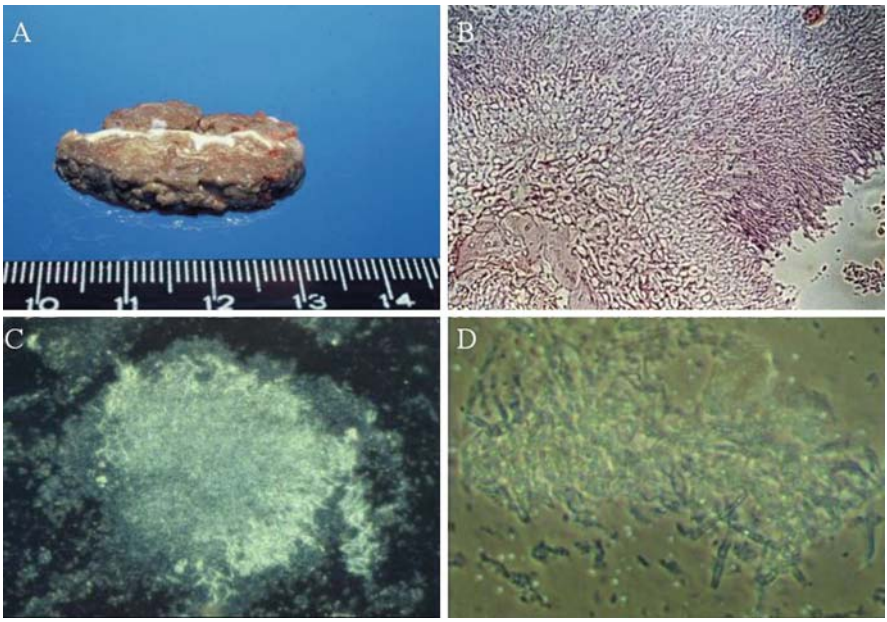

Fig. 2 (**a**) Macrocopic appearance of the zonation of hyphae growth of fungal ball; (**b**) A section of a fungal ball. Distorted, bulbous hyphae are mixed with typical forms; (**c**) Sputum with a fragmented fungal ball; (**d**) Microscopic appearance with distorted and typical hyphae

of the fungal mass [38]; and (vi) residual stage, abscesses containing fragments of mycelium. This concept has received broad approval [6, 45, 46, 51].

6 Fungal Ball and AIDS

Pulmonary aspergillosis is rarely found in patients infected with the HIV, in comparison to its prevalence in other conditions that produce similar degrees of immunosuppression. Aspergillosis occurring in HIV-infected patients is discussed elsewhere in this book.

Pulmonary fungal ball in HIV-infected patient differs in several features from those found in immunocompetent patients. Patients with HIV are particularly susceptible to cavitary lung diseases, including pneumatoceles secondary to *Pneumocystis jirovecii* pneumonia and tuberculosis, as well as necrotising bacterial pneumonias. These patients are less likely to have significant haemoptysis, which occurs in 73% of HIV-negative individuals, in comparison to 40% in HIV-infected patients. However, patients with HIV have a higher risk of CCPA progression, including the development of a semi-invasive aspergillosis (CNPA) [25].

Treatment with a combination of antiretroviral and antifungal therapy appears to be effective in improving the clinical and radiographic outcomes of patients with

HIV infection and pulmonary fungal ball [52]. Special attention should be given to drug–drug interactions in these patients.

7 Diagnosis

The most consistent diagnostic features of fungal balls are haemoptysis, chronic cough, which is usually productive, and a radiological imaging showing a mobile intra-cavitary mass with an air crescent in the lung periphery. That usually occurs in the upper lobes. Serum precipitating antibodies to *A. fumigatus* are found in virtually all patients [53]. Precipitins to other *Aspergillus* species such as *A. flavus* should be obtained in patients who are seronegative *A. fumigatus* [2]. In the series by Denning et al. [1], culture of sputum yielded positive results in 55% of cases – in all instances, *A. fumigatus* was the sole pathogen isolated. Positive culture results often occurred months or years after the patient first presented. Cytological examination of sputum revealed hyphae in only 5.5% of cases. A definitive diagnosis is established by microscopical demonstration of the septate hyphae and conidiophore of *Aspergillus* and/or culturing the *Aspergillus* from percutaneous aspirate/biopsy

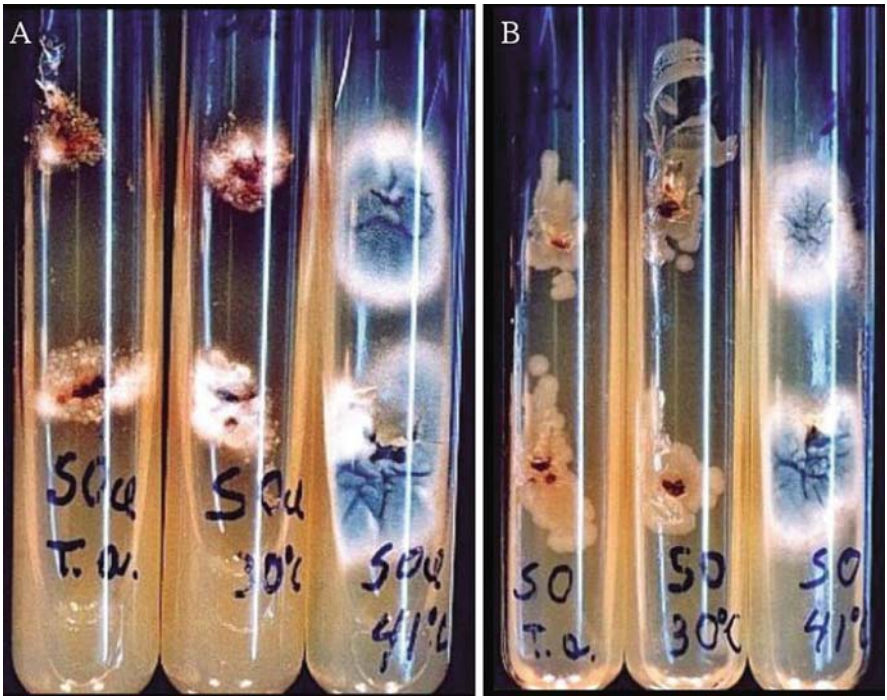

Fig. 3 Culture of a sample obtained from a pulmonary fungal ball. Incubation was performed in different media and temperature. (**a**) Sabouraud's agar cloramphenicol incubated at room temperature, 30 and 41°C; (**b**) Sabouraud's agar without antibiotic drugs, incubated the same temperatures. The pictures show that secondary bacterial colonisation can be inhibited by both adding antibiotic drugs in the media and by the use of high incubation temperatures

[28, 54], fiberoptic bronchoscopy and resected specimens of the cavity [55]. Sometimes the cultures from material taken from surgical specimens are negative because of non-viable organisms within the fungal mass and/or secondary bacterial colonisation (Fig. 3) [6, 45, 56].

7.1 Proposed Diagnostic Criteria

The diagnosis of CCPA is based on the following aspects:

(i) *Clinical data* – chronic pulmonary or systemic symptoms (usually cough, weight loss, fatigue and haemoptysis) lasting for ≥3 months in a patient with previous lung damage (usually residual cavities from previous tuberculosis). The presence of other pulmonary pathogens should be excluded and the patient should not suffer from any major immunosuppressive condition (except for CNPA, as discussed above);
(ii) *Radiology* – evidence of a slowly progressive (over several months) pulmonary lesions including cavities with surrounding inflammation. Not all CCPA patients manifest fungal balls – these are better evaluated radiologically by decubitus chest films or computed tomography (CT) cuts to demonstrate positional movements of the fungal ball;
(iii) *Bronchoscopy findings* – the fungal ball may be visualised and biopsied during fiberoptic bronchoscopy;
(iv) *Serology* – presence of precipitating (IgG) antibodies to *Aspergillus* species in serum (*A. fumigatus* precipitins test result >1:2 or detectable arcs on immunodiffusion);
(vi) *Serum inflammatory markers* – persistently elevated inflammatory markers in the serum (e.g., erythrocyte sedimentation rate, C-reactive protein or plasma viscosity);
(vi) *Cytology* – intra-cavitary percutaneous needle aspiration specimen revealing hyphae and coniophore of *Aspergillus* spp.;
(vii) *Pathological findings* – surgical/autopsy specimen revealing septated hyphae and conidiophore of *Aspergillus* spp.;
(viii) *Mycological findings* – positive results to *Aspergillus* species by microscopic/culture from respiratory tract samples

7.2 Radiological Findings

Although CCPA usually initiates in a previous lung cavity, disease progression in CCPA is usually manifested by expansion/coalescence of previous cavities and formation of new cavities and infiltrates. Initial radiological findings in these patients may include ill-defined areas of consolidation that tend to progress over time to form well-defined cavities. Multiples cavities of various sizes are commonly seen, particularly in patients with “complex fungal balls”. As mentioned before, fungal balls

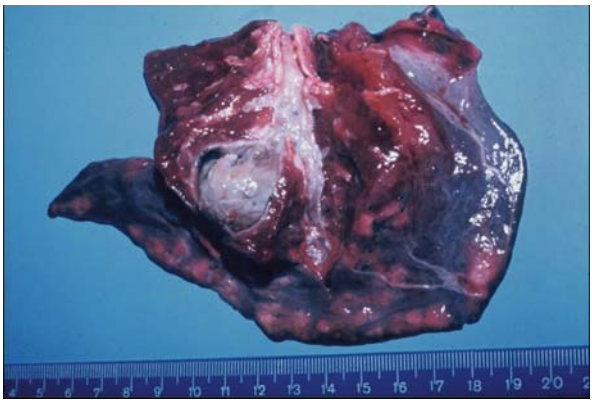

Fig. 4 Cut surface of the lower right lobe showing a single fungal ball complicating a thin-walled cyst of bronchial origin in which there was little abnormality in the surrounding lung tissue

may be divided into simple (Fig. 4) and complex (Figs. 5 and 6) types. Simple fungal ball occur in a cavity without surrounding parenchymal disease, whilst complex ones result in the formation of thick-walled cavities with surrounding parenchymal inflammation and have increased morbidity and mortality [57, 58].

Fungal ball are often found on routine chest radiograph in asymptomatic patients with a prior cavitary lung disease. Most fungal balls are located in the upper lobes reflecting the upper lobe predilection for tuberculous cavities (Fig. 7). Although a mobile mass in a pulmonary cavity that is partially surrounded by a crescent patch of air (Monod's sign) is commonly is due to fungal ball, alternative diagnoses should be considered. These include but are not restricted to debris within an abscess, intra-cavitary blood clots, a necrotic tumour, and hydatid cysts after the

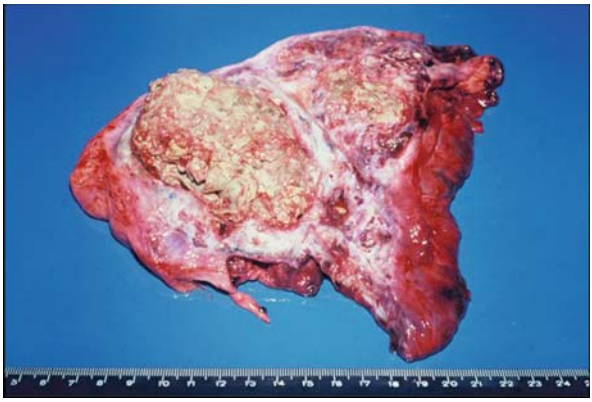

Fig. 5 Cut surface of right lung revealing a complex fungal ball, with significant inflammatory lesions in the lung tissues surrounding the cavity

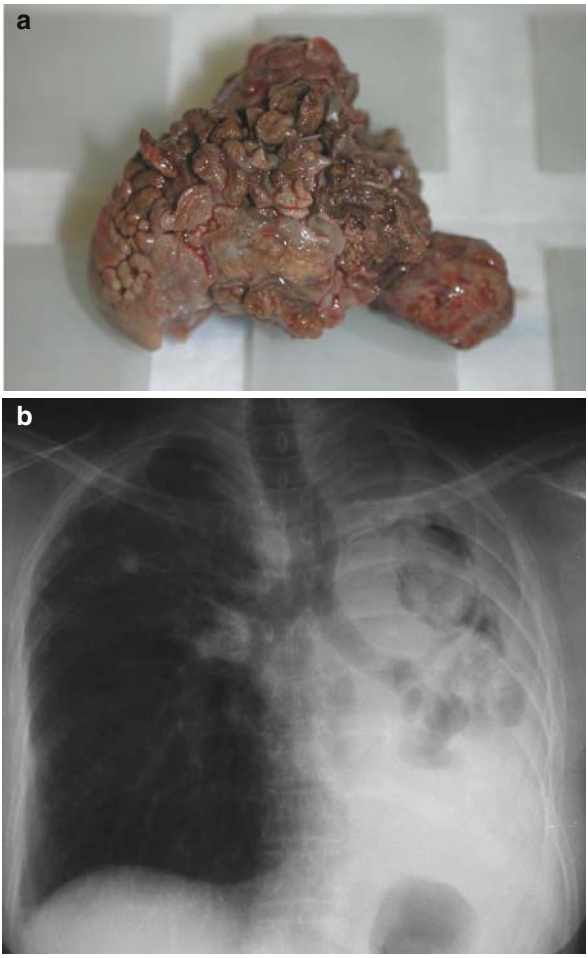

Fig. 6 A complex fungal ball removed from a patient who eventually died due to chronic cavitary pulmonary aspergillosis. Culture revealed multiple phenotypes of *A. fumitatus* showing different resistance patterns – the presence of multiple close-related genotypes were also confirmed by microsatellite typing. (**a**) Gross macroscopy; (**b**) Radiographic appearance. Courtesy of Drs. Pasqualotto and Denning

parasite has sloughed [26]. These have all very similar radiological appearances. The ball often moves as the patient changes position. If the fungal ball completely fills the pulmonary cavity, the air crescent sign may not be observed. Marked pleural thickening in areas adjacent to the cavity is common and may accompany or antedate the appearance of an intra-cavitary fungal ball, often better demonstrated by tomograms than by plain films [59]. Any new pleural thickening may be the earliest sign of fungal ball, for this reason attention to pleural changes in patients with cystic or cavitary disease in important. If such pleural thickening is recognised, tomograms and immunodiffusion test to *Aspergillus* should be made. Although fungal ball are usually single, bilateral lesions may also be present [14, 56, 60–62]. The pleural thickening is possibly due to the diffusion of toxic materials from the cavity or could result from an antibody-antigen reaction in the pleura [36].

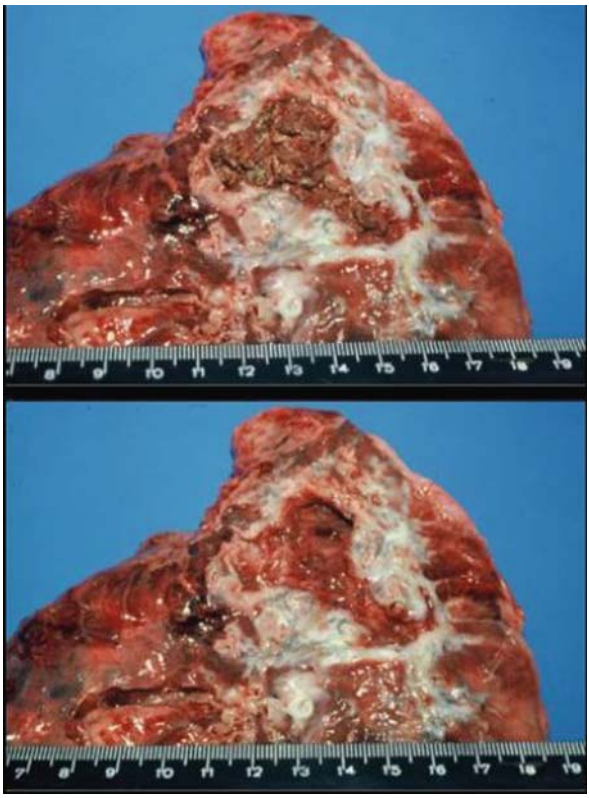

Fig. 7 Same patient as in Fig. 1. Gross pathology of the apex of the right lung reveals an old tuberculous cavity filled with a fungal ball (*upper image*). The image at the bottom shows the aspect of the same lung after fungal ball removal

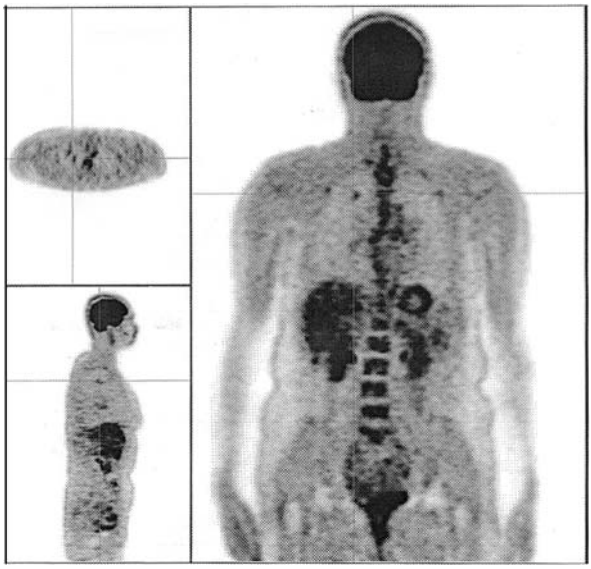

Fig. 8 Positron Emission Tomography scans in a patient with a simple fungal ball in the right upper lobe. Courtesy of Drs Pasqualotto and Denning

The chest X-ray in CCPA often shows a progressive infiltrative process with parenchymal necrosis. Unilateral or bilateral segmental areas of upper lobe consolidation that slowly cavitates over time and pleural thickening are the most frequent CT findings. One-half of the patients have intra-cavitary fungal balls. CCPA differs from CNPA since invasion of the lung tissue is observed in the latter [63, 64].

A Positron Emission Tomography (PET) scan performed on a patient with a fungal ball is shown in Fig. 8.

7.3 Sputum Examination

Cultures of *Aspergillus* from respiratory tract alone are not sufficient for the diagnosis of fungal ball. Repeatedly positive sputum cultures bring more clinical significance, especially when combined with typical chest roentgenographic findings. If fungal elements are seen on sputum microscopical examination, culture of the specimen is diagnostic [6].

7.4 Aspergillus *Precipitins*

Serological testing for detection of antibodies to *Aspergillus* antigens can be very helpful in the diagnosis of fungal ball. The fungal mass of the fungal ball provides abundant antigenic stimulation, for this reason, serum precipitating antibodies to *Aspergillus* antigen are found in 80–100% of immunocompetent patients who harbour a fungal ball, using the double diffusion (Immunodiffusion) technique in agar gel, according to Ouchterlony [65], using a culture filtrate or mycelial extract antigens of *A. fumigatus*, *A. flavus*, and *A. niger*. Most of the fungal ball cases do not

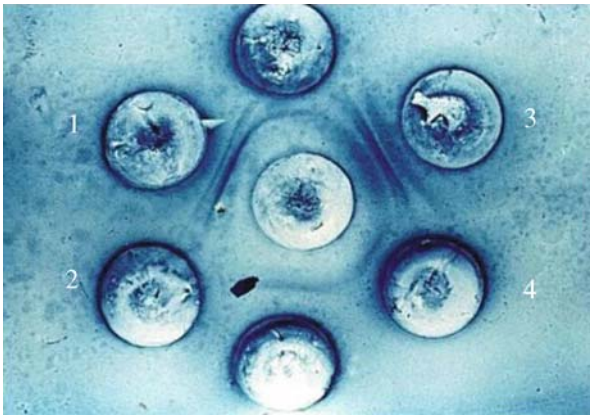

Fig. 9 In vitro detection of precipitating antibodies against *Aspergillus fumigatus*. Central well contains *A. fumigatus* culture filtrate antigen. Upper and bottom wells contain positive control sera. The other wells contain sera from patients. Wells 1, 3 and 4 show the specific precipitating lines. Multiple bands are seen in case 3 suggesting fungal ball – case 2 is negative

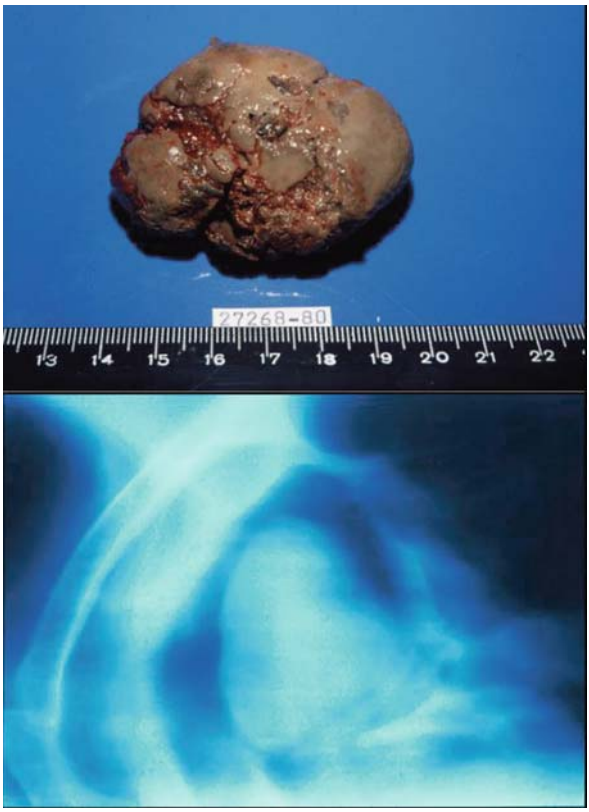

Fig. 10 Gross pathology (*upper image*) and radiologic appearance (*bottom image*) of a highly dense fungal ball composed of dead fungi

require serum concentration to demonstrate precipitin bands. The production of one or more lines of identity by a patient's serum interacting with antigen constitutes a positive reaction. Patients with fungal ball generally have one to four arcs of precipitation antibodies (Fig. 9), the presence of three or four is strong evidence of fungal ball. The absence of precipitating antibody in a small number of patients may be related to the presence of dead fungi (Fig. 10), gamma globulin deficiency, immunosuppressive therapy, or poor antigenicity of the pathogenic fungi. Patients with fungal balls usually do not become seronegative until several months after excision of the lesion. The immunodiffusion is widely used because of its ease of performance. In addition, no special expertise or costly equipment is required [26, 66–73]. The methodology involved in precipitin detection is discussed elsewhere in this book.

8 Treatment

Treatment of CCPA has to be individualised, depending on the severity of symptoms, extent and location of the pulmonary involvement, relationship to other diseases, and the patient's general condition. Thus, treatment of fungal ball must be

adapted specifically to each patient, with the entire clinical presentation dictating the appropriate management [13, 26, 27, 74–78].

8.1 Role of Surgery

The most frequent symptom indicating surgery is haemoptysis, because of the risk of massive and fatal bleeding. Surgical resection is the treatment of choice for symptomatic localised disease (i.e., simple fungal ball), in suitable candidates. Resection should only be undertaken if there is sufficient respiratory reserve and the resection removes minimal functioning pulmonary tissue [43, 53]. An important question is to determine how the remaining lung parenchyma is able to re-expand, in order to avoid postoperative space problems. However, surgical resection is often considered to be hazardous or impossible in individuals with complex fungal balls, because of underlying disease, impairment of respiratory function or old age. Surgery may be technically difficult in these patients, and most surgeons would opt for a lobectomy in a patient who has a reasonable operative risk [37, 67]. The surgical treatment is associated with relativity high mortality (8.2%) [79]. Bleeding, residual pleural space, bronchopleural fistula, and empyema are the most common surgical complications (24.5%) [43, 80–84]. Surgical options for the treatment of CCPA are discussed elsewhere in this book.

Intra-cavitary instillation of amphotericin B, in combination with cavernostomy has been described, although this only occasionally cures a fungal ball [44, 85, 86].

8.2 Antifungal Therapy

Due to the already discussed limitations of surgery in the treatment of CCPA, long-term antifungal therapy will be required for most patients. A number of medical approaches have been explored to treat CCPA, as discussed below.

8.2.1 Itraconazole

The benefit of therapy with oral itraconazole has been demonstrated in several small uncontrolled studies, with dosages ranging from 100 to 200 mg (once or twice daily) [87–91]. The overall response rate is ∼45% [1], which may be underestimated since many patients might have received lower itraconazole dosages than the currently recommended 200 mg twice daily. Administration of the oral itraconazole dose with a cola drink is advisable to ensure adequate absorption. Therapy is best monitored by the use of serum drug levels, which was not performed in most studies – therefore, underexposure to itraconazole cannot be ruled out. In addition, many patients were treated for short periods (i.e., 5–7 months only). In the absence of curative surgery, CCPA is regarded as an incurable disease requiring long-term (if not life-long) antifungal therapy, since many patients relapse after this is stopped [1]. Patients with suboptimal drug levels while on itraconazole capsules should be treated with itraconazole solution, which is associated with a better absorption.

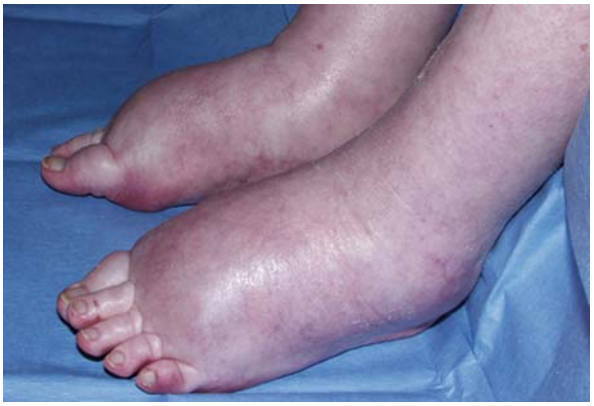

Fig. 11 Marked ankle oedema in a patient on treatment with itraconazole, a rare phenomenon. This effect is more pronounced in patients receiving concomitantly Calcium channel blockers. Itraconazole has negative inotropic effects and heart failure has to be excluded in these patients. Courtesy of Drs Pasqualotto and Denning

Itraconazole is the antifungal drug of choice for long-term treatment of CCPA. Its lipophilic characteristics may allow for great drug concentrations to be achieved in fungal balls. In fact, itraconazole achieves 10-fold higher concentrations in fungal ball than in the healthy lung parenchyma (837 ng/g versus 81 ng/g, respectively). Accordingly, Tsubura described that 63% of CCPA patients responded to itraconazole treatment (100–200 mg daily) in largest series described in the literature [89].

Long-term therapy with itraconazole frequently results in considerable drug toxicity (Fig. 11), which is discussed elsewhere in this book.

8.2.2 Voriconazole

Due basically to costs, voriconazole is reserved to patients failing therapy with oral itraconazole due to toxicity, persistently low serum levels or disease progression. Patients with persistently low itraconazole levels are at greater risk for drug resistance [92] – once that occurs, cross-resistance with voriconazole and other azoles such as posaconazole is frequently observed. The limited options for long-term oral treatment of CCPA are one of the major problems in treating this disease.

Voriconazole was first evaluated in an open non-comparative study involving 12 medical centres [93]. The study included CCPA patients and also patients with subacute invasive aspergillosis, but not simple fungal ball cases. A total of 36 patients were treated with voriconazole at 200 mg twice daily for 4–24 weeks. Voriconazole was given as primary therapy for 61% of patients. Overall response rate was 80% in the CCPA, which included partial responses and stable disease. Complete responses are rarely seen in CCPA. The second study [94] included 11 CCPA patients from Manchester (UK), all of which had been intolerant or failing itraconazole. Voriconazole was given in dosages ranging from 150 to 200 mg twice daily, which was

adjusted based on drug levels or tolerance. Most patients showed clinical and laboratorial improvement after 3 months of treatment – however, voriconazole resistance was detected in one patient after 18 months. The third study with voriconazole was a retrospective French cohort in whom 24 CCPA patients (15 with CNPA) were evaluated [95]. Clinical improvement and clinical and radiological improvement after 2–3 months of therapy occurred for 45 and 30% of patients, respectively. These rates were increased to 67 and 58% respectively after therapy for 10 months. Nearly all patients (95%) had negative sputum culture for *Aspergillus* spp. after 10 months of therapy with voriconazole.

Some patients failing therapy with voriconazole may still be treated with posaconazole, although a high risk for cross-resistance exists (Drs. Pasqualotto and Denning, unpublished data).

8.2.3 Intravenous Antifungal Therapy

Amphotericin B deoxycholate has poor penetration in fungal balls and intravenous treatment with intravenous amphotericin B has been regarded as ineffective [20]. Denning et al. however showed that short-lived responses to IV amphotericin B occurred in 80% of patients failing itraconazole therapy [1]. Admitting severely ill CCPA to the hospital for a course of IV antifungal drugs (e.g., for $\geq$2 weeks) may result in clinical benefit in the short term.

A small study [96] also evaluated the impact of micafungin therapy in CCPA. Nine patients were evaluated, 5 of which received concomitantly other antifungal drugs. After therapy for $\geq$4 weeks, most patients responded (77.8%). Moreover, no deterioration occurred, and micafungin was well tolerated.

8.2.4 Intra-Cavitary Use of Antifungal Drugs

Very limited data is available regarding intra-cavitary use of antifungal agents, the rational here being achieving high local levels of the antifungal compound with limited systemic toxicity. Most studies evaluated amphotericin B deoxycholate administration [86, 97–100], with some data also available for endoscopic intra-cavitary instillation of ketoconazole [101]. Hargis et al. reported their experience on treating 6 CCPA patients [101]. Amphotericin B deoxycholate was diluted in 10–20 ml of 5% dextrose water and given at 50 mg 2–3 times a week up to a dose of 500 mg. Clinical benefit was seen in four patients. It has been questioned if this benefit simply results from mechanical lavage of the cavity. Adverse events included a small pneumothorax and systemic reactions to amphotericin B (one patient each). Intracavitary amphotericin B requires close patient follow up, the placement of chronic indwelling catheter and proper patient compliance. This therapy should be reserved to cases in which other therapeutic approaches have failed. Other reports have also described the administration of amphotericin B by inhalation to patients with CCPA, including *Aspergillus* empyema [85, 102].

8.3 Bronchial Artery Embolisation and Haemoptysis Control

Bronchial artery embolisation has been used to occlude the vessel that supplies the bleeding site in patients experiencing haemoptysis [13, 103]. Unfortunately, bronchial artery embolisation is usually unsuccessful or only temporarily effective. Additional treatments for haemoptysis have included radiotherapy [13, 70, 104–106]. Tranexamic acid is also frequently used for this purpose.

8.4 Other Measures

Adequate treatment of the underlying pulmonary disease is also of great importance. Depending on the lung condition, treatment might include the use of inhaled steroids, bronchodilators and/or oxygen. Treating concomitant pulmonary infections is also essential.

Anecdotal data also exist regarding the use of interferon gamma (IFN-γ) in CCPA patients [1]. The basic principle would be driving the immune system in favour of an "antifungal" T helper-1 reaction instead of an "inappropriate" T helper-2 reaction. Kelleher et al. [107] stated that 2 CCPA patients with reduced blood levels of IFN-γ benefited from IFN-γ therapy. A benefit has also been demonstrated to a few patients with invasive aspergillosis [108–111]. Therapy with IFN-γ is highly limited by poor tolerability and by the lack of strong clinical data supporting its use.

8.5 Monitoring Therapy

Most patients effectively responding to antifungal therapy demonstrate clinical and laboratorial improvement in the first 8 weeks of treatment. The earliest parameters to be monitored include weight gain and energy levels, the latter being a bit subjective. Laboratorial tests include serum inflammatory markers and *Aspergillus* precipitins titres. Radiological improvement does not occur early in the curse of medical treatment. Some improvement is usually seen only after months of therapy, manifested by reduction in both inflammation surrounding cavities and cavity sizes [1].

Due to the possibility of secondary resistance to emerge during long-term treatment with antifungal drugs, sputum should be culture and azoles blood levels checked on a frequent basis. Special attention may be required on patients taking generic formulation of antifungal drugs [112]. All *Aspergillus* isolates recovered in culture should be identified at the species level – susceptibility to the antifungal drugs should be determined, including evaluation of the minimum inhibitory concentrations (MIC) and minimum effective concentrations (MEC). Antifungal drug toxicity including liver function tests should be routinely monitored.

9 Prevention

Environmental exposure to fungal conidia is a risk factor to fungal ball. For this reason the clinicians should counsel patients with all kind of lung cystic and cavitary parenchymal disease sequelae, especially old chronic tuberculosis cavities, to limit exposure to areas with building work, especially demolition, gardening, and with increased amounts of decaying vegetation such as composting facilities. Prophylactic excision of asymptomatic persistent pulmonary cavities for the prevention of fungal ball is not justified.

10 Comments About Our Study

Our group have been collecting information from over 305 CCPA patients (diagnosed by serologic and/or tissue examinations) over the last 10 years. The cases were classified as: *Aspergillus fumigatus* ($n = 246$); *A. niger* ($n = 23$); *A. flavus* ($n = 7$); *Pseudallescheria boydii* ($n = 4$); fungal infection not specified ($n = 21$) and Actinomycetes ($n = 4$). Due to their frequency and the scarcity of literature about *A. niger*, the groups *A. niger* (cases) and *A. fumigatus* (controls) were compared with respect to clinical and laboratorial variables [6, 12, 113, 114].

10.1 Fungal Ball Due to Aspergillus niger

During an 18-year period (1977–1995), 23 CCPA cases due to *A. niger* were diagnosed in our service. The diagnoses were all ascertained by a combination of the following criteria: (i) radiological features consistent with a fungal ball; (ii) detection of antibodies to *A. niger* by immunodiffusion test; (iii) presence of characteristic conidial head in the direct examination of specimen obtained from the intra-cavitary mass and/or in sections of the ball (Fig. 12); and (iv) isolation of *A. niger* from specimens obtained by transthoracic needle aspiration, surgery or necropsy [115].

Twenty-one patients (91.3%) were males and the mean age was 45.7 years old, ranging from 23 to 66 years (Table 1). Nineteen patients (82.6%) had tuberculosis, which in was found to be active in 4 patients (Fig. 13) [16]. Seven patients (30.4%) had diabetes mellitus. Cough, expectoration, and haemoptysis were the most frequent complaints (Table 2). The chest roentgenographic findings were typical of fungal ball in seventeen patients (73.9%). Associated conditions and laboratory variables can be seen in Tables 3 and 4. Serum precipitins (immunodiffusion) against *A. niger* antigens were positive in 18 patients (78%). Amongst the 8 patients with positive fungal identification, 6 (75%) presented with an *A. niger* conidial head in tissue sections (Fig. 12) [115].

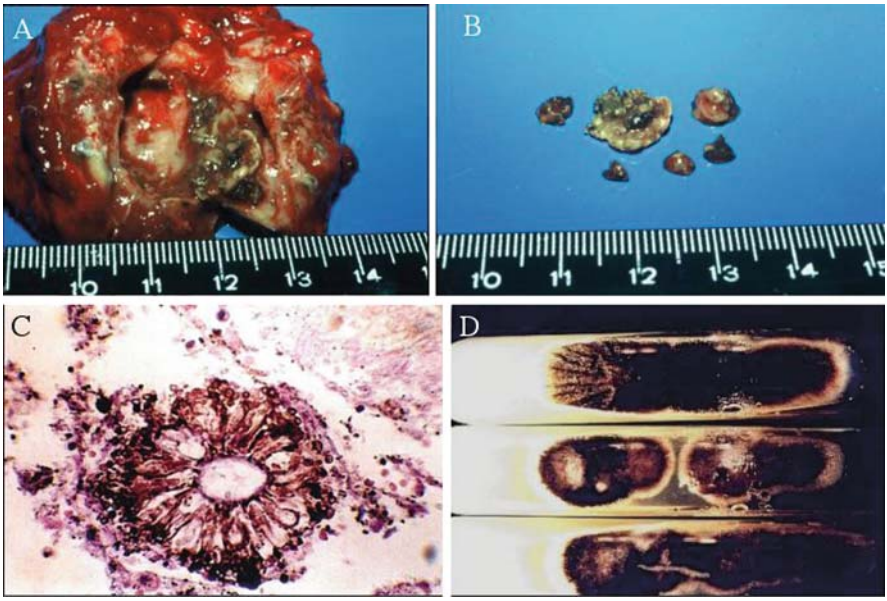

Fig. 12 (**a**) and (**b**) Black fragments obtained from a fungal ball that was removed by segmentectomy in a diabetic patient; (**c**) Histological section of a fungal ball showing typical conidial head of *A. niger*; (**d**) Culture from the fragments of that fungal ball revealing growth of *A. niger*. Reprint from reference [46] with permission from the publisher

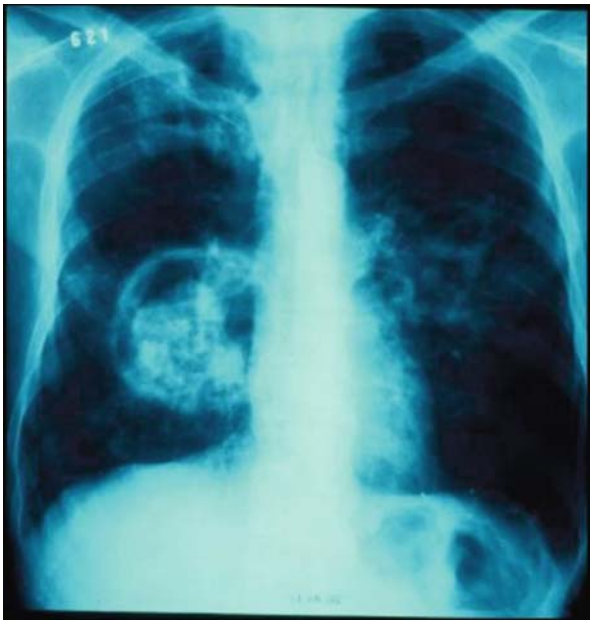

Fig. 13 Chest X-ray of a patient in whom active tuberculous lesions coexist with a fungal ball due to *A. niger*. Reprint from reference [46] with permission

Table 1 Clinical features of chronic cavitary pulmonary aspergillosis (CCPA) patients with *Aspergillus niger* infection [115]

No	Age, Sex	Underlying condition	Symptoms description (and duration) prior to diagnose	Therapy	Outcome
1	26, M	Cured TB	Cough, sputum production, weight loss (6 months)	Cavernostomy	Cured
2	60, M	Cured TB	Dyspnoea, fatal haemoptysis (2 months)	None	Cured
3	49, M	Active TB, DM	Cough, sputum production, massive haemoptysis, weight loss (3 months)	None	Died
4	56, M	Cured TB, COPD	Cough, sputum production, dyspnoea, slight haemoptysis (4 years)	None	Died
5	49, M	Active TB, COPD	Cough, sputum production, dyspnoea, slight haemoptysis (1 year)	None	Died
6	47, M	Cured TB	Cough, sputum production, dyspnoea, massive haemoptysis, weight loss (5 months)	Radiotherapy	Improve
7	38, M	Active TB, COPD	Cough, sputum production, dyspnoea, fatal haemoptysis (1 year)	Radiotherapy	Died
8	46, M	Cured TB	Cough, sputum production, massive haemoptysis (1 month)	Lobectomy	Cured
9	59, M	COPD, lung cancer	Cough, sputum production, weight loss (5 months)	None	Unknow
10	56, M	Cured TB	Cough, sputum production, slight haemoptysis (4 years)	None	Died
11	34, M	Active TB	Cough, sputum production, fever, massive haemoptysis (1 month)	None	Unknow
12	66, M	Cured TB, DM	Cough, sputum production, fever, slight haemoptysis, weight loss (1 year)	None	Died

Table 1 (continued)

No	Age, Sex	Underlying condition	Symptoms description (and duration) prior to diagnose	Therapy	Outcome
13	85, M	Cured TB	Cough, sputum production, weight loss (1 year)	None	Cured
14	36, M	Cured TB, renal failure	Cough, sputum production, weight loss (2 weeks)	None	Unknow
15	53, M	Cured TB	Cough, sputum production, dyspnoea, massive haemoptysis (2 months)	None	Improved
16	23, M	Cured TB	Cough, sputum production, slight haemoptysis, weight loss (3 months)	None	Unknow
17	55, M	Cured TB	Cough, sputum production, weight loss (2 weeks)	None	Died
18	38, M	Cured TB	Cough, sputum production, slight haemoptysis, weight loss (2 months)	Segmentectomy	Cured
19	51, F	Bronchiectais, DM	Cough, sputum production, mild haemoptysis (1 month)	Lobectomy	Cured
20	34, M	Cured TB	Cough, sputum production, mild haemoptysis, weight loss (2 months)	Segmentectomy	Cured
21	37, F	Lung abscess	Cough, sputum production, massive haemoptysis, weight loss (1 month)	None	Unknow
22	30, M	Active TB, DM	Cough, sputum production, fever, slight haemoptysis (5 years)	Lobectomy	Cured
23	66, M	DM	Cough, sputum production, slight haemoptysis (1 month)	Segmentectomy	Cured

Legend: COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; F, female; M, male; TB, tuberculosis.

Table 2 Comparison of signs and symptoms observed in chronic cavitary pulmonary aspergillosis (CCPA) patients affected by *Aspergillus niger*, as reported in the literature and in our series [115]

	Literature ($n = 40$)		Our series ($n = 23$)	
Symptoms and signs	n	% (95 CI)	n	% (95 CI)
Cough and expectoration	26	65.0 (48.3–78.9)	21	91.3 (70.5–98.5)
Haemoptysis	22	55.0 (38.7–70.4)	19	82.6 (60.5–94.3)
Fever	13	32.5 (19.1–49.2)	65	21.7 (8.3–44.2)
Weight loss	6	15.0 (6.2–30.5)	10	85.5 (24.0–65.1)
Weakness	3	7.5 (2.0–21.5)	6	26.1 (11.1–48.7)
Cachexia	2	5.0 (0.9–18.2)	2	8.7 (1.5–29.5)
Breathlessness	4	10.0 (3.3–24.6)	7	30.4 (14.1–53)
Cyanosis	1	2.5 (0.1–14.7)	1	4.3 (0.2–24.09)
Chest pain	1	2.5 (0.1–14.7)	4	17.4 (5.7–39.6)
Anorexia	4	10.0 (3.3–24.6)	0	0.0 (0.0–17.8)

Legend: CI, Confidence interval.

Table 3 Conditions associated with chronic cavitary pulmonary aspergillosis (CCPA) caused by *Aspergillus niger* [115]

	Literature ($n = 40$)		Our series ($n = 23$)	
Condition	n	% (95% CI)	n	% (95% CI)
Pulmonary oxalosis	10	25.0 (13.2–41.5)	6	26.1 (11.1–48.7)
Systemic oxalosis	1	2.5 (0.1–14.7)	1	4.3 (0.2–24.0)
Acute invasive aspergillosis	2	5.0 (0.9–18.2)	0	0.0 (0.0–17.8)
Chronic necrotising pulmonary aspergillosis	4	10.0 (3.3–24.6)	2	8.7 (1.5–29.5)
Uraemia	1	2.5 (0.1–14.7)	1	4.3 (0.2–24.0)
Allergic bronchopulmonary aspergillosis	1	2.5 (0.1–14.7)	0	0.0 (0.0–17.8)

Legend: CI, Confidence interval.

Fourteen patients did not receive any treatment and 7 patients underwent surgical resection. The remaining 2 patients (Cases 6 and 7) with massive haemoptysis had no conditions for surgery and were submitted to radiotherapy. Ten patients survived: 7 were considered cured by surgery; 1 had spontaneous lysis of the ball (Case 13) and the remaining two patients improved slightly. Eight patients died, 2 as a result of haemoptysis (Cases 2 and 7), 1 of systemic oxalosis (case 17), and the remaining 5 patients of unknown causes. Five patients were lost to follow-up (Table 5) [115].

In comparison to *A. fumigatus* infection, *A. niger* infection was significantly associated with male sex, nosocomial infections, active tuberculosis, diabetes mellitus

Table 4 Laboratory variables in *Aspergillus niger* chronic cavitary pulmonary aspergillosis (CCPA) [115]

	Literature ($n = 40$)		Our series ($n = 23$)	
Diagnostic findings	N	% (95% CI)[a]	n	% (95% CI)[a]
A. niger immunodiffusion	7	17.5 (7.9–33.4)	18	78.3 (55.8–91.7)
Sputum				
Fragments of fungal ball	–	65.0 (48.3–78.9)	1	4.3 (0.2–24.0)
A. niger isolation	26		–[b]	
Bronchial secretion				
A. niger isolation	9	22.5 (11.4–38.9)	–	–
Transthoracic biopsy				
A. niger conidial head	–		1	4.3 (0.2–24.0)
A. niger isolation	2	5.0 (0.9–18.2)	2	8.7 (1.5–29.5)
Tissue section				
Biopsy	1	2.5 (0.1–4.7)	2	8.7 (1.5–29.5)
Surgery	6	15.0 (6.2–30.5)	6	26.1 (11.1–48.7)
Necropsy	9	22.5 (11.4–38.9)	1	4.3 (0.2–24.0)
Fungal identification				
A. niger conidial head	8	20.0 (9.6–36.1)	5	21.7 (8.3–44.2)
A. niger isolation	15	37.5 (23.2–54.2)	4	17.4 (5.7–39.5)

[a]95% Confidence interval.
[b]Isolation of *A. niger* from sputum was not utilised as a diagnostic criteria in our series.

Table 5 Treatment and outcome in *Aspergillus niger* chronic cavitary pulmonary aspergillosis (CCPA) [115]

	Literature ($n = 40$)		Our series ($n = 23$)	
	n	% (95% CI)	N	% (95% CI)
Treatment[a]				
Surgery				
Monaldi drainage	1	2.5 (0.1–14.7)	–	
Cavernostomy	–		1	4.3 (0.2–24.0)
Lobectomy	6	15.0 (6.2–30.5)	6	26.1 (11.1–48.7)
Pneumonectomy	2	5.0 (0.9–18.2)	–	
Antifungal therapy				
Topical treatment	3	7.5 (2.0–21.5)	1	4.3 (0.2–24.0)
Systemic administration				
Amphotericin B	2	5.0 (0.9–18.2)	1	4.3 (0.2–24.0)
Fluconazole	1		–	
Radiotherapy	–		2	8.7 (1.5–29.5)
None	6	15.0 (6.2–30.5)	14	60.9 (38.8–79.5)
Not recorded	15	37.5 (23.2–54.2)	–	
Outcome				
Survived	14	35.0 (21.1–51.7)	10	85.5 (23.9–65.2)
Died	11	27.5 (15.1–44.1)	8	34.8 (17.2–57.2)
Unknown	12	30.0 (817.1–46.7)	5	21.7 (8.3–44.2)

[a]Some patients received more than one drug.
Legend: CI, Confidence interval.

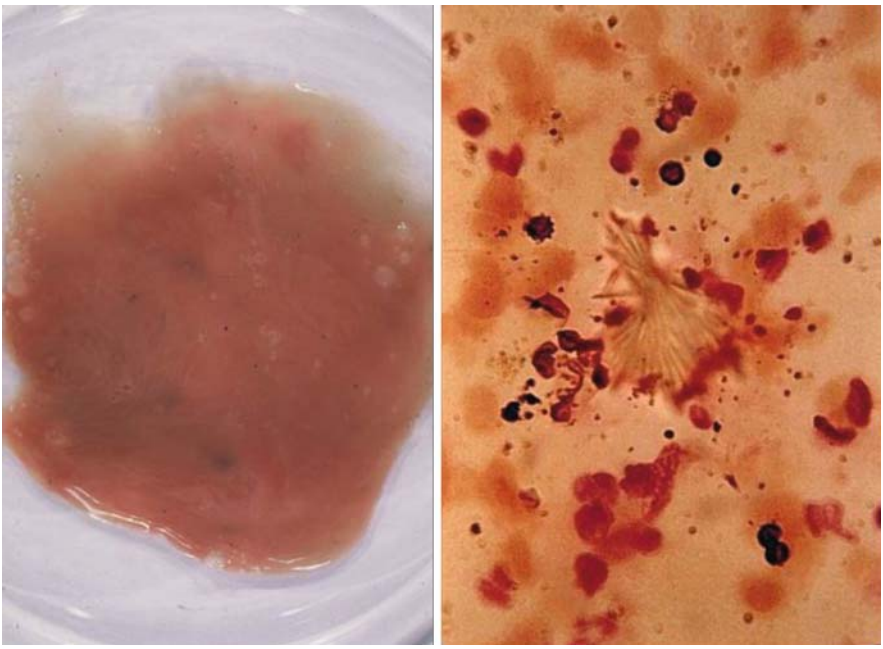

Fig. 14 Sputum analysis from a patient with haemoptysis showing *A. niger* conidia and calcium oxalate crystals

and a lethal outcome. In addition, systemic oxalosis or the presence of calcium oxalate crystals in sputum were only observed in patients with *A. niger* infections (Fig. 14). Oxalosis (Figs. 15 and 16) occurred more frequently in diabetic patients with CCPA (60%) than in CCPA patients without diabetes (13%) ($p<0.001$ for the comparison). Data obtained from this series of 23 cases as well as 40 other cases collected from the literature are shown in Tables 1, 2, 3, 4 and 5. Initially there was no substantial demographic difference between the series. Table shows that there were no statistically significant differences regarding pulmonary symptoms and overall complaints ($p>0.05$). Although reported in both groups, tuberculosis was statistically more common in our series as a predisposing factor than in the literature (82.6 and 27.5%, respectively; $p<0.05$). Regarding associated conditions there were no statistically significant differences. However, dystrophic oxalosis was by far the most common associated condition reported in both series [6, 115–117].

10.2 Fungal Ball Due to Scedosporium apiospermum (Pseudallescheria boydii)

S. apiospermum is classified as an ascomycetes; the homothallic teleomorph state is called *P. boydii*, previously named as *Allescheria* and *Petriellidium*. This fungus is ubiquitously found worldwide, occurring in nutrient-rich, poorly aerated environment, such as polluted water [114].

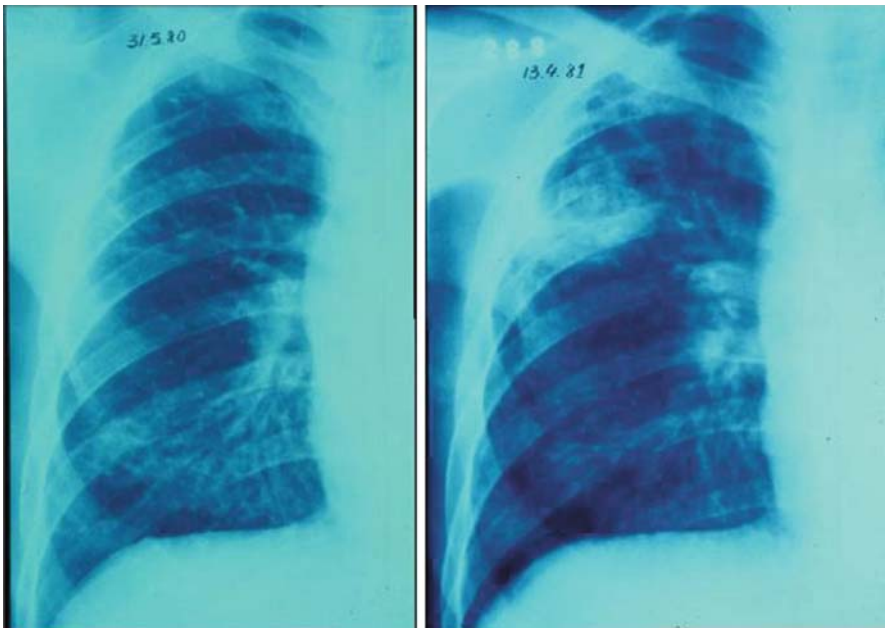

Fig. 15 Chest X-ray showing a fungal ball within a cavity that appeared in a healthy lung parenchyma. This diabetic patient presented with cavity wall invasion characterizing chronic necrotising pulmonary aspergillosis (sub-acute invasive aspergillosis or CNPA). Reprint from reference [46] with permission

The spectrum of disease in the respiratory tract is similar in terms of variety and severity to those caused by *Aspergillus* (Fig. 17). Differential diagnosis is mandatory because of the frequent resistance of the *Scedosporium* species to a variety of commonly used anti-mycotic agents [114].

The definitive aetiological characterisation of *S. apiospermum* as the agent of fungal balls requires recovery of the organism by culture from the cavity. Table 6 condenses five cases of fungal ball due to *S. apiospermum* emphasising the need for a careful search for histological demonstration of conidia, specially in culture-negative cases and in the absence of *Aspergillus* conidial heads, since some cases of scedosporiosis were mistakenly diagnosed on histological examination as aspergillosis [114]. Serum precipitins may be of aid in diagnosis and can be used as a screening test [12].

Other microscopically distinctive features are the production of anneloconidia and chlamydospores in the fungal ball (Fig. 18). Anneloconidia represents an actively growing element of the fungus deeply in the zonation of hyphal growth. The peripheral zone is composed of densely packed aggregation of large, thick-walled chlamydospores. Amongst these, it can observe an intermediate zone with both fungal elements. These findings raise the hypothesis that the chlamydospores constitute a phase of the anneloconidia that comes up in contact with an air space [114].

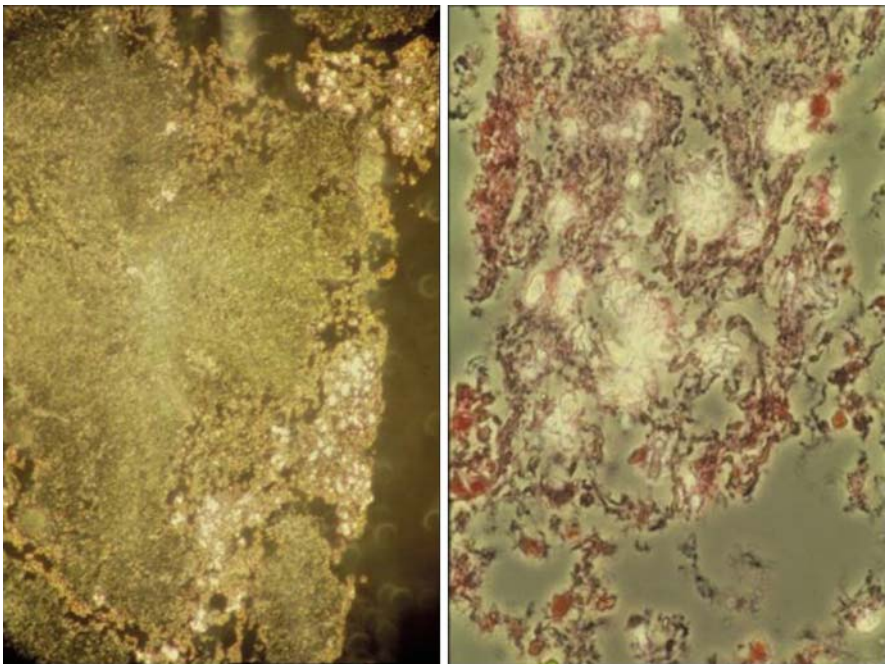

Fig. 16 Oxalosis associated with *A. niger* fungal ball. Oxalete crystals in the cavity wall. Reprint from reference [117] with permission

Surgical removal of the fungal ball is the most successful method of *S. apiospermum* fungal balls, as observed in our patients [114].

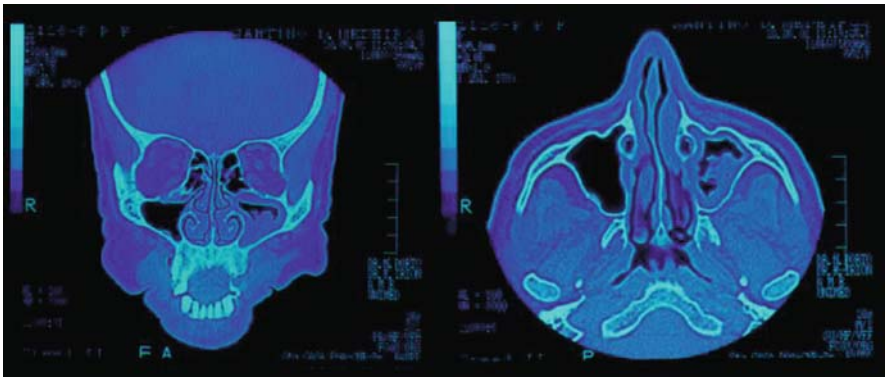

Fig. 17 A computed tomography scan showing partial opacification of the left maxillary sinus along with thickening of the sinus mucosa with irregular soft tissue mass, with anfractous contours of a fungal ball due to *S. apiospermum*

Table 6 Demographics and clinical manifestations of four patients with fungal balls due to *Scedosporium apiospermum* infection [11, 118]

Case	Sex, age	Primary disease; previous treatment	Site	Pathology; culture	Treatment; outcome
1	M, 45	Rheumatic arthritis; therapy with steroids	Lung: right upper lobe	Conidia; *S. apiospermum*	Surgery; recovered
2	F, 36	Diabetes mellitus	Lung: right lower lobe	Conidia; *S. apiospermum*	Surgery; recovered
3	F, 57	Healed tuberculosis	Lung: left upper lobe	Conidia; *S. apiospermum* (*P. boydii*)	Surgery; recovered
4	M, 66	None	Maxillary sinus	Conidia;	Surgery; recovered
5	M, 65	Active tuberculosis	Right upper lobe and right lower lobe	*S. apiospermum* *S. apiospermum* (*P. boydii*)	Died

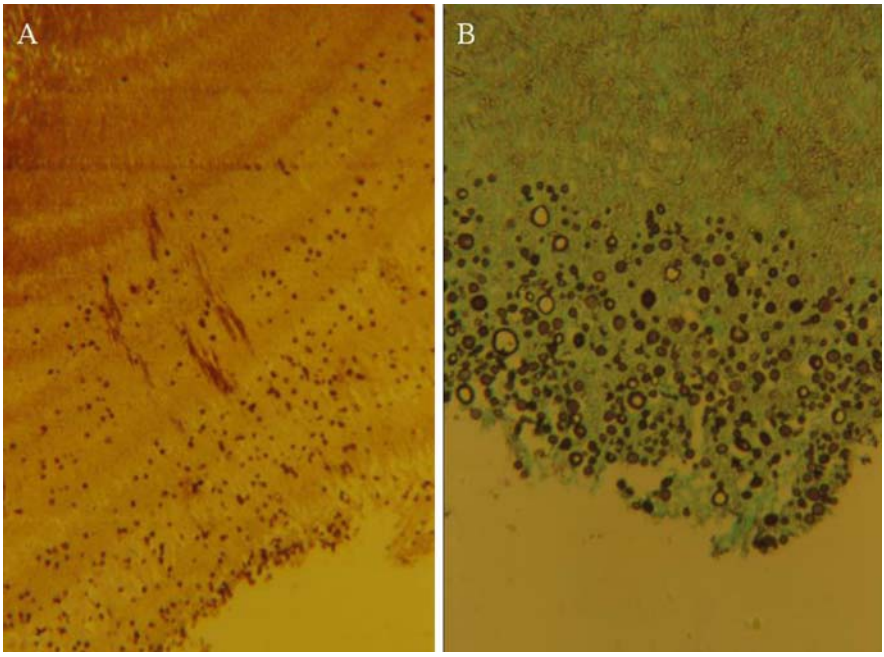

Fig. 18 (**a**) Anneloconidia of *S. apiospermum* (growing elements); (**b**) Chlamydospores of *S. apiospermum* (resistance structure). These are typical fungal elements of *Scedosporium* in the infected aerate cavity that differs it from the conidiophores of *Aspergillus*

Table 7 Actinomycotic intra-cavitary lung lesions – clinical findings [113]

Case	Age, sex	Symptoms	Predisposing condition	Site of the lesion
1	42, M	Haemoptysis, purulent expectoration (1 year)	No	Right upper lobe
2	85, M	Haemoptysis, anorexia, weakness, dyspnoea, chest pain (8 years)	Diabetes melitus	Left upper lobe
3	64, M	Haemoptysis, foul-smelling purulent expectoration (1 year)	Diabetes; 7 years prior, lung abscess in the same position	Left lower lobe
4	65, M	Haemoptysis, dyspnoea (5 years), purulent expectoration	Diabetes mellitus; bronchopulmonary obstructive disease	Right upper lobe

Legend: F, female; M, male.

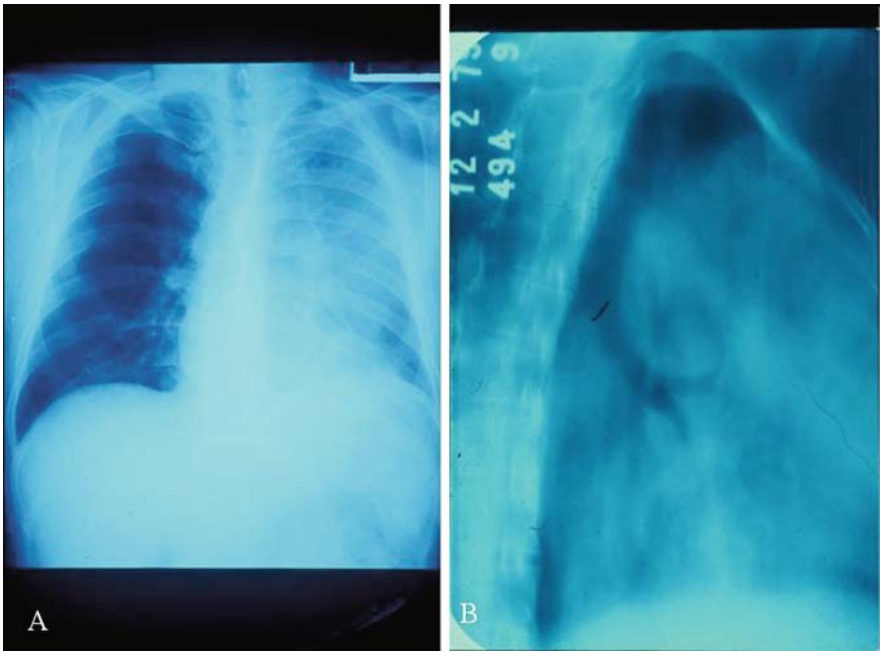

Fig. 19 (**a**) Chest X-ray showing a fibroatelectasic retraction of left upper lobe; (**b**) Bronchoestenosis

10.3 Actinomycotic Intra-Cavitary Lung Lesions

Four adult male patients with chronic lung symptoms were referred to us for diagnostic investigation (Table 7). The clinical symptom (haemoptysis) and radiologic feature (pulmonary air meniscus) suggested fungal ball but the double diffusion tests for aspergillosis were negative in all cases. All patients were submitted to surgery (lobectomy). A cavity partially filled by balls of granular material was found in all cases (Fig. 19). Gram staining of the balls showed them to consist of numerous gram-positive branched filaments <1 μm, arranged in confluent granules (Fig. 20). Modified acid-fast (Kinyoun) staining for *Nocardia* spp. was negative. Cultures were negative both for aerobes and anaerobes. After surgery the patients received penicillin and the post-operative courses were without complications. In case 2 the problem recurred with the same symptoms 5 years later. He was submitted again to surgery and a ball of 610 g actinomycotic material was found within a cavity in the remaining lung. The fluorescent antibody test in fixed tissue identified the organism as *A. israellii* (CDC 84041811). The patient has remained well, after 3 years [6, 113].

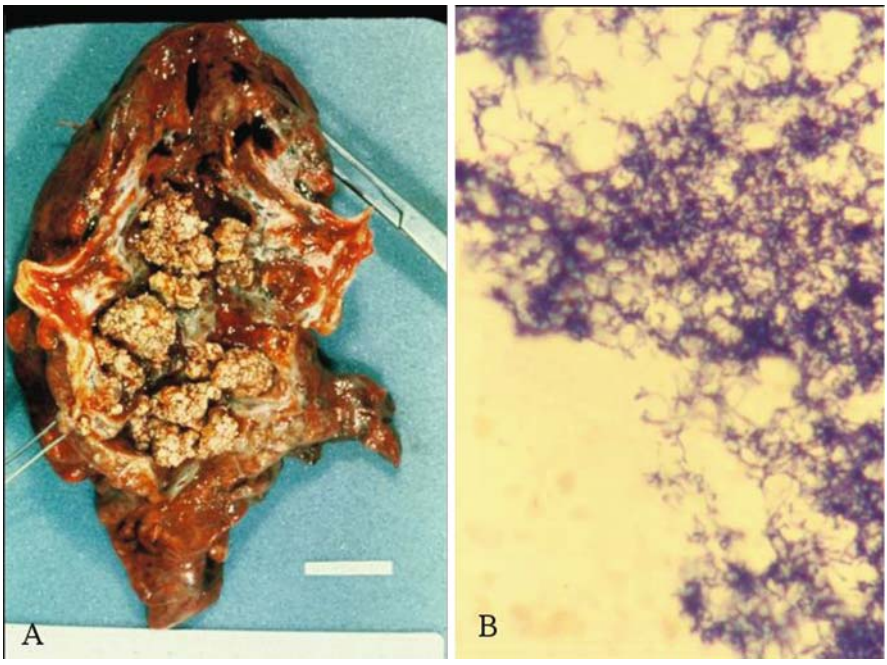

Fig. 20 (**a**) Numerous actinomycotic balls within the cavity of left upper lobe; (**b**) Gram-stained section of granules showing thin branched filaments

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Pathogenesis of Chronic Cavitary Pulmonary Aspergillosis: The Importance of the Host Immune System

Helen Sambatakou

Abstract *Aspergillus* species cause a wide spectrum of illnesses in humans, including severe invasive infection in those with immunocompromised states, allergy in atopic patients and indolent progressive infection in those without major immunodeficiency but prior pulmonary insults. This spectrum of disease offers a special opportunity to understand the relationship of the host and pathogen. Chronic cavitary pulmonary aspergillosis (CCPA) is an unusual manifestation of aspergillosis that usually affects middle-aged persons who are mildly or not immunosuppressed. Subtle immune defects in innate immunity (mannose binding protein polymorphisms and more recently in surfactant) have been previously identified in these patients, suggesting that many of the differing manifestations of these conditions might be genetically determined. The role of functional polymorphisms in certain candidate cytokine genes – derived from pathways of Th1 and Th2 immune response that are critical for effective antifungal activity – that could contribute to the risk of developing CCPA has only recently begun to be elucidated. Host immune status and the presence of underlying lung disease are important in determining the type of pulmonary involvement. These novel findings of non-cellular, immunogenetic, qualitative defects in patients with CCPA could explain the devastating consequences of the disease in otherwise healthy individuals without any obvious immune defect.

Keywords Chronic cavitary pulmonary aspergillosis · Cytokines · Genetic polymorphisms · Lectins

H. Sambatakou (✉)
Second Department of Internal Medicine and Infectious Diseases Unit, Hippokration General Hospital, The University of Athens, 11527 Athens, Greece
e-mail: helensambatakou@msn.com

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Contents

1 Introduction

Despite the ubiquitous distribution of the environmental *Aspergillus* species, only a subset of individuals develops any form of aspergillosis. The inherent resistance to diseases caused by *Aspergillus fumigatus* suggests the occurrence of regulatory mechanisms that provide the host with adequate defence without necessarily eliminating the fungus or causing unacceptable levels of host damage. The lung is constantly exposed to *Aspergillus* spp. that usually only colonise the host without damage, but may cause disease, sometimes leading to fatal outcomes. A variety of host defence mechanisms, which comprise physical, anatomical and mechanical barriers are developed to control the resident microbiota. A plethora of molecules, particularly cytokines and chemokines produced either by monocytes/macrophages or by epithelial cells, are involved in defence against *A. fumigatus*. Many recent reviews describe these mediators and the respective role of these cells in host defence against this pathogen. One of the most important roles is probably played by tumour necrosis factor-alpha (TNF-α) that stimulates the antifungal effector function of neutrophils and macrophages and induces the expression of a number of other protective cytokines including interferon-gamma (IFN-γ), interleukin (IL)-1, IL-6 and IL-12.

Although the pathogenesis of fungal infections involves several potential virulence factors that allow fungal survival and persistence [1], virulence is secondary mostly to the immune status of the host. The impressive disease spectrum of aspergillosis offers a special opportunity to understand host-pathogen relationship. Immune system is a major determinant of which particular form of disease will develop, if any, after exposure to the ubiquitous fungi or wether transition from commensalism to infection will occur. Mechanisms of host defence are numerous, and range from protective machinery that is present early (innate immunity) to sophisticated adaptive mechanisms, which are specifically induced during infection (adaptive immunity). These two systems are sequentially activated during infection and work cooperatively to eradicate the fungus. Most host defence

mechanisms, however, are inducible after infection through receptors, called pattern recognition receptors (PRR) because they recognise fungal components known as pathogen-associated molecular patterns (PAMP). There is a strong evidence of a collaborative recognition of *Aspergillus* by different classes of innate immune receptors. The immune response, following recognition in the lung, induces a pro-inflammatory response and limits damage to the alveolar surface. Alveolar macrophages and dendritic cells are the first cellular line of defence in the alveoli and their surface, are rich in PRR. There is accumulating evidence that soluble and cell associated C-type (Ca^{++}-dependent) lectins play a key role in regulating the innate response in the lung.

In contrast to invasive aspergillosis (IA), chronic cavitary pulmonary aspergillosis (CCPA) is a subacute form of pulmonary aspergillosis that most commonly causes a slowly progressive inflammatory destruction of lung tissue in patients with underlying lung disease with minor or no immunocompromising factors [2, 3]. Although "immunocompromise" is a vague term, until recently no clear immunological defect has been identified in patients with CCPA. One or more pulmonary cavities, which may or may not contain fungal balls, in association with pulmonary and systemic symptoms, raised inflammatory markers and detectable serum *Aspergillus* antibodies are characteristic of CCPA. Over years, untreated, these cavities enlarge, and fungal balls (aspergillomas) may appear or disappear. Most patients have underlying pulmonary insults as a result of previous cavitary tuberculosis or histoplasmosis, fibrocystic sarcoidosis, bullous emphysema, or fibrotic lung disease. Amongst the serious complications of CCPA are potentially life-threatening haemoptysis, extensive pulmonary fibrosis, and rarely locally, IA.

Development of new or expansion of existing pulmonary cavities with surrounding paracavitary shadowing is the hallmark of CCPA. CCPA tends to affect middle-aged persons who are mildly or not immunosuppressed, with a predominance of males. It has an indolent course lasting for years. In some cases, there is some evidence of impairment of host defences such as diabetes mellitus, a connective tissue disorder, poor nutrition, chronic obstructive lung disease or low dose therapy with steroids. Moreover there is considerable diversity in clinical course and radiological findings amongst these patients. Denning et al. [4] have described three distinct syndromes of CCPA with distinct radiological patterns of infection, based on the review of 18 patients seen over 10 years. The reader is referred to that reference for more detail on this.

The diagnosis of CCPA is usually made clinically and radiographically without a lung biopsy. When a large proportion of the lung is removed in patients with CCPA, hyphae were usually visualised as well as erosion of the cavity wall – however, these findings are restricted to the lung cavity, which contrasts with invasive aspergillosis cases. In immediate proximity to cavities, there is typically chronic inflammation with fibrosis, sometimes with granulomatous formation. The major utility of a biopsy in this context is the exclusion of other major conditions, such as carcinoma and mycobacterial infection and not for the establishment of a CCPA diagnosis. The absence of hyphae does not exclude the diagnosis of CCPA because of the paucity of hyphae in CCPA and the possibility of sampling error. Therefore, CCPA

is characterised histologically by chronic inflammation, with or without granuloma formation, and subsequently fibrosis. It is most likely the result of complex interactions between environmental and host genetic factors. Granulomatous inflammation is the hallmark of cell-mediated immunity.

Although inflammation is an essential component of the protective response to fungi and is required for a prompt control of fungal infections, its dysregulation may significantly worsen fungal diseases. Prolonged inflammation is a hallmark of a wide range of chronic diseases and autoimmunity, such as CCPA. For *Aspergillus*, the association of persistent inflammation with intractable infection is common in non-neutropenic patients after allogeneic haematopoietic stem cell transplantation. For the last two decades, the immunopathogenesis of fungal infections and associated inflammatory diseases has been explained primarily in terms of Th1/Th2 balance and as affected by a combination of different types of regulatory T (Treg) cells.

Moreover, in damaged lungs, due to previous pulmonary diseases, respiratory tract colonisation may occur, which may be temporary or semi-permanent. Defective mucociliary clearance appears to be a key defect in CCPA. Although generalised immunological defects exist in patients with CCPA, local defects from structural abnormalities (previous pulmonary pathology) may be sufficient for the disease to be initiated and the latter hypothesis explains the local expansion of disease in the lung over time without dissemination in distant organs.

Antibodies against *Aspergillus* species are common because of the ubiquitous nature of the organism, although they are not protective nor are they useful in the diagnosis of infection in high-risk patients owing to the lack of consistent seroconversion following exposure of infection. Serum antibodies are more commonly seen amongst CCPA patients than amongst those with invasive disease, a finding that likely reflects differences in the predisposing conditions between these two forms of aspergillosis. The presence of *Aspergillus* species within lung cavities causes a brisk IgG antibody reaction even though invasion of the cavity wall is rarely observed.

Experimental studies can also provide much insight to elucidate the pathogenesis of CCPA – however, in contrast to IA a CCPA model is extremely difficult to be reproduced.

2 Innate Defence Mechanisms Against CCPA

Innate antifungal defence is mediated by cells, cellular receptors and several humoral factors. Professional phagocytes consisting of neutrophils, mononuclear leukocytes (monocytes and macrophages) and dendritic cells have an essential role.

Normal pulmonary defence mechanisms are usually able to contain the organism in a host with intact pulmonary defences. The first line of defence against *Aspergillus* is ciliary clearance of the organism from the airways and limited access to the alveoli due to conidial size. After conidia reach the alveoli, the first line of cellular defence includes alveolar macrophages, which ingest conidia, kill germinating cells, and secrete cytokines and chemokines to coordinate secondary cellular defences [5–9]. Efficacy of killing may be enhanced by conidial opsonisation

by complement and/or collectins such as mannose binding lectin and surfactant proteins [10, 11], which indicate that the innate and adaptive immune systems do not work in independently. Alveolar macrophages express several immune receptors, including Fc-γ receptors and complement receptors, and particularly high levels of PRR, such as mannose receptor, Dectin-1 (β-glucan receptor), scavenger receptors, and toll-like receptors (TLR). During inflammation, immigrating neutrophils also contribute to the inflammatory response. Despite constant stimulation by inhaled conidia, alveolar macrophages display an anti-inflammatory phenotype, which includes altered cytokine responses (increased IL-10 and TGF-β) reduced oxidant production in response to stimuli and reduced microbicidal activity. Thus, alveolar macrophages seem best adapted for removal of small airborne particles with minimal induction of inflammatory immune responses. Germinating conidia and hyphae that escape macrophage surveillance are destroyed primarily by neutrophils and monocytes [12, 13].

Polymorphonuclear (PMN) neutrophils are typical cells of the innate immune system. Their importance is well established in host defence mechanisms, and the relationship of neutropenia with IA has been demonstrated in neutropenic mice inoculated with conidia of *A. fumigatus*. These cells possess their own array of PRR and express significant quantities of cationic antimicrobial peptides. Dendritic cells play also a key role in the adaptive immune response. One way these cells can regulate this immune response is, also, through the expression of major pattern recognition receptors.

Complement, collectins and antibodies promote opsonisation and recognition of fungi by various receptors. A member of the collectin family, pentraxin 3 (PTX3) is required for prompt handling of *Aspergillus* conidia by alveolar macrophages, such as that its deficiency is linked to the susceptibility to infection of otherwise immunocompetent mice. Conidia, but not hyphae, bind the soluble receptor PTX3 within terminal airways via galactomannan, an interaction that enhances uptake by alveolar macrophages and dendritic cells. In vivo, PTX3 drives the pulmonary production of protective Th1 cytokines, such as IL-12 and IFN-γ, and enhances survival in a murine bone marrow transplant model. Complement, collectins and antibodies also have an impact on the inflammatory and adaptive immune responses, through several mechanisms, including regulation of cytokine secretion and co-stimulatory molecule expression by phagocytes. Antibodies contribute to the activation of the complement system by fungi and complement is essential for antibody-mediated protection. However, both protective and non-protective antibodies are induced during infection.

3 The Importance of Collectins

Collectins are another component of the innate defence against infection. Efficacy of host defences against the organism may be enhanced by opsonisation of conidia with complement or other molecules such as mannose binding protein and surfactant proteins.

3.1 Mannose Binding Lectin (MBL)

Mannose binding lectin (MBL) is a collagenous lectin produced by the liver with at least two important roles in host defence: it binds to sugars, particularly N-acetylglycosamine and mannose, on the surface of the pathogens. Like other collectins, MBL serves as a PRR for microorganisms, facilitating their opsonisation by macrophages. Opsonisation can be further enhanced by complement activation via MBL-associated serine protease (MASP)-1 and MASP-2, of which the latter cleaves C4 and C2 to generate a C3 convertase independently of immunoglobulin. In addition, MBL may interact directly with phagocytic cells to regulate their function.

Surprisingly, inactivating mutations of the MBL gene are quite prevalent, with frequencies of up to 40% in various populations. Serum concentrations of MBL are genetically determined via 3 structural gene mutations and 3 promotor region polymorphisms. Three single amino acid changes are found at codons 52, 54, and 57, each of which leads to a substantial reduction in MBL concentration in heterozygotes. Homozygotes for these variants have absent or extremely low MBL levels in serum (median of 2 μg/ml to < 0.01 μg/ml). In the heterozygous state, serum levels are also profoundly reduced to –0.4 μg/ml. Variation in the promoter of the gene has less marked functional effects. MBL deficiency may be associated with an increased risk of exacerbation of chronic obstructive pulmonary disease. *A. fumigatus* produces a complement inhibitor, which may increase its pathogenicity.

There is increased evidence that polymorphisms in the MBL are associated with different types of infections such as HIV, cryptosporidiosis, meningococcal disease and tuberculosis. A mutation in the structurally encoding region of the MBL gene that leads to dramatically reduced serum levels of the functional protein could result in impaired immune clearance of *Aspergillus* species. Five functional single-nucleotide polymorphisms exist within the MBL gene, each affecting serum levels of the protein. Two polymorphisms located within the promoter region of the gene (H or L [G→C] at −550 and Y or X [G→C] at −221) affect transcriptional activity of the basal-promoter complex and reduce levels of circulating MBL. The remaining 3 functional polymorphisms at codons 52 (W/M52 [i.e., wild-type/mutant allele at codon 52], type D), 54 (W/M54, type B), and 57 (W/M57, type C) of the MBL gene result in single amino acid substitutions that dramatically reduce functional levels by causing structural defects in the MBL protein.

These structurally encoding mutations have dominant genetic consequences. The presence of one mutant allele (heterozygote) decreases functional MBL to almost the same extent as that in homozygous individuals. A rat model of MBL variant strains recently demonstrated that all mutations affecting the architecture of the MBL molecule significantly affect MBL activity, which implicates a major contribution to an immunodeficient phenotype in these individuals.

Distinct MBL genotypes have been linked with CCPA [14, 15]. Vaid et al. [14] observed a significant association of mutant "T" allele of codon 52 and a high frequency of MBL defects (66.67%) in CCPA patients. The gene encoding MBL protein and surfactant are in proximity on chromosome 10, which could imply linkage of defects. In addition, an intronic polymorphism in the MBL gene was found to

underlie elevated levels of MBL and greater disease severity in ABPA patients [16]. In a small study, Crosdale et al. [15] investigated whether MBL deficiency could account for CCPA cases. Wild and mutant alleles were detected at MBL codons 52, 54, and 57 by the use of polymerase chain reaction (PCR), using sequence-specific primers. Eight of 11 patients with CCPA (73%) had low MBL genotypes at codon 52, compared to 82 white control subjects ($p = 0.05$). Wild-type levels and genotypes were found in 4 additional patients with other forms of aspergillosis (chronic invasive *Aspergillus* sinusitis, $n = 2$; simple fungal ball, $n = 1$; IA, $n = 1$). Seventy percent of patients with chronic necrotising pulmonary aspergillosis (CNPA, a more severe form of CCPA) had MBL haplotypes that encode for low levels of the protein, compared with 25.6% of the white control subjects ($p = 0.004$). Presence of the codon 52 mutation was particularly common in patients with CNPA ($p = 0.015$), which suggests a greater involvement of this mutation. Genotype, haplotype, and allele frequencies for codon 54 and 57 mutations of the CNPA patients were all statistically similar to those for control subjects. Based on this study, it was hypothesised that possession of a codon 52 mutation might be a causative factor for the susceptibility to develop CNPA, possibly because of defective immune clearance of *Aspergillus* species or, alternatively, this might be a founder haplotype, carrying another disease susceptibility gene further along chromosome 10, encoding surfactant proteins A and D.

In a more recent study we have compared CCPA patient ($n = 15$) with patients with allergic aspergillosis ($n = 7$) for their association to polymorphisms in the collagen region of surfactant proteins (see below) SP-A1, SP-A2 and MBL. It was found that C allele at positions 1492 and 1649 of SP-A2 and T allele and CT genotype at codon 52 (position 868) of MBL were associated with CCPA. The gene encoding MBL and surfactant are in proximity on chromosome 10, which could imply linkage of defects. Further analysis of genotype combinations at position 1649 of SP-A2 and at 868 of MBL between patient groups showed that both CC/CC and CC/CT SP-A2/MBL were found only in CCPA patients, whilst GG/CT SP-A2/MBL was significantly higher in CCPA patients compared to ABPA patients. The study infers that alleles of host genetic factors predisposing individuals to clinically different forms of aspergillosis are distinct and confirmed the results of the earlier study. Especially, the presence of “T” allele and “CT” genotype at codon 52 of MBL and the genotype combination GG/CT of codon 52 of MBL and codon 91 of SP-A2 increases susceptibility to CCPA (Fig. 1).

3.2 Lung Surfactant Proteins

The alveolar space consists of the lining cells or type I cells important in gas exchanged and type II cells that produce and secrete a mixture of proteins and phospholipids that comprise surfactant. Pulmonary surfactant enhances pathogen clearance and regulates adaptive and innate immune-cell functions. Five surfactant proteins have been described in humans, SP-A1, SP-A2, SP-B, SP-C and SP-D, all belonging to the collectin family.

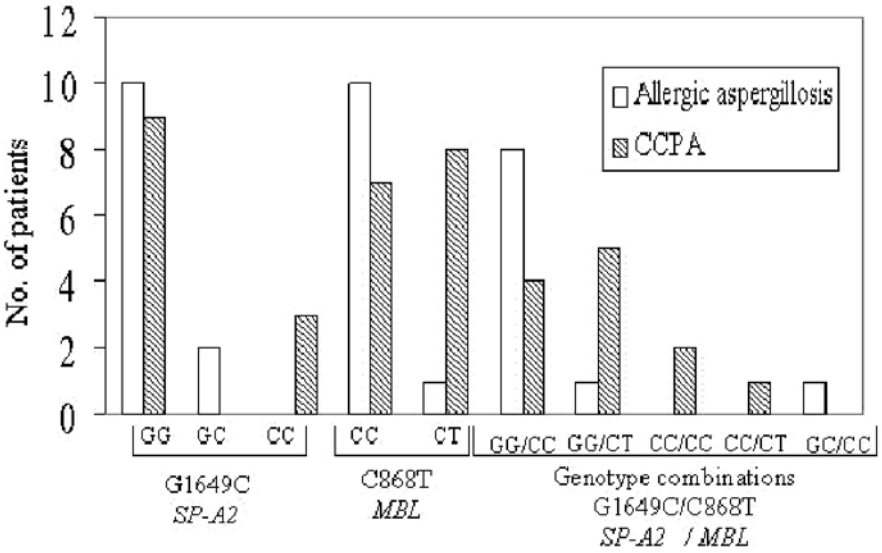

Fig. 1 Genotypic comparison patterns of surfactant A protein (SP-A) and mannose binding lectin (MBL) between patients with allergic aspergillosis and chronic pulmonary aspergillosis (CCPA) (see text for a detail explanation)

SP-A and SP-D are essential players of the lung innate immune system. They participate in the agglutination and opsonisation of microbes and enhance phagocytosis through different mechanisms, whilst modulating the immune response to prevent excessive inflammation. SP-A and SP-D are secreted collectins of the C-type lectin superfamily. These proteins contain an N-terminal collagen-like region important in the formation of trimers and a carbohydrate recognition domain (CRD) at the C-terminus. Lung surfactant proteins A and D bind oligosaccharides on the surface of a variety of pathogens (including fungi) via CRD, regulating interactions between microbes and host cellular components (Fig. 2). After binding of the collectins to the microorganism surface, effector mechanisms such as agglutination, neutralising or opsonisation of the pathogen for phagocytosis are initiated. SP-A enhances the uptake of microbes through Fc receptors and complement receptor I on alveolar macrophages. There is growing evidence that SP-A and SP-D increase the expression of other phagocytic receptors of macrophages, such as mannose receptor, regardless of microbial binding. The nature of the macrophage-collectin interactions and mechanisms implicated in the up-regulation of phagocytosis continue to be uncovered.

The genes encoding SP-A and SP-D contain several polymorphic sites. SP-A is encoded by two highly polymorphic genes. Some of the single nucleotide polymorphisms (SNP) occur with relative frequency in the general population. SP-A gene polymorphisms are associated with neonatal respiratory distress syndrome. SP-A and SP-D polymorphisms are also associated with tuberculosis and respiratory syncytial virus infection, and a SP-A2 gene polymorphism in the CRD of SP-A

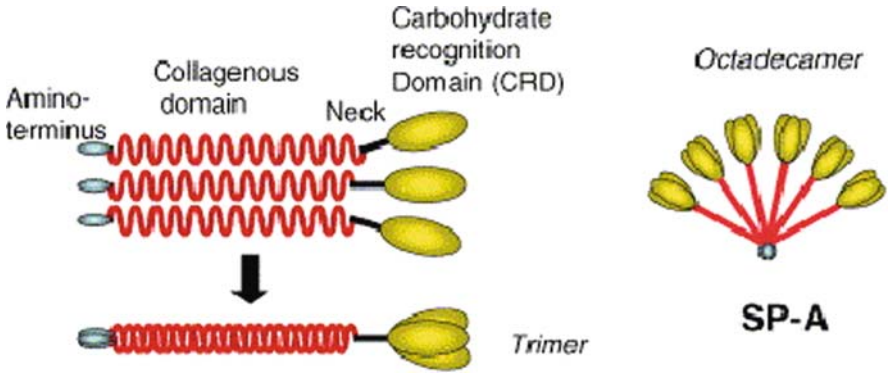

Fig. 2 Surfactant protein A (SP-A) structure

has been associated with increased susceptibility and risk of death in patients with meningococcal disease, supporting their involvement in innate immunity. SP-A and SP-D polymorphisms are important determinants for respiratory distress syndrome [17, 18]. Exogenous administration of SP-D has been protective against murine IA. Allele variants in a gene encoding SP-A have been associated with increased susceptibility to both allergic bronchopulmonary aspergillosis and CCPA [14].

Surfactant proteins B and C have important biological properties that result in lowering surface tension and preventing the alveoli from collapsing. A deficiency of these proteins in premature infants leads to infant respiratory distress syndrome, for which administration of exogenous surfactant is one of the treatments. Mutation in the SP-B gene is responsible for SP-B deficiency in congenital alveolar proteinosis, although the nature of the protein defect of SP-B deficiency is currently unknown [19, 20]. The SP-B single open reading frame polymorphism Thr131Ile is associated with acute respiratory distress syndrome.

Because uncontrolled inflammation within the lung can be lethal, mechanisms to modulate the inflammatory response are necessary to alert the host of the presence of pathogens, whilst avoiding excessive inflammation. The role of collectins in modulation of inflammation has been demonstrated in SP-A–/– mice which produced more proinflammatory cytokines and nitric oxide compared to SPA+/+ mice after endotracheal instillation of lipopolysaccharides (LPS). SP-A may interact with TLR2 decreasing the inflammatory response elicited by peptidoglycan. Finally, SP-A has been shown to down-regulate the oxidative response to agonists in macrophages.

3.3 Pentraxin 3

The collectin pentraxin 3 (PTX3), a member of the collectin family, is an essential component of host resistance to pulmonary aspergillosis and is required for prompt handling of *Aspergillus* conidia by alveolar macrophages, such that its deficiency

is linked to the susceptibility to infection of otherwise immunocompetent mice. In a murine model of PTX3-null mice was shown that were susceptible to invasive pulmonary aspergillosis [21] due to defective recognition of conidia by alveolar macrophages and dendritic cells, as well as inappropriate induction of an adaptive type 2 responses. IFN-γ and IL-12 levels were reduced and IL-4 levels increased in lungs of PTX3–/– mice. This defect was reverted by the administration of PTX3. In another experimental study [22] the researchers examined the protective effects of administration of PTX3 alone or together with deoxycholate amphotericin B or liposomal amphotericin B against IA in a murine model of allogeneic haematological stem cell transplantation. PTX3, alone or in combination with the polyenes, was given intranasally or parenterally either before, in concomitance with, or after the intranasal infection with *A. fumigatus* conidia. Mice were monitored for resistance to infection and parameters of innate and adaptive T helper immunity. The results showed the following: (i) complete resistance to infection and reinfection was observed in mice treated with PTX3 alone; (ii) the protective effect of PTX3 was similar or superior to that observed with liposomal amphotericin B or deoxycholate amphotericin B, respectively; (iii) protection was associated with accelerated recovery of lung phagocytic cells and Th1 lymphocytes and concomitant decrease of inflammatory pathology; and (iv) PTX3 potentiated the therapeutic efficacy of suboptimal doses of either antimycotic drug. Together, these data suggest the potential therapeutic use of PTX3 either alone or as an adjunctive therapy in *A. fumigatus* infections.

Based on the above data, polymorphisms in the PTX3 gene were evaluated in CCPA patients. However, neither serum PTX3 levels nor any particular SNP were associated with CCPA (Dr Pasqualotto, unpublished data).

4 Cytokine and Cellular Immunity Against CCPA

Although in general phagocytes have intrinsic antifungal activity, this activity can be increased by opsonins- and T cell-derived cytokines. T cell responses to *Aspergillus* antigens are present in healthy individuals, likely due to universal exposure to the organism. Serological and skin reactivity assays indicate that fungal infections are common, but clinical disease is rare, consistent with the development of acquired immunity. For many fungal infections the effective tissue response to invasion is granulomatous inflammation, a hallmark of cell-mediated immunity.

Increasing understanding of the pleiotropic regulatory role of various cytokines in immune defence has led to analyses of the role of cytokine genes in several infectious diseases. Several point mutations are found in the promoter of extensively studied cytokine genes, such as TNF, which may affect the level of cytokine production (promoter variant associated with increased levels of TNF gene expression). Direct evidence that disease-associated promoter variants have functional effects is recently emerging. These genetic associations with promoter variants of the TNF gene have supported attempts to modulate the severity of various diseases using a variety of anti-TNF reagents.

Lymphocytes seem to play an important role in the chronic forms of aspergillosis, including CCPA. T cells may mediate the effector function of macrophages inducing IL-1 on the macrophage and thereafter producing a cavalcade of chemotactic factors resulting in chronic inflammation and/or granulomatosis. Interferon-γ increases the expression of MHC Class II molecules on macrophages and activates macrophage receptors, carrying the Fc fraction of IgG to potentiate their ability to phagocytise. Under the impact of transforming and platelet-derived growth factor existing granuloma progresses to fibrosis [23, 24]. Immunohistochemistry has revealed a continuing role for fibronectin, collagen, integrin receptors and transforming growth factors in the formation of fibrosis.

Besides the innate host factors, acquired cellular immunity seems to play a crucial role as well. Th1 cells, through the production of IL-2, IFN-γ and TNF-α participate in the delayed-type hypersensitivity response; whilst Th2 cells producing IL-4, IL-5, IL-6, IL-9 and IL-10, lead to B-cells expansion, IgE production and eosinophilia. There are experimental data suggesting the protective role of Th1 CD4+ predominance against IA in the imbalance of Th1/Th2 response [7, 25, 26]. Cytokines and anti-cytokines that promote this pathway may be protective in vivo. It appears that whilst phagocytic cells clearly play a central role, adaptive cellular and humoral immunity are important determinants as well. Thus multiple lines of defence work in a co-ordinated way to protect the host and eradicate the fungus. This is explored in detail in the chapter by Dr Romani elsewhere in this book.

Studies in a murine model have suggested a role for cellular immunity, in which Th1 response induced by administration of soluble IL-4 was associated with a favourable response. In experimental IA, certain inbred mouse stains are substantially more susceptible (notably DBA/2 ($H2^d$)) compared with others such as BALB/c ($H2^d$) and 57BL/6 ($H2^d$).The difference in susceptibility appear to be determined primarily by the balance between CD4+, Th1 and Th2 responses [7, 25]. Th1 cell responses are associated with resistance and onset of protective immunity whilst Th2 responses are associated with progressive disease, more tissue damage and poor survival. Th1 produced cytokines (including IFN-γ, IL-6, IL-12, TNF-α, and IL-1) activate neutrophils and pulmonary macrophages, the key effector cells in IA, whereas Th2 cytokines, notably IL-4 and IL-10, are associated with reduced IL-12 and TNF-α and worse outcome. IL-10 knock out mice survive an otherwise lethal challenge and IL-4 mice knock out mice show deficient Th2 responses and are more resistant to infection [27]. Additional work supports this hypothesis as antagonism of TNF-α resulted in increased death in experimentally infected mice and more fungal burden in the lungs, whereas a TNF-α agonist peptide improved survival [28]. Granulocyte macrophage colony-stimulating factor (GM-CSF) may also be important [29]. All the experimental data is consistent with the key role of Th1 mediated responses, but no human data exist. In addition to these data, even more recent information indicate that certain chemokines are important as well. Antibody depletion of macrophage inflammatory protein-1α leads to a 6-fold increase in mortality in neutropenic mice [28]. Blockage of the CXC chemokine receptor-2 rendered non-neutropenic mice as susceptible as neutropenic mice [30]. Chemokines appear to enhance the recruitment and effector functions of lymphocytes and macrophages and contribute to granuloma formation and fibrosis.

According to recent data [31], distinct Treg capable of selectively inhibiting aspects of innate and adaptive immunity and mediating anti-inflammatory or tolerogenic effects are co-ordinately induced by the exposure of mice to *Aspergillus* conidia. Their function depends on IDO protein expression, elucidating the role of this enzyme in the pathogenesis of aspergillosis. Survival of experimental model was significantly reduced upon concomitant blockage of both IDO and Treg.

Functional polymorphisms in certain cytokines in patients with CCPA with respect to candidate pro- and anti-inflammatory cytokines, have also recently studied, which might influence the development and outcome of CCPA. Patients infected with *Aspergillus* appear to be higher producers of IL-15, a Th2 promoting cytokine, and lower producers of TNF-α, a cytokine central in protective responses. In a case-control association study, functional cytokine polymorphisms of IL-10, IL-15, TGF-β1, TNF-α and IFN-γ in patients with CCPA ($n = 24$) were compared with other forms of aspergillosis (mostly ABPA) ($n = 15$) and with ethnically-matched controls ($n = 130$–660) [32]. The results showed that CCPA occurs in patients who are genetically lower producers of both IL-10 and TGF-β1. As these cytokines are regulatory and anti-inflammatory, CCPA may be a consequence of poor inflammatory response control in the lung. However, the impact of cytokine genetic polymorphisms in each clinical phenotype of CCPA was not examined. Moreover, CCPA patients had 32 and 22% frequency of two mutations in SP-A2, compared with normal controls (18 and 11%) ($p = 0.021$ and $p = 0.044$, respectively). Patients with different forms of aspergillosis had a higher frequency of the +13689 IL-15 A allele and A/A genotype, in comparison to normal controls [32]. Aspergillosis was also associated with lower frequency of the TNF-α -308 A/A genotype, which was not associated with any particular allele. There was no association with IL-10, IFN-γ or TGF-β1 genotype or allele. IL-15 (+13689) alleles were not differently distributed in the two forms of aspergillosis. However, the aspergillosis patients were significantly different from controls. The +13689 T allele, encoding lower production of the cytokine, was less frequent in aspergillosis patients. The frequency of the TNF-α high producer −308A allele, which is central in protective responses, was lower in the patients with CCPA, as in any form of aspergillosis. One possible interpretation is that a Th2 response, which is not protective, predisposes to aspergillosis.

Within patients with CCPA compared to those with allergic aspergillosis, CCPA was associated with lower frequency of the IL-10 -1082G allele and G/G genotype, but not with the −592 allele [32]. Although CCPA is characterised histologically by various degrees of fibrosis, was associated with a lower frequency of the TGF-β1 +869 T allele and T/T genotype compared with allergic forms of aspergillosis and normal controls, indicating that CCPA occurs in patients who are genetically lower producers of TGF-β1. Since TGF-β1 is anti-inflammatory cytokine, CCPA may be a consequence of failing to control the inflammatory responses. There was no association with IL-15, IFN-γ or TNF-α genotype or allele. Inheritance of the IL-10 -1082A allele (lower IL-10 production by lymphocytes) is associated with the development of CCPA (Fig. 3). Because IL-10 is anti-inflammatory, such patients may be prone

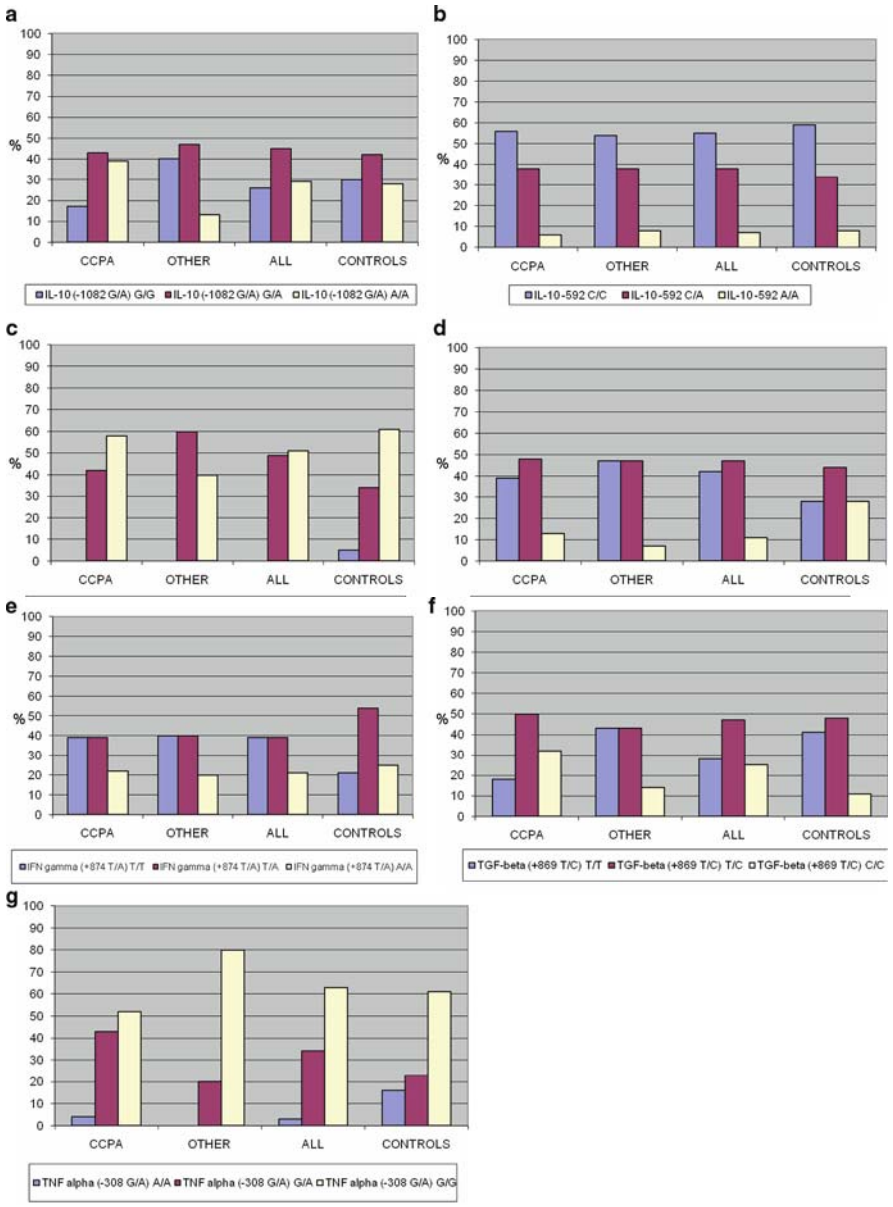

Fig. 3 Genotypic comparison amongst patients with chronic cavitary pulmonary aspergillosis (CCPA), allergic forms of aspergillosis (referred to as "other" patients), and normal control regarding (**a**) Interleukin-10 (–1082 allele); (**b**) Interleukin-10 (–592 allele); (**c**) Interleukin-15 (at position -80); (**d**) Interleukin-15 (+13689 allele); (**e**) Interferon (IFN)-gamma (+874 allele); (**f**) Transforming growth factor (TGF)-β1 (+869 allele); and (**g**) Tumour necrosis factor (TNF)-alpha (–308 allele). In comparison to patients with allergic aspergillosis, CCPA was associated with a lower frequency of the genotype G/G in IL-10 (–1082 allele) ($p<0.001$) and a higher frequency of genotype

to necrotic sequelae. By contrast, non-CCPA patients expressed the G allele (higher producers).

Surprisingly, no associations were seen between IFN-γ genotypes and aspergillosis [32]. TGF-β and IL-10 are potent immunosuppressive cytokines, with beneficial and detrimental effects on host responses to fungi. High levels of IL-10 are also detected in chronic candidosis, severe forms of endemic mycoses, and in non-neutropenic patients with aspergillosis. However, it is unclear if this reflects causality or consequence.

Studies in murine models of IA in mice deficient in IL-10 revealed increased resistance to *A. fumigatus* infection, whereas mice expressing high serum IL-10 levels show decreased resistance to aspergillosis and were protected by neutralisation of this cytokine [7, 33].

The study of fibrosing factors is essential as well, since CNPA is characterised histologically by fibrosis through the time. Transforming growth factor-β (TGF-β) is an important regulator of fibrosis. Three isoforms of TGF-β exist in mammals that have similar in vitro activities. The isoform TGF-β1 is most often implicated in inflammation and fibrosis up-regulating collagen synthesis and gene transcription in fibroblasts. It is also stimulates production of fibronectin and proteoglycans by these cells. Other fibrosing factors may also be implicated in the pathogenesis of the disease.

Several clinical observations indicate an inverse relationship between IFN-γ and IL-10 production in patients with fungal infections. High levels of IL-10 negatively affecting IFN-γ production are detected in neutropenic patients with aspergillosis and chronic candidal diseases (opposite to the ratio of these cytokines in the present study of CCPA). However, later in the course of an infection, high level production of IL-10 might be beneficial by contributing to resolution of the inflammatory response. Circumstantial evidence indicates that IL-10 produced by cells of the innate immune system is responsible for the prevention of excessive activation of innate effector function, whereas IL-10 secreted by Treg cells is mainly responsible for the establishment of commensalism and, perhaps, fungal latency and persistence. Th1-cell responses successfully eradicate pathogen, but often also cause immunopathology. To minimise the deleterious side-effects, the anti-inflammatory cytokine IL-10 is produced. IL-10 pushes towards Th2 phenotype, which is deleterious in aspergillosis. Although IL-10 was originally isolated from Th2 cells, it is now known to be produced by many cell types. There is recent evidence that Th1 cells are the main source of IL-10 that controls the immune response against *Leishmania major* and *Toxoplasma gondii* infection [34]. IFN-γ might not work in a Th2 setting, a finding that underscores the requirement for critical interpretation of the levels of

Fig. 3 (continued) T/C in TGF-β1 (+869 allele) ($p < 0.001$). CCPA patients showed a lower frequency of the genotype T/T in TGF-β1 (+869 allele) than controls ($p < 0.001$). Comparing all patients with aspergillosis with controls, a higher frequency of the genotype A/A in IL-15 (+13689 allele) ($p < 0.001$) and a lower frequency of the genotype T/A in IFN-gamma ($p < 0.02$) were observed. No difference amongst groups were observed for IL-10 (-592) and IL-15 (-80). Adapted from reference [32]

IFN-γ production in clinical settings. This highlights the importance of a panel of cytokine polymorphisms in the pathogenesis of the disease and the profound role that genetic factors can play in CCPA.

Recently the Th17 pathway is an emerging subset [35]. According to these results, IL-23 can have a protective role in fungal infections in the absence of IFN-γ. IL-23/IL17 dependent pathway may provide some antifungal resistance in condition of IFN-γ deficiency. A cross regulation exists between the Th1 and Th17 pathways. The current results might aid in the development of strategies for immune therapy of fungal infections that attempt to limit inflammation to stimulate an effective immune response. As IL-23 driven inflammation promotes infection and impairs antifungal resistance, modulation of the inflammatory response represents a potential strategy to stimulate protective immune responses to fungi. IL-23 production by dendritic cells is regulated by TLR and T cells and is associated with Th17 cell activation. The results of the present study show that the Th17 pathway, and not an uncontrolled Th1 response, is associated with effective pathogen clearance, failure to resolve inflammation and to initiate protective immune responses to *Candida* and *Aspergillus* species. Thus, the new findings may serve to accommodate the paradoxical association of chronic inflammatory responses with intractable forms of fungal infections where fungal persistence occurs in the face of an ongoing inflammation. Both IL-23 and IL-17 impaired the antifungal effector activities of PMN even in the presence of IFN-γ, a finding suggesting that the Th17 effector pathway prevails over the Th1 pathway. IFN-γ is known to regulate the induction of Th17. The IL-23 and IL-17 production were indeed heightened in condition of IFN-γ deficiency in both infections, and the number of IFN-γ producing cells increased upon IL17 neutralisation. Also, the finding that IL-23 is produced in response to fungi in condition of high threat inflammation (i.e., by inflammatory dendritic cells in response to high yeast numbers through the TLR/MyD88 pathway) has important implications. Not only does it point to IL-23 as an important molecular link between the inflammatory processes and fungal virulence, but it also establishes a scenario whereby a vicious circle may be at work.

5 Toll-Like Receptors

Since their recent discovery, less than a decade ago, TLR have been shown to be crucial in host protection, but also in homoestasis of the normal microbiota. They recognise specific fungal ligands with the use of distinct adaptor molecules, activating different signalling pathways that in turn trigger subsequent inflammatory and immune response that allows a immediate response towards infection and the initiation of the long lasting adaptive immunity through the production of pro-inflammatory mediators, including chemokines and cytokines. TLR are type I transmembrane proteins that are characterised by an extracellular leucin-rich domain (LRR) and an intracellular or cytoplasmic domain homologous to the interleukin-I receptor (IL-1R) and therefore called Toll/IL-1 receptor (TIR) domain (Fig. 4). The homology between TLR and IL-1R is restricted to their cytoplasmic domain, whilst

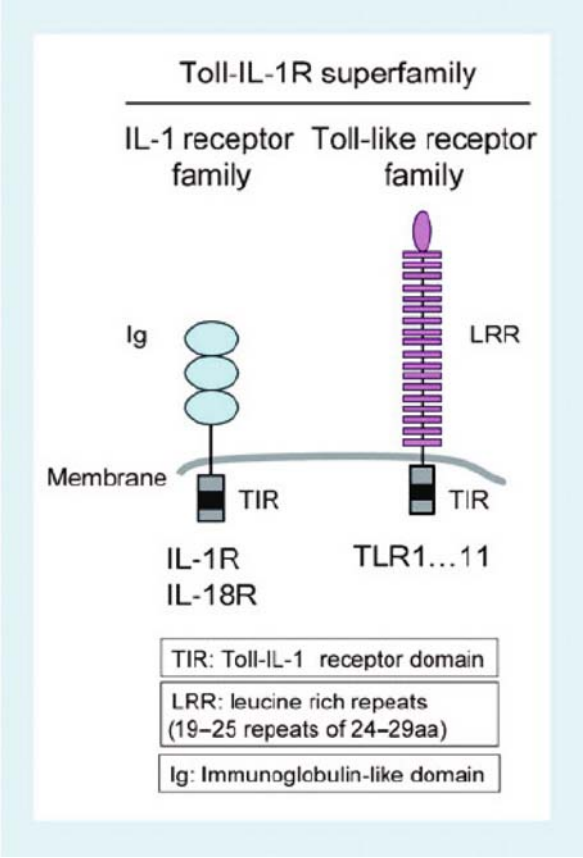

Fig. 4 Schematic representation of the transmembrane proteins toll-like receptors and interleukin-1

their extracellular domains are remarkably different. The LRR domains are responsible for the recognition of PAMP from bacteria, parasites, viruses and fungi. At least one ligand for each TLR has been identified so far. Human express at least 10 TLR involved in the recognition of various PAMP, including LPS, a major component of Gram negative bacteria, as well as lipopeptides produced mainly by Gram positive bacteria. Ligand-TLR interactions, except those involving TLR3, trigger the binding of at least one adaptor molecule, MyD88, to the intracellular domain of the TLR.

TLR4 is the most extensively studied PRR and it recognises a variety of ligands, but is mostly known as the LPS receptor. TLR4 is not localised at the cell surface but rather at the Golgi apparatus requiring internalisation of LPS to activate signalling. TLR2 also recognises a broad range of ligands, including lipopeptides. The variety of ligands recognised is believed to be due to heterodimer formation of TLR2 with two other TLR, TLR1 and TLR6. The expression of TLR differs with cell types and cellular localisation on numerous myeloid cells (macrophages, dendritic cells, neutrophils, T and B cells, and epithelial cells). Upon recognition of their

ligands, TLR dimerise and initiate a signalling cascade that leads to the activation of a pro-inflammatory response. Ligand binding induces two signalling pathways, one is MyD88-dependent and the other is MyD88-independent inducing the production of pro-inflammatory cytokines and type I interferons.

TLR contribute to macrophage recognition of *A. fumigatus*, but TLR are also important pattern recognition receptors for PMN. PMN express all the known TLR, except TLR3. Specific TLR ligands trigger or prime cytokine release and superoxide generation in PMN, particularly important for the fungicidal activity of these cells. Findings from in vitro experiments suggest that TLR control PMN activity against *A. fumigatus*. Especially, signalling through TLR2 promotes fungicidal activity and release of pro-inflammatory cytokines. TLR4-dependent signalling also favours fungicidal activity, but triggers production of the anti-inflammatory cytokine IL-10. Similar to macrophages and PMN, *A. fumigatus* recognition by dendritic cells, whether of mouse or human origin, depends on the presence of TLR2 and TLR4. The respiratory epithelial cell, a nonprofessional immune cell, is believed to actively participate in innate defence against pathogens. They express all known TLR and produce various cytokines and chemokines needed for recruitment of cells of the innate defence, including PMN. However, the epithelial cell response seems to be less intense than that of leukocytes. The interaction between *A. fumigatus* and epithelial cells leads to the production of comparatively modest levels of IL-6 and IL-8. This may be explained, in part, by different distribution of the TLR. Unlike leukocytes, respiratory epithelial cells do not express various pattern-recognition receptors involved in *A. fumigatus* sensing on their cell surfaces, including TLR4.

Concerning the recognition of *A. fumigatus*, conflicting data have been published. Wang et al. [36] showed that activation of human macrophages mainly depends on TLR4, whereas Mambula et al. [37] reported that mouse macrophages released TNF-α in a TLR2- but not TLR4-dependent manner. In addition to these opposing results, Meier et al. [38] used trasfected HEK cells expressing TLR1-10 to show that TLR2 and TLR4, and not other TLR, recognised *A. fumigatus*. In agreement with the latter, Netea et al. [39] indicated that conidia and hyphae activated murine peritoneal macrophages through TLR2 and TLR4. Moreover, macrophages responded differently to conidia and hyphae. It appears that whereas TLR2 recognises both conidia and hyphae, TLR4 only detects conidia. Moreover, TLR2 activation by hyphae induces the production of IL-10. Thus, the authors speculated that the phenotypic switching during germination is an important escape mechanism for *A. fumigatus*, enabling it to evade host defence. This is the consequence of the expression by conidia and by hyphae of different cell wall components constituting PAMP that is most probably differentially recognised by TLR2 and TLR4. A better understanding of the specific PAMP produced by the fungus and sensed by TLR is required is an area where more research is needed.

In a recent study the association of polymorphisms in the TLR2, TLR4 and TLR9 genes and susceptibility to CCPA was investigated, in comparison to allergic forms of aspergillosis and healthy controls [40]. No association was observed between TLR2 polymorphisms and susceptibility to CCPA, as was previously described for patients with IA [41]. Interestingly, a significant association was observed between

allele G on Asp299Gly (TLR4) and CCPA (odds ratio 3.46; $p < 0.01$). Since the presence of this SNP does not ultimately affect signal transduction and cytokine production in mononuclear cells challenged with *A. fumigatus,* a putative mechanism for disease association would be an abnormal TLR4 extracellular domain, which hampered its function by disrupting microbial recognition. Conversely, susceptibility to ABPA was associated with allele C on T-1237C (TLR9). A recent study indicated that TLR1 and TLR6 SNP were associated with susceptibility to IA after allogeneic stem cell transplantation [42]. By contrast, no association has been found between IA and two classical TLR4 polymorphisms, Asp299Gy and Thr399lle, thus questioning the relevance of the in vitro findings indicating a role of TLR4 in the recognition of *A. fumigatus*.

6 Concluding Remarks

Based on the results of the above genetic case-control studies, both innate and T helper immunity play a central role in CCPA. However, genetic investigation of each clinical phenotype in large-scale studies is required, in order to explain the differences in the clinical, radiological ground and outcome in the CCPA subgroups. Other non-MHC related genes or HLA-Class II genes/gene products may also be important, but are more difficult to assess for statistical and technical reasons.

Increased knowledge both in human genomics and in host inflammatory response has led to increasing interest in infectious disease genetics over the last 5 years, although twin studies have suggested the hypothesis many years ago. In recent years, the methodology and techniques available for analysing human genetic variation have advanced rapidly leading to the identification of a large number of genes associated with altered susceptibility to infectious pathogens. The availability of new genetic markers with a defined position on the human genome sequence is central to the mapping of susceptibility genes and defining their functional variants.

Polymorphisms in genes regulating innate and adaptive immune function are likely important determinants of host susceptibility to fungal infection and may become critically important during times of immunosuppression. Human cohort studies are using genetic association studies to correlate clinical outcomes with genetic background. Generally, in case-control association studies, the frequencies of marker alleles in groups of patients and healthy controls are compared and the difference is subjected to statistical analysis. Establishing the role of a particular SNP in a disease process requires large case control studies. It is important that patients from the same gene pool are compared; otherwise very different allele frequencies could produce a false positive result. The number of patients required for such studies depends on the frequency of any particular mutation. These studies have met with only modest success in identifying disease-causing genes, in part because in the difficulty in selecting from amongst the many candidates and the likely modest effect of any single disease susceptibility gene. The difficulty in identifying a perfectly matched control group creates an additional limitation. Furthermore, even

when cases and controls are adequately matched, most study designs involve relatively small sample sizes that lack the statistical power to detect small or moderate gene effects

More recently, research on fungi has entered the era of genomics. The emerging genomic sequence has allowed the application of genomic technologies for the study of fungal pathogenesis, virulence and new antifungal discovery. Despite sequencing the genome [43], the intrinsic reason for the remarkable pathogenicity and allergenicity of *A. fumigatus* compared with any other member of the *Aspergillus* genus is not yet well understood. According to recent data gene polymorphisms of specific host immune defence appear to be of major importance in the risk for CCPA. However, we are still far from understanding why despite the constant exposure to *Aspergillus*, only a small number of individuals suffer from CCPA. Are these individuals affected in their innate immune response by previous infections or because of the abnormal lung architecture?

The therapeutic efficacy of antifungals is limited in CCPA and probably is required support of host immune reactivity. Manipulation of the immune system could be a candidate for future strategies aimed at preventing or treating fungal infections in susceptible patients. A beneficial effect with adjuvant IFN-γ administration in some cases of CCPA has been described [4]. Various cytokines, including chemokines and growth factors, have proved to be beneficial in experimental and human fungal infections. For more than 25 years surfactant therapy has been successfully used in neonates with respiratory distress syndrome, facilitating alveolar gas interchange. Surfactant therapy, however, has been use without benefit in adults with acute respiratory distress syndrome. Surfactant replacement therapy, of note, has been based on the biochemical properties of surfactant rather than its biological properties. Neither SP-A nor SP-D are components of artificial surfactant. Since the levels of these collectins are decreased in CCPA, replacement therapy may be useful in this clinical setting. In one study, the use of recombinant SP-D was protective in mice sensitised to allergens of *A. fumigatus*. The Th1/Th2-cell balance itself can be the target of immunotherapy. The discovery of TLR, which are now targets of antifungal drugs, the new roles for antibodies are achievements in the field of fungal immunology, which might offer new grounds for a better understanding of the immune pathways and for the manipulation of patients with or at risk of fungal infections.

Although several genes have already been associated with susceptibility to CCPA, it is likely that those represent only a small fraction of all the relevant genes.

The rationale for studying gene polymorphisms in CCPA can aid:

- To enhance the understanding of the aetiology and pathology of the disease;
- To identify potential markers of susceptibility, severity and clinical outcome;
- To identify potential markers for responders in therapeutic trials;
- To identify targets for therapeutic intervention;
- To identify novel strategies to prevent disease.

Understanding the role of host genetics in the complex phenotype of CCPA can lead to numerous clinical benefits and hopefully improved outcomes. An understanding of genetic susceptibility profile will permit the use of specific prophylaxis strategies in high risk patients, and potentially novel therapies. Tools are available to uncover and validate the role of genetic polymorphisms in determining disease risk. This field however is in its infancy and much validation specific to CCPA is needed. One problem is the difficulty to contact a large-scale multicentre study, due to racial differences in gene polymorphisms and the rarity of the disease.

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Current Surgical Strategies for Chronic Cavitary Pulmonary Aspergillosis

Gerard Babatasi, Hashmatullah Nawabi, and Mambun Socheat

Abstract Treatment of pulmonary aspergillosis has to be individualized. Surgery is usually the treatment of choice for immunocompetent patients with a single fungal ball and adequate lung function. Surgery in patients with complex fungal ball could also be done for patients who persist with symptoms despite medical therapy or in cases where a lung tumour or other diagnoses are being considered. Pulmonary resection may also be indicated in patients with massive haemoptysis. To diminish operative complications in patients with complex fungal ball, cavernostomy and obliteration of the cavity by a thoracoplasty using intrathoracic transposition of extrathoracic skeletal muscle should be performed. These techniques may be proposed for patients in whom respiratory failure does not allow for a pulmonary resection to be performed. These operations are rather exceptional, but the results in those categories of high-operative risk patients are reasonable. Additional studies are mandatory to substantiate these results.

Keywords Aspergilloma · Cavernostomy · Fungal ball · Latissimus dorsi · Thoracoplasty · Open-window thoracostomy

Contents

G. Babatasi (✉)
Department of Cardiothoracic Surgery, University Hospital, Caen Cote de Nacre, France
e-mail: babatasi-g@chu-caen.fr

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1 Introduction

Since pulmonary aspergillosis had been reported in 1842 by Bennet [1], the optimal therapeutic strategy for patients suffering from these conditions is a topic of great concern. Surgical decision-making for chronic pulmonary aspergillosis is one of the major dilemmas shared by chest physicians and thoracic surgeons. In addition, different surgical strategies have been proposed for the treatment of fungal balls (aspergillomas). The first operative resection of a fungal ball seems to have been carried out by Gestl et al. [2]. Although successful lobectomy for pulmonary aspergilloma has been described [2], surgical resection is frequently not performed because of the high incidence of complications and mortality.

The most common predisposing factor for chronic cavitary pulmonary aspergillosis (CCPA) is previous tuberculosis, and at least 25% of such patients reporting a history of this disease [3]. Many series have reported that ~80% of fungal balls occur following treatment for cavitary tuberculosis. In the cavity, normal clearance mechanisms are impaired or absent, which may permit adherence and germination of fungal conidia and lead to the development of a fungal ball. Fraser [3] speculated that the initial saprotroph nature of *Aspergillus* pulmonary colonisation may evolve to form new cavities as the hyphae advance, which may be facilitated by the secretion of toxins, enzymes or inflammatory mediators derived from the inflammatory reaction surrounding the cavity.

By definition, a fungal ball [4] is formed by a tangled mass of *Aspergillus* mycelia. *Aspergillus fumigatus* is the usual aetiological agent in CCPA being able to colonise any intrathoracic cavity [5]. The fungal ball consists of both living and dead fungal elements, fibrin, inflammatory cells, epithelial cells debris and mucus [6]. Fungi within cavities may remain quiescent and may grow for months [7] or even years without invading pulmonary tissue (Fig. 1). CCPA treatment is usually conservative unless massive haemoptysis occurs which may indicate the need for surgical resection.

The mortality rate associated with CCPA surgery has ranged between 7 and 8% [8]. Early post-operative mortality in patients with complex fungal balls reach 34% [9]. Moreover, at least 25% of patients suffer from complications following surgery, including extension of the fungal infection to the chest wall [9, 10], bacterial sepsis, respiratory failure, and the development of pleural thickening, bronchopleural fistulas, and *Aspergillus* empyema [9]. Despite the wide variety of antifungal agents available for treating CCPA, in our opinion no consistent results have been obtained so far. Two important factors should be taken into consideration when analysing the literature on the subject. First of all, drugs such as amphotericin B

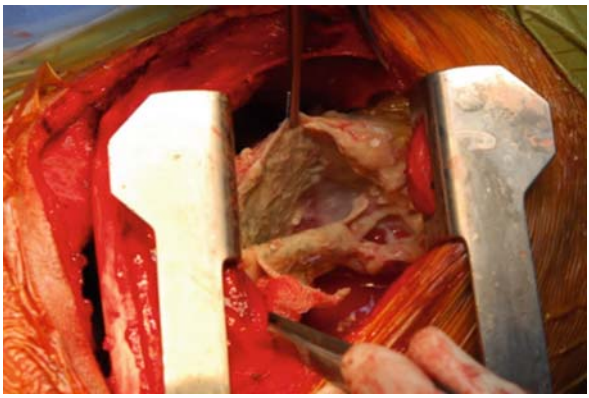

Fig. 1 Complicated pulmonary fungal ball requiring double thoracotomy followed with cavernostomy

given intravenously demonstrate very limit penetration in the fungal ball. In addition, a successful treatment of CCPA may be confused with spontaneous regression, which occurs in about 7–10% of cases. Several therapeutic strategies have been used in CCPA treatment. Systemic antifungal therapy with ketoconazole [11] seems to be ineffective. Itraconazole at 200–400 mg daily [12–14] seems to offer some symptomatic relief but little radiological benefit [13]. Tsubura [12] has demonstrated that itraconazole accumulates in fungal balls and cavities but responses are usually incomplete. Long-term therapy with antifungal drugs may increase drug efficacy and show radiological improvement, though cure should not be expected with medical treatment alone.

One solution to increase antifungal penetration into cavities would involve direct instillation of drugs into cavities [11]. This however has not been properly evaluated. Repeated instillations of nystatin or amphotericin B into the cavity have yielded some benefit in some cases (especially with amphotericin B [15]). Since communication usually occurs between the cavity and the airways, the instilled agent frequently leaks into the airways, leading to toxicity. Repeated instillations are labour intensive and are of limited efficacy for complex or bilateral fungal balls. All patients with chronic necrotising pulmonary aspergillosis (CNPA) require systemic antifungal therapy, since tissue invasion may occur in these patients.

2 Surgical Outcomes and Options

As already mentioned, surgery may be appropriate for CCPA patients with haemoptysis, particularly in the presence of simple fungal balls. One study showed that surgery offered a higher 5-year survival, in comparison to conservative therapy (84% versus 41%, respectively) [16]. Recent series have showed substantially lower mortality rates, particularly in carefully selected patients [10, 17, 18]. The extent of surgery is widely determined by the degree of involvement of the lung parenchyma and the functional respiratory reserve of the patients. The decision of patient selection for surgery depends on the balance between the risk of disease and risk of

surgery. We do not hesitate to propose surgical resection for simple fungal balls because the risk of haemoptysis is present and the surgical risk is minimal. However, owing to a higher surgical risk in complex fungal balls, we carefully evaluate the patients and only proposed surgery for low-risk patients. Bronchial artery embolisation is now the recommended technique in high-risk patients [19, 20]. In rare cases when the procedure fails, cavernostomy with muscle flap transposition can be considered [21]. Adhesions could create difficulties for surgery particularly when the right upper and middle lobes are involved. Sometimes removing both upper and middle lobes is mandatory [22].

The spirometric threshold of resectional surgery is primarily based on the forced vital capacity, the forced expiratory volume in one second (FEV1), and the maximum voluntary ventilation. Thoracotomy is usually recommended only for patients with a predicted post-operative FEV1 exceeded 40% [23].

Cavernoplasty may be considered for poor-risk candidates based on spirometric data and computed tomography (CT) findings. Our group has no experience on percutaneous cavernostomy, though it may be a better option in these patients as it carries less risk of post-operative respiratory failure. But few cases have been published [23]. The resected specimens should routinely be sent for histopathological examination and fungal culture, in order to confirm the diagnosis of CCPA. Average blood loss during this type of surgery range from 300 to 2,000 ml with a mean of 80 ml during regular pneumonectomy (500 ml for lobectomy). The mean amount of draining over first 24 h is 600 ml after lobectomy and 800 ml after pneumonectomy. Massive haemorrhage is usually defined as an excessive blood loss up to 1,500 ml a day [24].

Lobectomy is the most frequent initial procedure for CCPA [25]. Wedge resection of the lung is generally performed in patients with either small peripheral lesions or poor respiratory functional reserve [26]. Open-window thoracostomy and cavernostomy may be considered as an ultimate proposition for patients in which respiratory insufficiency does not allow pulmonary resection [24]. These interventions are rather exceptional in CCPA series (though it was a standard treatment ∼30 years ago [27]).

The usual plombage with omental flap [28] or muscle flap plombage to close the defect is sometimes completed with skin flap. We reserve muscle flap plombage only to open-window thoracostomies that do not heal spontaneously after 6 months. Patients with poor general status and low pulmonary condition precluding any parenchymal resection are candidates for external drainage through thoracostomy. After cavernostomy and thoracostomy, patients have daily bandage with insertion of gauze, impregnated with amphotericin B in the cavity for almost 4 weeks.

3 Localisation of Cavernostomy

Most of the cavernostomies are opened through a posterior approach, high, in the vertebro-speculae space. Some are opened via an anterior approach, high, subclavicular, through pectoralis muscle. In other rare cases, incision occurs via an axillary way, high or very low sus-diaphragmatic.

4 Open-Window Thoracostomy

The majority of open-window thoracostomy (OWT) cases are axillary, more or less high, with a few regarding diaphragm. The opening required some rules [21, 28]:

- Wide opening to the skin of the pleural cavity by resection of "the dead space" of the pleural cavity;
- Resection of the top of the dead zone including all thoracic wall (ribs, intercostals spaces, muscles). This wide resection need to be extended to the exact size of the pocket, which is usually larger than initially demonstrated on the preoperative CT scan;

The extent of the pleural space to epidermidize, the importance of the empyema, the number and the size of the multiple fistulas rather than the infection explain why it takes a very long time before spontaneous healing occurs. Instillation of amphotericin B is undertaken daily for ~3 months. Thereafter, the omentum is passed through the diaphragm and wrapped over the surface of the exposed lung, successfully closing the fistulas. There is no contraindication for these operations. The omentum has a sufficient blood supply to occlude the bronchopleural fistulas produced by *Aspergillus* species invasion. Some of these procedures are performed in patients in very bad condition (cachexia). It is critical to obtain complete detersion of septical areas of lung parenchyma (particularly in cases of tuberculosis). Daily changes of gauzes and local instillation of amphotericin B are usually required, in addition to repeated sampling for bacteriological and mycological examination.

Bronchial fistulas resulting from the cavernostomy need to be closed or diminished by use of silver gauzes or acidification. The periscapulae muscles need to be trained by using physiotherapy or electric or ultra-sound stimulation to gain maximum of size and vascularization.

5 Preoperative Preparation

Preoperative preparation of the patient requires cessation of smoking and improvement of nutritional status. A short course of antibiotics will also be required for most patients. Preoperative antifungal drugs are usually not prescribed. Basically all patients including patients undergoing cavernoplasty are operated on under general anaesthesia – lung isolation is ensured by the use of a double-lumen endobronchial tube.

Surgical procedures were performed through postero-lateral thoracotomy, pleural space was entered through the 5th space, minimal lysis of adhesions is done to allow placement of a limited blade retractor avoiding haemorrhage. Further lysis is done using electrocautery.

6 Surgical Techniques

6.1 Preparation of Muscle Flap

The fibrosed and sclerotic tissues are resected until tissue with good vascularization is detected. This includes resecting skin edges, muscular edges and more often bone edges of the cavernostomy.

6.2 Muscle Flap

The muscular graft needs to be large, thick, well-vascularized and mobile, without any tension. For these reasons, our preference goes to the latissimus muscle. Harvesting of the muscle is done through posterior approach, even in patients that had previous thoracotomy. The muscle is pediculized avoiding harvesting the junctions with the last four ribs where vascularization is preponderant. We cut the muscle from the scapulae angle and from there we can easily dissect in the axillary area a thick piece of muscle that ends by an insertion in biceps area. A contra-incision, high in axillary region is necessary to mobilize and cut the insertion on humeral roots when the muscle is prepared through posterior approach. Then an adequate and large piece of well-vascularized muscle is harvested even in case of redo thoracotomy.

To obliterate the "dead space" due to axillary areas, we can use the latissimus dorsi, although the Teres major or Teres minor can also be harvested. Other muscles can also be harvested: pectoralis muscle for anterior speleotomies by desinserting of the inferior part from biceps brachii insertion.

In posterior speleotomies, mobilization of latissimus dorsi is not mandatory. In those situations, it could be a better alternative to pediculize one or more intercostal spaces. When it is not available to harvest intercostal spaces, after thoracoplasty or extended pariectomy, we can harvest subscapularis muscle or supraspinatus muscle that give a huge and well-vascularized mass. With that technique, Hertzog et al. [28] could easily obliterate wide defects of upper spaces. This technique request cut of the inferior part of the scapulae, which does not give any sequelae.

Regarding the site and the type of the dead space to obliterate, enclosed find the list of the harvesting muscles:

- Anterior speleotomies: pectoralis major;
- Axillary speleotomies: latissimus dorsi with or whithout the Teres major;
- Posterior speleotomies: majority of latissimus dorsi, intercostal muscles and others.

6.3 How to Insert the Muscle Graft

The muscle graft can be implanted directly in the defect to cause obliteration without any traction or tension. It is mandatory to have enough graft even more than needed to close all the area with some interrupted sutures with low resorption materials.

6.4 Cover Plan

A second aponeurotic and muscular stage is implanted in order to ensure adequate compression and adequate stasis of the scapulae. The trapezius muscle is used in the cover plan for the half upper and posterior part.

6.5 Cutaneous Plan and Drainage

Harvesting of muscular graft always necessitate wide subcutaneous dissections. At the end of operation, there is an excess of cutaneous tissue requiring resection to avoid subcutaneous dead space. Aspiration and suction with drainage using redon tube is used. The bandage should be compressive.

7 Conclusions

Surgery remains to date the mainstay of treatment of fungal balls [29], being more effective than antifungal treatment particularly for the resolution of simple aspergillomas [30]. However, most CCPA patients are poor candidates for pulmonary resection. In order to minimise the rate of post-surgical complications, patients should be carefully selected and evaluated before surgery is performed. The best surgical approach depends on the lesions and the patient being operated on. Pleuro-pneumonectomy should be reserved for patients with diffuse lung destruction (Fig. 2). A less aggressive surgical management such as cavernostomy should always be considered before pleuro-pneumonectomy is considered. Open-window thoracostomy followed by daily insertion of gauze impregnated with amphotericin B is a good alternative option in the treatment for *Aspergillus* empyema. As for cavernostomy the thoracostomy has to be secondarily closed by a muscle flap.

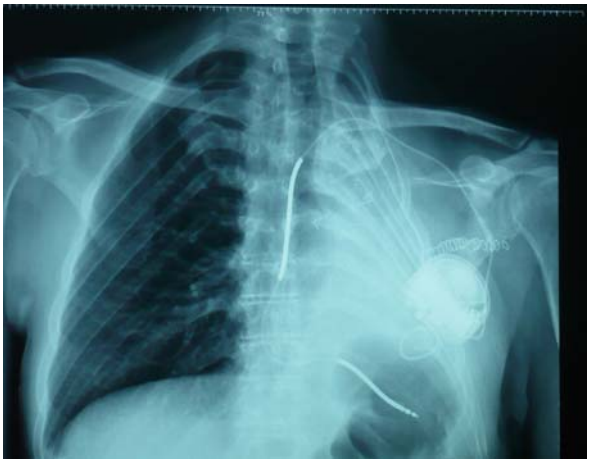

Fig. 2 Twenty-five years follow-up after pleuropneumonectomy and thoracoplasty for complicated pulmonary fungal ball

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PART IV
ALLERGIC PULMONARY SYNDROMES ASSOCIATED WITH ASPERGILLOSIS

Overview of *Aspergillus* Allergens

R. Crameri, A.G. Glaser, M. Vilhelmsson, S. Zeller, and C. Rhyner

Abstract Fungi in general and, *Aspergillus fumigatus* (*A. fumigatus*) in particular, are able to produce complex patterns of IgE-binding molecules. Robotics-based high throughput screening of *A. fumigatus* cDNA libraries displayed on phage surfaces revealed at last 81 different sequences encoding structures potentially able to bind to serum IgE of sensitised individuals suffering from *A. fumigatus*-related complications. Although not all of these allergens have been characterised in detail, *A. fumigatus* still represents the best investigated allergenic source. A total of 23 *A. fumigatus* allergens are recorded by the official allergen list of the International Union of Immunological Societies (www.allergen.org) and this is by far the longest allergen list reported for a single allergenic source. The IgE-binding molecules include species-specific as well as phylogenetically highly conserved cross-reactive structures and such with unknown function. A subset of cDNAs have been used to produce and characterise the corresponding recombinant allergens which have proven to be useful diagnostic reagents allowing specific detection of *A. fumigatus* sensitisation and differential diagnosis of allergic bronchopulmonary aspergillosis. Structures highly conserved through different species like manganese-dependent superoxide dismutase, P_2 acidic ribosomal protein, cyclophilins and thioredoxins induce, beyond sensitisation, IgE antibodies able to cross-react with the corresponding homologous self-antigens. The frequently observed cross-reactivity is traceable back to shared discontinuous B-cell epitopes as shown by detailed analyses of the crystal structures.

Keywords *Aspergillus* · Allergy · Crystal structures · Fungal allergens · IgE-mediated Autoreactivity

R. Crameri (✉)
Molecular Allergology Swiss Institute of Allergy and Asthma Research (SIAF), CH-7270 Davos, Switzerland
e-mail: crameri@siaf.uzh.ch

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Contents

1 Introduction

In contrast to other allergenic sources like pollens or mites, which are primarily associated with IgE-mediated type I allergic reactions, fungi are also associated with a broad range of other partly life-threatening diseases [1]. Primary and secondary fungal infections have become an important medical problem, concomitantly with the increasing number of patients undergoing immunosuppressive medical treatment, chemotherapy, and with increases in congenital and acquired immunodeficiency [2]. Colonisation with fungi is a common finding. The spectrum of fungal diseases in humans ranges from superficial skin infections, allergy and asthma, to life-threatening diseases like invasive systemic mycoses or allergic bronchopulmonary aspergillosis (ABPA) [3]. However, fungal infections rarely affect immunocompetent hosts, and patients at high risk to be affected by fungal mycoses are those with significant reduction in the number or function of granulocytes such as leukaemia patients undergoing chemotherapy [4], patients on long-term treatment with immunosuppressive drugs (e.g. organ transplant patients) [5], or patients under high dosed steroid treatment [6]. The fatality rate of invasive mycoses is high and the majority of the patients die despite of anti-mycotic treatment [7].

In contrast fungal allergy is rarely a life-threatening disease but the dimension of the problem might be underestimated [8]. Three groups of fungi include most of the species relevant for allergic disease: *Zygomycota*, *Ascomycota*, and *Basidiomycota*. Allergens from these species are associated with fungal allergic asthma, sinusitis, rhinitis, hypersensitivity pneumonitis, allergic bronchopulmonary mycoses and atopic eczema [1]. However, recombinant allergens have so far only been reported for the two groups *Ascomycota* and *Basidiomycota* (Table 1). Fungal allergy in general (and allergy to *A. fumigatus* in particular) has been shown to be associated with strong humoral responses to fungal antigens [2, 3]. Skin test surveys indicate that at least 3–10% of the worldwide population might be affected by fungal sensitisation [9]. Unfortunately the prevalence of fungal sensitisation has not yet been reliably established because the available reports of skin test reactivity to fungi vary from 3 to 90% depending on the study population, the fungal species tested, and especially

Table 1 Number of cloned fungal allergens and most prominent cross-reactive structures

Ascomycota	Cloned allergens	Pan-allergens
Alternaria alternate	10	Ribosomal P_1 protein (Alt a 12); Ribosomal P_2 protein (Alt a 5); Heat shock protein (Alt a 3); Aldehyde dehydrogenase (Alt a 10)
Cladosporium cladosporioides	1	Serine protease (Cla c 9)
Cladosporium herbarum	8	Ribosomal P_1 protein (Cla h 12); Ribosomal P_2 progein (Cla h 5); Enolase (cla h 6); Serine protease (Cla h 9); Aldehyde dehydrogenase (Cla h 10)
Curvularia lunata	3	Serine protease (Cur l 1); Enolase (Cur l 2); Cytochrome c (Cur l 3)
Aspergillus flavus	1	Serine protease (Asp fl 13)
Aspergillus fumigatus	23	Peroxysomal protein (Asp f 3); MnSOD (Asp f 6); Ribosomal P_2 protein (Asp f 8); Heat shock protein (Asp f 12); Serine proteases (Asp f 13, Asp f 18); Enolase (Asp f 22); Cyclophilins (Asp f 11, Asp f 27); Thioredoxins (Asp f 28, Asp f 29)
Aspergillus niger	3	Serine protease (Asp n 18)
Aspergillus oryzae	2	Serine protease (Asp o 13)
Penicillium brevicompactum	2	Serine protease (Pen b 13); Ribosomal P_1 protein (Pen b 26)
Penicillium chrysogenum	5	Serine proteases (Pen ch 13, Pen ch 18)
Penicillium citrinum	7	Peroxysomal protein (Pen c 3); Serine protease (Pen c 3); Enolase (Pen c 22); Catalase (pen c 30)
Penicillium oxalicum	1	Serine protease (Pen o 18)
Fusarium culmorum	2	Ribosomal P_2 protein (Fus c 1); Thioredoxin homologous (Fus c 2)
Trichophyton rubum	2	Alkaline protease (Tri r 2); Serine protease (Tri r 4)
Trichophyton tonsurans	2	Serine protease (Trit 4)
Candida albicans	2	Alcohol dehydrogenase (Cand a 1); Peroxysomal protein (Cand a 2)
Candida boidinii	2	Peroxysomal membrane protein (Cand b 2)
Epicoccum purpurascens	1	Serine protease (Epi p 1)
Basidiomycota		
Coprinus comatus	5	Thioredoxin (Cop c 2)
Rhodotorula mucilaginosa	2	Enolase (Rho m 1); Serine Protease (Rho m 2)
Psilocybe cubensis	2	Cyclophilin (Psi c 2)
Malassezia furfur	3	Peroxysomal membrane proteins (Mala f 2; Mala f 3)
Malassezia sympodialis	10	Cyclophilin (Mala s 6); MnSOD (Mala s 11); Thioredoxin (Mala s 13)

from the fungal extract used [9, 10]. One of the major reasons for this huge variation is due to the fact that even commercially available fungal extracts used for skin testing show an extremely high variation in their (major) allergen content [6, 7]. A second important factor complicating the diagnosis of fungal allergy results from an extended cross-reactivity detectable between different fungal species, which is partly traceable back to the presence of structurally related allergens [11]. Cloning, sequencing, and expression of these allergens as recombinant proteins and characterisation of their cross-reactive profiles might contribute to considerably improve this unsatisfactory situation.

2 The World of Fungal Allergens

During the past 20 decades an impressive number of allergens from different sources have been cloned, sequenced, produced as recombinant proteins, and partly evaluated for their diagnostic [10], and therapeutic value [12, 13]. Behind plant pollens and mites with 184 and 164 reported single allergens, respectively, fungi with 100 allergens represent the third group of organisms from where the largest number of allergens has been cloned and characterised (Table 1). The spectrum of fungal allergens has been recently reviewed in detail, including also allergens reported in the literature but not included in the official allergen nomenclature list [1]. According to this work the fungal allergens cloned so far are 206. Of course as for the other allergenic sources not all cloned fungal allergens match to unique structures, because cross-reactivity between homologous structures present in fungal extracts derived from different species is a quite common phenomenon (Table 1).

Basically fungal allergens can be subdivided in species-specific allergens which are specific to a species or at least to a genus [11] and phylogenetically highly conserved structures which represent allergens showing cross-reactivity not only with fungal proteins of many fungal species [14, 15], but also with homologous proteins of other allergenic sources [16, 17], including human proteins [18].

3 The Allergen Repertoire of *Aspergillus fumigatus*

All fungal allergen repertoires described so fare are quite large, but those of *A. fumigatus* is by far the most complex one. It spans at least 81 different cDNA gene products ranging from 10 to more than 65 kDa in size [19], and 23 *A. fumigatus* allergens are reported by the official allergen list (Table 2). The recently sequenced 29.4 megabase *A. fumigatus* genome contains 9,926 predicted genes [20, 21] indicating that only a very small portion to the fungal proteins are able to induce a switch towards production of specific IgE in B cells. However, the availability of the complete genome sequence allowed to verify all reported cDNA sequences encoding *A. fumigatus* allergens for consistency and for the presence of homologous and orthologous allergens within the fungal kingdom [11, 22, 23]. The detailed comparison between genome sequence and published cDNA sequences [23] revealed several

Table 2 Cloned *Aspergillus fumigatus* allergens (www.allergen.org)

Allergen	Size (kDa)	CDS	Function/sequence similarity	IgE-binding in vitro (%)[a]	SPT	Access. no.	References
Asp f 1	16.9	C	Ribotoxin	66	pos.	S889330	[34]
Asp f 2	37.0	C	Unknown	ND	NT	U56938	[78]
Asp f 3	18.5	C	Peroxisomal protein	88	pos.	U58050	[79]
Asp f 4	30.0	P	Unknown	42	pos.	AJ001732	[45]
Asp f 5	42.1	C	Metalloprotease	85	pos.	Z30424	[27]
Asp f 6	23.0	C	MnSOD	35	pos.	U53561	[58]
Asp f 7	11.6	P	Unknown	39	pos.	AJ223315	[27]
Asp f 8	11.1	C	P_2 ribosomal protein	10	pos.	AJ224333	[59]
Asp f 9	32.3	P	Unknown	66	pos.	AJ223327	[27]
Asp f 10	34.4	C	Aspartic protease	18	pos.	X85092	[27]
Asp f 11	18.8	C	Cyclophilin	50	pos.	AJ006689	[60]
Asp f 12	65.0	P	Heat shock protein	ND	NT	U92465	[80]
Asp f 13	34.0	C	Alkaline serine protease	ND	NT	Z11580	[25]
Asp f 15	19.5	C	Serine protease?	29	pos.	AJ002026	[24]
Asp f 16	43.0	C	Unknown	ND	NT	G3643813	[26]
Asp f 17	19.4	P	Unknown	43	pos.	AJ224865	[24]
Asp f 18	34.0	C	Vacuolar serine protease	ND	NT	Y13338	[81]
Asp f 22	46.0	C	Enolase	ND	NT	AF284645	[82]
Asp f 23	44.0	C	L3 ribosomal protein	ND	NT	AF464911	[83]
Asp f 27	18.0	C	Cyclophilin	75	pos.	AJ937743	[30]
Asp f 28	11.9	C	Thioredoxin	16	pos.	AJ937744	[29]
Asp f 29	11.9	C	Thioredoxin	26	pos.	AJ937745	[29]
Asp f 34	19.3	C	Phi A cell wall protein	94	pos.	AM496018	Glaser et al. (unpublished)

[a]Incidence amongst patients sensitised to *A. fumigatus*.
Legend: C, complete; CDS, coding sequences; ND, not determined; NT, not tested; pos., positive; P, partial.

inconsistencies: (i) Asp f 15 [24] and Asp f 13 [25] are identical; (ii) Asp f 16 [26] does not appear to be encoded by the *A. fumigatus* AF293 genome but shows a high degree of homology with Asp f 9 [27], which is present in the genome. Therefore it can be concluded that the Asp f 9 cDNA sequence is correct, and that the published Asp f 16 peptide sequence results from frameshift, other sequencing errors, or derives from a different *A. fumigatus* strain; (iii) Asp f 17, originally described as a cDNA encoding a 191 amino acid protein [24], is incomplete and the complete gene encodes a protein of 284 amino acids; and (iv) the postulated sequence of the Asp f 56 kDa allergen not included in the official allergen database, which was derived from a short peptide obtained from a biochemically purified protein [28] is not predicted to be encoded in any of the sequenced *Aspergillus* genomes.

All other *A. fumigatus* allergens reported in the official allergen database as well as the sequences of the recently cloned thioredoxins (Asp f 28, Asp f 29) [29], cyclophilins (Asp f 11, Asp f 27) [30], and Phi A cell wall protein (Asp f 34, Glaser unpublished) sequences showed virtually a 100% identity with the genome sequence, confirming at genomic level the validity of cDNA cloning approaches for the elucidation of allergen repertoires. The whole repertoire of *A. fumigatus* allergens included in the official allergen list reported in Table 2 have been produced as highly pure recombinant allergens and evaluated for their IgE-binding capacity in vitro. The majority of these proteins have also been tested for their ability to elicit positive skin tests in vivo [27, 29–32].

Although these detailed clinical investigations clearly show that the cloned cDNA sequences encode biologically active and clinically relevant allergens, the reason for the allergenicity of these specific proteins remains unknown. Even the solution of the three dimensional structure of an array of fungal allergens (Table 3), and intensive modelling approaches based on homologous structures (Table 4), have not yet contributed to understand the reasons why some proteins are able to induce a switch to allergen-specific IgE production and some others are not [15, 33].

Table 3 Solved crystal structures of fungal allergen

Allergen	Size (kDa)	Basic structure	Coordinates[a]	References
Asp f 1 *(A. fumigatus)*	16.9	Ribotoxin	1ONE	[84]
Asp f 6 *(A. fumigatus)*	23	MnSOD	1KKC	[65]
Asp f 11 *(A. fumigatus)*	13.7	Cyclophilin	2C3B	[61]
Mala s 1 *(M. sympodialis)*	22.9	Unknown function	1ABM	[67]
Mala s 6 *(M. sympodialis)*	17.2	Cyclophilin	2CFE	[30]
Mala s 13 *(M. sympodialis)*	11.9	Thioredoxin	2J23	[29]

[a]PDB-coordinates of the structure.

Table 4 Modelled fungal allergen structures

Allergen	Size (kDa)	Basic structure	Coordinates[a]	References
Cla h 6 (C. herbarum)	47.5	Enolase	1ONE	[85]
NTF 2 (C. herbarum)	14.2	Nuclear transport factor 2	1 OUN	[86]
Cla h 8 (C. herbarum)	8.1	Cold shock protein	3MFE	[87]
NTF 2 (A. alternata)	13.7	Nuclear transport factor 2	1OUN	[86]
MnSOD (S. cerevisiae)	22.9	MnSOD	1ABM	[65]
Asp f 22 *(A. fumigatus)*	46	Enolase	4ENL	[11]
Asp f 27 *(A. fumigatus)*	18	Cyclophilin	2CFE	Glaser et al. (unpublished)
Asp f 28 *(A. fumigatus)*	11.9	Thioredoxin	2TRX	Glaser et al. (unpublished)
Asp f 29 *(A. fumigatus)*	11.9	Thioredoxin	2J23	Glaser et al. (unpublished)
Pen ch 18 (chrysogenum)		Serine protease	1IC6A	[11]

[a]PDB-coordinates of the structure used for modelling.

Beside Asp f 4, Asp f 7 Asp f 9 and Asp f 16 which lack to show a relevant sequence similarity to proteins deposited in the Swissprot database, all but one allergen show extended sequence similarities with known fungal and non-fungal proteins (Table 2). The exception is Asp f 1, a potent fungal ribotoxin which was the first fungal allergen cloned [34] and the first recombinant allergen tested in humans at all [31]. Asp f 1 reveals sequence identity to restrictocin, an 18 kDa ribotoxin produced by *Aspergillus restrictus* never described as allergen [35] that is >99% identical to Asp f 1 sequences derived from different clinical isolates of *A. fumigatus* [36, 37]. All these 18 kDa proteins are related to ribotoxins such as α-sarcin [38] and mitogillin [39]. They represent the most potent ribonucleolytic enzymes found in nature [40, 41] and are restricted to the genus *Aspergillus* as recently demonstrated by a genome wide analysis involving all fungal genomes sequenced so far [11].

4 Diagnostic Value of Recombinant *A. fumigatus* Allergens

As already mentioned allergen extracts, and in particular *A. fumigatus* extracts, are complex mixtures of allergenic and non allergenic proteins and as such difficult to standardise [42]. The best example thereof is delivered by a skin test study with four different commercially available *A. fumigatus* extracts conducted in cystic fibrosis patients sensitised to *A. fumigatus* [43]. This study shows that none of the four tested commercial extracts is able to detect all of the 31 *A. fumigatus*-sensitised patients in skin prick test, clearly demonstrating that the diagnostic result strongly depends on the quality of the extract used. An additional complication in the diagnosis of *A. fumigatus* sensitisation results from the fact that the extracts immobilised on the ImmunoCAP system, which is still considered as the "golden standard" for the in vitro detection of *A. fumigatus*-sensitisation, is not available for skin prick tests (the author is referred to the chapter on *Aspergillus* IgE testing, in this book). This fact might partly explain the huge discrepancy between skin prick test and in vitro incidence of the prevalence of *A. fumigatus* sensitisation reported in the literature, because both methods depend on the quality of the used extracts. In contrast, recombinant allergens can be produced as perfectly standardised protein preparations [44]. As shown in Table 2, rAsp f 1, 3, 4–11, 15, 17, 27–29, and 34 have been investigated in skin test studies. The experience accumulated performing skin tests and serology with different recombinant *A. fumigatus* allergens involving more than 600 individuals suffering from various *A. fumigatus*-related diseases and over 100 healthy controls allows to draw the following firm conclusions: (i) only individuals with detectable serum IgE levels against a tested recombinant allergen showed positive skin test reactions with the specific allergen [44]; (ii) the overall positive skin test response of individuals sensitised to *A. fumigatus* extracts to recombinant allergens are highly variable from allergen to allergen ranging from around 70% for the major allergen rAsp f 1 [27] to a few percentage for minor allergens like Asp f 8 or Asp f 10 [24]; (iii) no reactivity to any of the recombinant allergens could be detected in any of the more than 100 healthy controls tested; (iv) rAsp f 4 and rAsp f 6 are predominantly recognised by patients suffering from ABPA [32, 45, 46]; (v) no

discrepancy between serology and skin test results based on recombinant allergens could be detected in any of the subjects tested [44]; and (vi) the recombinant allergens can be immobilised to ImmunoCAPs without loss of specificity or sensitivity allowing a fully automated serologic diagnosis of sensitisation against each single allergen [32, 47, 48], and thus a component-resolved diagnosis of allergic conditions related to *A. fumigatus* sensitisation.

Notably, skin tests and serologic investigations showed that two recombinant allergens, Asp f 4 and Asp f 6, can be used to confirm or reject an ABPA suspected from clinical signs [32, 45, 46, 49, 50]. ABPA is a clinical syndrome that is difficult to diagnose, especially in patients suffering from cystic fibrosis [51], which should be excluded in all individuals sensitised to *A. fumigatus* to avoid an irreversible deterioration of the lung function [52].

5 Cross- and Autoreactivity

Many of the *A. fumigatus* allergens cloned, characterised, produced, and evaluated for their allergenicity in vivo (Table 2) represent phylogenetically-conserved proteins widespread in basically every living organism. The presence of cross-reactive structures like enolase and MnSOD, which are also described as latex allergens [16, 17], cyclophilin known as a birch pollen allergen [53], thioredoxin described as a prominent food allergen [54], and serine proteases which play an important role in house dust mite allergy [55] indicate that the whole repertoire of allergenic molecules is highly redundant and, thus, limited to a discrete number of structures [56]. In contrast to polysensitisation which is easy to demonstrate by simple determination of the RAST values against different allergen extracts [8], cross-reactivity between fungal extracts is a poorly investigated phenomenon which, however, could contribute to a better understanding of polysensitisation. Basically cross-reactivity can be traced back to conserved proteins sharing common B and T cell epitopes as shown for many allergen structures [33]. Amongst these MnSOD (Asp f 6) [57, 58], P_2 acidic ribosomal protein (Asp f 8) [59], cyclophilins (Asp f 11 and Asp f 27) [30, 60, 61] and thioredoxins (Asp f 28 and Asp f 29) [29] have been shown to belong to families of cross-reactive pan-allergens. Per definition pan-allergens share a high degree of sequence identity at primary structure level, thus belonging to protein families showing similar structural folding [33, 62]. Interestingly some of these proteins show cross-reactivity also with their homologous human proteins at both, humoral and cellular level. The best investigated structures at this level are human MnSOD [57, 58], cyclophilin [30, 61] and thioredoxin [29], which were shown to bind to serum IgE from *A. fumigatus* sensitised patients in vitro, to elicit specific skin test reactions in vivo, and to be potentially involved in the pathogenesis of ABPA and atopic eczema [18, 63]. Although the role played by IgE-mediated autoreactivity to self-antigens remains controversial, the fact that the application of human MnSOD in patch test on healthy skin areas of atopic eczema patients sensitised to fungal MnSOD is sufficient to induce an eczematous reaction [57, 63] strongly indicates that autoreactivity could contribute to the exacerbation of the symptoms in the

absence of external exposure to environmental allergens. For self-antigens showing a high degree of sequence identity/homology to environmental allergens, these reactions can be clearly explained by cross-reactive IgE antibodies primarily raised against external allergens. However, also self-antigens without sequence homology with any known allergen but able to bind serum IgE from atopic eczema patients have been described [64]. For these structures it is still not clear if they are able to directly induce a B cell switch to IgE production, or if the observed IgE-binding capability is due to cross-reactivity with not yet identified environmental allergen structures [18].

6 Structural Aspects of Fungal Allergens

The list of known allergen crystal structures with coordinates deposited in the Protein Data Bank (PDB, http://www.rcsb.org/pdb/) report about 40 structures. The best investigated allergenic source in structural terms is the timothy grass *Phleum pratense* where five structures have been solved, followed by profilins of different species [15]. However, only three crystal structures derived from fungal allergens, ribotoxin (Asp f 1) [45, 46], MnSOD (Asp f 6) [65] from *A. fumigatus* and a Xylanase from *Paecilomyces variotii* [66] have been reported so far. Recently the crystal structures of a cyclophilin of *A. fumigatus* (Asp f 11) [61] and *Malassezia sympodialis* (*M. sympodialis*) (Mala s 6) [30], as well as the crystal structure of thioredoxin of *M. sympodialis* (Mala s 13) [29], and Mala s 1, a major allergen of *M. sympodialis* with unknown function [67] have been determined at high resolution.

These structures are per se not new, nor are the solved allergen crystal structures in general [62]. Therefore it is unlikely that crystal structures will help us to progress in our understanding of the basic immunologic mechanisms leading to allergy. In contrast, elucidation of the 3D structure of allergens has essentially contributed to our understanding of cross-reactivity amongst homologous structures derived from phylogenetically distant allergen sources [29, 30, 68]. Based on the coordinates of a known homologous crystal structure of a sufficiently homologous protein it is easy to model a structure starting from the known amino acid sequence of an allergen (Table 3) and this work will strongly contribute to predict fungal proteins that have the potential to be allergens derived from comparative genomic approaches [11]. Progress in this field will have a strong impact for understanding fungal sensitisation, cross-reactivity, interpretation of diagnostic outcomes and management of fungal allergy.

7 Therapy of Fungal Allergy

Severe symptoms due to fungal sensitisation, especially in patients with ABPA, are usually treated with systemic steroids [69]. The discussion on the benefits of antifungal therapy for these patients is beyond the scope of this article. Allergen-specific immunotherapy, the only treatment able to cure allergy, is not recommended for

mould allergy because standardised extracts are not available [70], and because the therapy is associated with frequent occurrence of severe side effects [71]. In fact only a few studies describing immunotherapy with fungal extracts are available from the literature. The use of recombinant allergens for allergen-specific immunotherapy is claimed to solve problems related to the standardisation of extracts [72, 73]. Indeed some studies report the successful immunotherapy of pollen allergic patients with recombinant allergens [12, 74–76]. Therefore, the use of recombinant allergens for the immunotherapy of fungal allergies may be a future aim [1]. However, in view of the large number of allergens produced by fungi it is questionable if such an approach will be feasible, especially if the financial aspects for the production of cGMP conform multi-component vaccines based on single recombinant allergens are considered [77].

8 Conclusions

There is no doubt that *A. fumigatus* plays a relevant role in invasive aspergillosis as well as in many forms of allergy. Amongst the *A. fumigatus*-related allergic complications ABPA is, although infrequent, the most severe one, and should be ruled out in all asthmatic and cystic fibrosis patients sensitised to the fungus to avoid irreversible deterioration of the lung function. The diagnosis of ABPA is challenging as it is based on distinct clinical and laboratory findings that are rarely present at the same time during the different phases of the disease. Amongst the laboratory findings serology is of outmost importance for the diagnosis of ABPA, although strongly limited by the quality of the extracts used. Cloning, expression, characterisation, and production of recombinant *A. fumigatus* allergens have contributed to improve a component resolved diagnosis of the disease. Large scale serological studies showed that specific IgE responses to at least four *A. fumigatus* allergens (Asp f 2, 4, 6, and 8) are quite specific for ABPA. These disease-specific allergens allow a serological discrimination between ABPA and *A. fumigatus*-sensitisation with almost 100% specificity and around 90% sensitivity. Such discrimination is not possible using conventional fungal extracts, highlighting the potential of recombinant allergens for diagnostic purposes. However, to reconstruct the whole allergenicity of the fungal extract, more of the IgE-binding cDNA sequences identified by high-throughput phage surface display screenings need to be cloned and used to produce and clinically evaluate the encoded recombinant allergens. The availability of the complete *A. fumigatus* genome sequence will facilitate this task and in addition contribute to identify cross-reactive IgE-binding proteins widespread among the fungal kingdom. Together with the rapidly growing knowledge about 3D structures a complete allergen repertoire will allow to gain deep knowledge about the role of each single structure in the pathophysiology of ABPA and, perhaps, to develop a panel of allergens for the immunotherapy of the disease.

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Epidemiology of Allergic Bronchopulmonary Aspergillosis

Ritesh Agarwal and Arunaloke Chakrabarti

Abstract Though allergic bronchopulmonary aspergillosis (ABPA) has a worldwide distribution, there are likely to be significant geographic differences in the prevalence of ABPA. In this chapter, we summarise the available data on the epidemiology of ABPA. We have classified the review into the following headings: (i) environmental and genetic factors influencing the epidemiology of ABPA; (ii) prevalence of *Aspergillus* hypersensitivity and ABPA in patients with asthma; (iii) prevalence of ABPA in patients with cystic fibrosis; and (iv) occurrence of ABPA in certain special situations. For the purpose of this review we have performed a systematic search of the electronic databases – MEDLINE and EMBASE for relevant studies published from 1965 till current date using the free text terms: allergic bronchopulmonary aspergillosis. In addition, we reviewed our personal files. A total of 208 articles were reviewed in detail for the purpose of this review.

Keywords ABPA · Allergic bronchopulmonary aspergillosis · Epidemiology

Contents

R. Agarwal (✉)
Department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India
e-mail: riteshpgi@gmail.com

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1 Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a pulmonary disorder attributed to hypersensitivity reactions to *Aspergillus* species mainly *A. fumigatus* [1]. There are approximately 250 species of *Aspergillus* but only a few are known to cause disease in humans [2]. *A. fumigatus*, *A. flavus*, and *A. niger* are the most commonly encountered pathogenic species, but other species, like *A. terreus*, *A. clavatus*, and *A. nidulans* are rarely reported as human pathogens [3, 4]. It is of interest to note that only pathogenic species (for example *A. fumigatus*, *A. flavus*, *A. terreus, A. niger, A. nidulans*) can grow at 35–37°C (95–99°F). *Aspergillus* was first catalogued in 1729 by an Italian priest and biologist Pietro Antonio Micheli. Viewing the fungi under a microscope, Micheli thought of the shape of an aspergillum (holy water sprinkler), and named the genus accordingly. *A. fumigatus* was described as a species in 1863 by Johann Baptist Georg Wolfgang Fresenius. *Aspergillus* species are ubiquitous mould found in organic debris, dust, compost, foods, spices, and rotted plants. In addition, they are common indoor moulds especially in attic and basements, bedding, curtains, floor mats and house dust [5]. Worldwide surveys have found that *Aspergillus* species represent ~0.1–22% of the total air spores sampled [6]. *Aspergillus* species are thermotolerant and are capable of growing at a wide range of temperatures ranging from 15 to 53°C. These fungi can be cultured easily using Sabouraud agar medium at 37°C in contrast of many other fungi whose growth is inhibited beyond 35°C. The conidia of *A. fumigatus* are 2.0–3.5 μm in size which are in the respirable range, and thus the inhaled spores easily enter the airways. In susceptible persons, *Aspergillus* species might grow from conidia to hyphae and colonise the bronchi. *Aspergillus* spp. are recognised in tissue preparations, exudates and sputum as filamentous, septate hyphae, which are approximately 3–7 μm wide and show acute angled branching. The reader is referred to the first chapters of this book for more on the identification of the *Aspergilli* at the species level.

The clinical, radiological and histological manifestations of ABPA are a complex interplay between the number and virulence of the organisms and the patient's immune response [7]. These are discussed in more detail elsewhere in this book. Depending on the host immunity and the organism virulence the respiratory diseases caused by *Aspergillus* species can be broadly classified as allergic (allergic *Aspergillus* sinusitis, ABPA, severe asthma with fungal sensitisation, SAFS, and hypersensitivity pneumonias), chronic non-invasive (e.g., chronic sinusitis or pulmonary cavitary aspergillosis, with or without fungal balls), and invasive conditions

[8]. Some overlap however does occur, as discussed by Dr. Soubani in a distinct chapter.

Patients with ABPA present with a history of asthma and are generally atopic [9]. The major diagnostic features of ABPA can easily be remembered by a mnemonic ARTEPICS that include (A) history of *a*sthma; (R) *r*adiological pulmonary opacities- transient or fixed; (T) immediate cutaneous reaction to an intradermal skin *t*est against *Aspergillus* antigen; (E) peripheral blood *e*osinophilia; (P) presence of serum *p*recipitins against *A. fumigatus*; (I) elevated serum *I*gE; (C) *c*entral bronchiectasis on high-resolution computed tomography (HRCT) of the chest; and (S) elevated serum *s*pecific IgG and IgE levels against *A. fumigatus*. Dr. Shah reviews diagnostic features of ABPA in a separate chapter.

Based on the presence or absence of central bronchiectasis (CB) on HRCT, ABPA can be further classified as ABPA-CB or seropositive ABPA (ABPA-S, in the absence of CB) [10]. Based on the radiological and serologic findings, ABPA has also been classified as mild, moderate and severe [11]. Whilst most patients with ABPA-S have a mild disease, ABPA-CB is usually moderate in intensity in comparison to ABPA-CB associated to other radiological findings (ORF). In a subsequent large study of 126 patients with ABPA (34 patients with ABPA-S, 42 with ABPA-CB, and 50 with ABPA-CB-ORF) it was shown that there were no significant differences between the stages of ABPA (either staging the disease as ABPA-S and ABPA-CB or dividing the disease into three categories as ABPA-S, ABPA-CB and ABPA-CB-ORF) and the duration of illness, the severity of asthma, and the serologic findings (i.e., absolute eosinophil count, total and *A. fumigatus* specific IgE levels) [12].

Despite significant advances in understanding of the pathophysiology of the disorder, the exact prevalence of ABPA still remains unknown although it is now diagnosed with greater frequency and certainty. The disease may well have its onset in childhood [13] and may go unrecognised for years or even decades [14]. It is unlikely that true population prevalence of ABPA can be determined by analysis of the data only from reference centres such as institutions or chest clinics with a special interest in the disease. Furthermore, ABPA is not recognised in the international classification of diseases (including the tenth revision of 2007) [15]; ABPA has to be classified as either B44.1 (“Other pulmonary aspergillosis”) or B44.9 (“Aspergillosis, unspecified”), making it difficult to retrieve data from hospital records. Also the diagnostic criteria of ABPA lack clinical uniformity which makes it further difficult to compare data reported from various centres. The rest of this article focuses on the prevalence of ABPA in different scenarios, and the factors influencing the epidemiology of ABPA.

2 Environmental Factors Influencing the Occurrence of ABPA

The *Aspergillus* group of fungi is found worldwide in soil and decaying vegetable matter, with peak atmospheric concentrations of conidia occurring during the winter season [16]. It is unclear whether the size of the inoculum (i.e. number of

conidia inhaled) is a factor in the causation of ABPA. It is important to note that high conidia concentrations alone do not account for the high incidence of sensitisation to this fungus as higher concentrations of other fungi are also found in both indoor and outdoor environment. There are certain specific characteristics of the fungi that allow for this predisposition. These include the small size of the spores (2.5–6 μm), which are in the respirable range and favour deposition in the peripheral lung or paranasal sinuses. A second important feature is the property of thermotolerance which enables them to grow at body temperature and accounts for the higher numbers in specimens from bronchi sampled at post-mortem relative to other atmospheric fungi [17]. The conidia of *A. fumigatus* have also been shown to inhibit phagocytosis of antibody coated radiolabeled sheep red blood cells by primed mouse phagocytic cells whereas conidia of another fungus, *Penicillium ochrochloron* had no such effect. This suggests that conidia of *A. fumigatus* when bound to the surface of phagocytes are able to release a substance that inhibits their ingestion while having little or no effect on surface binding [18]. Production of reactive oxygen intermediates in response to *A. fumigatus* is also significantly lower in comparison to other fungi suggesting that conidia of *A. fumigatus* fail to trigger and also inhibit the production of reactive oxygen intermediates by phagocytic cells [19].

Some authors have suggested that exposure to large concentrations of conidia of *A. fumigatus* may be related to the disease and the fact that it occurs more commonly in agricultural conditions [20, 21]. In fact, exposure to high concentrations of *A. fumigatus* conidia from garbage dump sites, bird droppings, and smoking mouldy marijuana have been reported to cause ABPA [22–24]. Other investigators have found a correlation of worsening airway obstruction in patients with ABPA with an increase in *A. fumigatus* conidia in the immediate outside environment [25], whereas others did not find such an association [26]. The reader is referred to the chapter by Dr Akiyama for more detail on the occurrence of ABPA as an occupational disease. Unlike hypersensitivity pneumonitis where environmental factors are central to the pathogenesis, the environmental factors are currently not considered to be the main pathogenic factors in causation of ABPA because not all asthmatics develop ABPA despite being exposed to the same environment.

3 Genetic Factors Influencing the Epidemiology of ABPA

The current understanding of ABPA is that the host immune/genetic status and subsequent response to *A. fumigatus* are more important than environmental/occupational factors in the causation of ABPA. In fact, in the last decade several genetic factors [27–40] have been identified, which influence the development of ABPA in patients with asthma and cystic fibrosis (Table 1). A greater detail on the pathogenesis and current understanding on the genetic influences on the pathogenesis, natural history and course is discussed in another chapter in this book.

Table 1 Genetic factors implicated in the occurrence of allergic bronchopulmonary aspergillosis

HLA-DR2 polymorphisms [27–30]
Interleukin-10 promoter polymorphisms [31]
Surfactant protein A2 gene polymorphisms [32, 33]
Polymorphisms of mannose-binding lectin [33]
Interleukin-15 polymorphisms [34]
Tumour necrosis factor (TNF)-α polymorphisms [34]
Interleukin-4 receptor polymorphisms [35]
Interleukin-13 polymorphisms [36]
CFTR gene mutation [37–39]
Tool-like receptor 9 polymorphisms [40]

Legend: CFTR, cystic fibrosis transmembrane conductance regulator; HLA, human leukocyte antigen.

4 Prevalence of *Aspergillus* Hypersensitivity in Patients with Asthma

Aspergillus hypersensitivity is defined for this chapter as the presence of an immediate-type cutaneous reaction to commercially- or indigenously-prepared extracts of *A. fumigatus* in the laboratory [41]. The exact incidence of *Aspergillus* hypersensitivity is not known because of absence of population-based data for this purpose. Most data is available from specialised clinics where there is likely to be a selection bias. The reported prevalence of *Aspergillus* hypersensitivity in asthma varies from 13 to 45% in different parts of the world, and is summarised in Table 2 [42–50].

The significance of *Aspergillus* hypersensitivity, however, remains unclear. In the recently published European Community Respiratory Health Survey, the frequency of sensitisation to *Alternaria* or *Cladosporium*, or both, was a powerful risk factor for severe asthma in adults [51]. Previous studies have shown that exposure to

Table 2 Prevalence of *Aspergillus* hypersensitivity reported from different countries

Country	Year	Study	Patients with asthma (n)	*Aspergillus* skin test	AH prevalence (%)
England	1964	Campbell et al. [42]	239	SPT	45
	1975	Hendrick et al. [45]	656	SPT	13
United States of America	1973	Hoehne et al. [44]	105	ID	22
	1991	Schwartz et al. [68]	100	ID	38
Australia	1971	Adiseshan et al. [43]	79	ID	33
South Africa	1980	Benatar et al. [48]	500	SPT	22
Saudi Arabia	2001	Al-Mobeireek et al. [49]	53	SPT	23
India	1976	Khan et al. [46]	367	ID	16
	2007	Agarwal et al. [50]	755	ID	39

Legend: CI, confidence intervals; ID, intradermal; SPT, skin prick test; AH, *Aspergillus* hypersensitivity.

environmental fungi increased the risk of death from asthma [52] and also acute attacks of asthma requiring intensive care unit admission [53]. However, none of these studies specifically evaluated *Aspergillus* hypersensitivity with severity of asthma. In a study published in 1978, 28% of the asthmatics from Cleveland (United States of America, USA) and 23% from London (United Kingdom, UK) had immediate skin reactivity to *Aspergillus* [47]. Interestingly, *Aspergillus* skin test reactivity was related to the severity of airway obstruction [47]. In another study published from India, 28.5% of asthmatics subjects had positive skin reactivity to *Aspergillus* antigens [54]. Although the duration of asthma was longer and age of onset of asthma was significantly earlier in patients with *Aspergillus* hypersensitivity, the lung function test did not vary significantly for patients with or without *Aspergillus* hypersensitivity [54]. In a retrospective analysis, treatment with itraconazole of *Aspergillus*-hypersensitive and severe asthmatic patients was associated with a decline in hospital admissions and steroid courses, but not in the total and specific IgE [55]. The importance of antifungal therapy for patients with SAFS is discussed by Drs. Prys-Picard and Niven elsewhere in this book.

5 Prevalence of ABPA in Patients with Asthma

The first case of ABPA was reported in 1952 by Hinson from the UK [21] whereas in the USA it was identified in 1967 [56]. The first case description from Australia came in 1967 [57] and from India in 1971 [58]. There was an initial belief that the disorder was rare in North America [59] but subsequent reports disproved this myth [44, 60]. In fact, one of the three largest series of ABPA is from the USA. The disorder is much more common than previously thought and truly has a global presence. In fact, a high frequency of ABPA has been reported in the last two decades [12, 49, 50, 61, 62]. Many are the reasons for that – primarily it is because of heightened physician awareness and improvements in laboratory support.

The population prevalence of ABPA in patients with asthma is currently not known. Three studies did try to address this issue [63, 64]. In the published study from Ireland (which included a catchment area population of around half a million), 14 patients with allergic bronchopulmonary mycosis were identified from a total of 1,390 new referrals estimating a period prevalence of just >1% [63]. In a questionnaire-based survey carried out in 2.4 million people belonging to Orange County, California (USA) there were 143 cases of ABPA under the care of Pulmonary and Allergy specialists [64]. In 1991, the ABPA committee of the American Academy of Allergy, Asthma and Immunology (USA) conducted another survey amongst the members of academy to find out the number of ABPA cases under their treatment. Of the 33% respondents, it was observed that 703 patients with ABPA were under current care, and other physicians shared 49% of them. If the same case rate is assumed amongst non-respondents, then this figure might increase to 2,000. Both the above surveys carried out in the USA projected the maximum number of ABPA patients in 1991 to be around 11,000 (in a 260 million USA population),

and represented 1% of the estimated 12 million asthma patients in the USA. This is equivalent to one ABPA patient per 24,000 population [64].

All other data on the prevalence of ABPA comes from specialised chest clinics and probably cannot be extrapolated to the general population. In 1959 Pepys et al. demonstrated the clinical and immunologic significance of *A. fumigatus* in the sputum of 2,080 patients with different chest disorders admitted in the ward. Sputum revealed growth of *A. fumigatus* in 147 patients [65]. Of the 147 patients, 59 were asthmatics and 86 belonged to other lung diseases (including bronchiectasis, lung cancer, lung abscess, sarcoidosis, and pneumonia), and 27 patients were finally diagnosed with ABPA. That study showed that the prevalence of *A. fumigatus* in sputum was far higher in asthmatics when compared to other lung diseases [65]. The first large data on the prevalence of ABPA in asthma was described by Campbell et al. in 1964 and this study disclosed a prevalence of ~32% [42]. Until 1969 this disease was considered to be a North American rarity [59, 66], but in 1973 Hoehne et al. described the prevalence of *Aspergillus* sensitivity in 105 adults and 20 children, and gave a clinical description of 14 cases of ABPA [44]. The prevalence of ABPA in asthma in different case series from single centres across the globe ranges from 4.5 to 20% [42, 46, 50, 54, 66–70].

6 Prevalence of ABPA in Cystic Fibrosis

The association of ABPA and cystic fibrosis (CF) was first reported in 1965 by Mearns et al. who reported two cases of ABPA in CF [71]. Subsequently, a high prevalence of precipitating antibodies (31%) and cutaneous hypersensitivity (33%) to *A. fumigatus* was reported in patients with CF [72]. CF is characterised by impaired mucus clearance and airway obstruction. This may favour germination of conidia of *A. fumigatus* and release of antigens with resultant complex immune response by the host. The prevalence of ABPA in CF has been variably reported between 1 and 15% [73], and the disease is known to complicate CF in children below 2 years of age [74].

However, unlike asthma, there are several problems in the diagnosis of ABPA in CF. Presence of wheezing (due to intercurrent infections), transient pulmonary infiltrates, bronchiectasis and mucus plugging are common manifestations of CF-related pulmonary disease, regardless of ABPA. Moreover, the prevalence of *Aspergillus* hypersensitivity in patients with CF is unusually high, varying from 29 to 53% [75–79]. That is defined by immediate cutaneous hypersensitivity to *Aspergillus* antigen or a positive specific IgE in serum against *A. fumigatus* and/or increased specific IgE >9.6 EU/ml in serum against recombinant *Aspergillus* allergen rAsp f 1 (normal values for rAsp f 4 are <8.4 EU/ml and rAsp f 6 <7.2 EU/ml). In addition, a number of patients with CF develop an increase in various immunologic parameters including sensitisation to *A. fumigatus*, in the form of skin test reactions, an increase in total IgE, specific IgE, and IgG to *A. fumigatus*. However, the surprising aspect is that many CF patients without ABPA, over time, demonstrate a spontaneous decrease in several immunologic parameters including IgE level [80]. It has been

suggested that the diagnosis of ABPA in CF should not be based solely on serology and skin test results as patients with CF may demonstrate variable responses to *A. fumigatus* [80].

Since many clinical, radiographic, microbiologic and immunologic features of ABPA often be observed in uncomplicated CF, the exact prevalence of ABPA in CF patients may be quite difficult to ascertain [81–84]. The development of ABPA in CF may be detrimental for patients as it has been shown that deterioration of lung function is most strikingly pronounced in patients with ABPA compared to a control group of CF patients not sensitised to *A. fumigatus* [85, 86], though different observations were made in one study [87]. In patients with ABPA, higher rates of microbial colonisation, pneumothorax, massive haemoptysis and poorer nutritional status were also observed [88]. Three large studies examining the prevalence of ABPA in CF have been published and are summarised in Table 3 [87–89].

In a survey conducted in 58 CF centres in the UK, marked non-conformity in the diagnostic and treatment criteria occurred in 45 centres. Amongst the major criteria that are generally used for diagnosis, *Aspergillus*-specific IgE was used in just 54%, total serum IgE (>1,000 ng/ml) in 45%, *Aspergillus* precipitins in 42% and skin test in 11% of centres [90]. Finally, most centres would treat patients for ABPA even in the absence of clinical deterioration. These findings highlight the wide differences in the reported incidences of ABPA in the literature. To overcome these difficulties, an international consensus conference has laid down recommendations for assistance in diagnosis and management of ABPA in CF [73]. This is discussed in more detail in the chapter by Dr. Shah in this book.

7 Occurrence of ABPA Without Asthma

The most common situation in which ABPA occurs is in the setting of a pre-existing asthma. In fact, in most studies asthma has been considered an essential diagnostic criterion and has an important role in the natural history of the disease. However, there are cases on record in which ABPA has occurred without asthma or CF. A systematic review of the literature revealed 15 articles that have reported this occurrence [9, 91–106]. Glancy et al. first reported this finding in 1981 [92]. Even before that period there are descriptions of the occurrence of ABPA without asthma in at least nine cases [9, 91, 107]. Subsequently there are numerous cases of ABPA which have been described without coexisting asthma [93–106, 108]. In total they include 36 cases reported across the globe; two cases demonstrated bronchodilator reversibility [92] and one showed airway hyperresponsiveness to methacholine challenge [95]. Importantly, asthma diagnosis was not excluded by bronchoprovocation tests in most studies. Thus it is probable that many of these patients might develop asthma later during the course of their lives. All had hypersensitivity to *A. fumigatus* but three cases showed hypersensitivity to *Helminthosporium* [92] and one case each to *A. niger* [98, 102]. Because of absence of asthma these cases are often mistaken initially for other disorders especially bronchogenic carcinoma [93, 102, 103].

Table 3 Prevalence of *Aspergillus* hypersensitivity and allergic bronchopulmonary aspergillosis in patients with cystic fibrosis

Study	ABPA prevalence	Criteria used for diagnosis
Geller et al. [89]	281/14210	Two of three criteria required: (i) immediate skin reactivity to Af antigen; (ii) precipitating antibodies to Af antigen; and (iii) total serum IgE >1,000 IU/ml. At least two of the following were also required: (i) bronchoconstriction; (ii) eosinophilia (>1,000/μl); (iii) history of pulmonary infiltrates; (iv) elevated serum IgE/IgG specific to Af; (v) Af in sputum; and (vi) response to steroids.
Mastella et al. [88]	967/12447	Immediate (type 1) skin test reaction to Af, serum precipitins to Af, total serum IgE level >1,000 U/ml, and physician's suspicion that the patient had ABPA based on one or more of the following: (i) reversible bronchoconstriction or asthma; (ii) pulmonary infiltrates; (iii) elevated serum IgE and/or IgG specific for Af; (iv) peripheral eosinophilia (>1,000/μl); (v) recovery of Af from sputum or hyphae present on smear; and (vi) response to steroids. In the absence of validated diagnostic criteria in CF, the physician's suspicion of ABPA based on multiple clinical, radiological and additional immunological conditions was considered a reasonable compromise to overcome the problem that the usual clinical and radiological criteria of ABPA are not selective in CF.
Taccetti et al. [87]	191/3089	Major criteria: (i) episodic bronchial obstruction; (ii) raised serum IgE (>2 SD for age or >1,000 IU/ml in adults); (iii) specific IgE anti-Af (≥ class 2); (iv) positive immediate skin test reaction to Af; (v) positive serum precipitins to Af; (vi) blood eosinophilia (>500/mm^3); (vii) pulmonary infiltrates; and (viii) central bronchiectasis. Minor criteria: (i) positive culture of sputum for Af; (ii) brown plugs in sputum; and (iii) late skin test reaction. In 10.5% of cases diagnosis was based on <4 criteria whereas in 89.5% it was based ≥4. Total IgE and IgE-Af were elevated in 84.5 and 81.6% respectively. Positive prick test was only seen in 68.3%.

Legend: Af, *Aspergillus fumigatus*; ABPA, allergic bronchopulmonary aspergillosis; CF, cystic fibrosis; IgE, Immunoglobulin E; SD, standard deviations.

8 Occurrence of ABPA in Other Situations

As already stated, ABPA is a disease primarily occurring in patients with asthma or CF. Occasionally, ABPA has been reported to complicate other conditions like bronchiectasis (Table 4) – either idiopathic [109], post-tubercular [110], or secondary to Kartagener's syndrome [111]. It has also been reported in a patient with chronic obstructive pulmonary disease [112] and in patients with chronic granulomatous disease and hyper IgE syndrome [113]. However, these are single patient case reports or small cohort of patients. Larger observations are required to definitely establish an association.

Table 4 Other conditions known to be complicated by allergic bronchopulmonary aspergillosis

Idiopathic bronchiectasis [109]
Bronchiectasis secondary to Kartagener's syndrome [111]
Post-tubercular bronchiectasis [110]
Chronic granulomatous disease [113]
Hyper immunoglobulin E (IgE) syndrome [113]
Chronic obstructive pulmonary disease [112]

9 Coexistence of ABPA and Fungal Balls

An ABPA-like syndrome has also been reported in patients with fungal balls (aspergillomas) [114–124] and chronic necrotising pulmonary aspergillosis [125]. This syndrome can represent true hypersensitivity reaction consequent to the development of fugal balls in long-standing pulmonary cavities due to pulmonary diseases such as tuberculosis or sarcoidosis [114, 115]. It has been postulated that a fungal ball may function as a nidus for antigenic stimulation due to continuous release of *Aspergillus* antigens in a genetically predisposed individual, which may lead to ABPA. An alternative explanation would be saprotrophic *Aspergillus* colonisation with subsequent fungal ball formation in bronchiectatic cavities resulting from ABPA [122, 123]. Whatever the predisposing cause, most patients show brisk response to steroids [114–116, 123, 124].

10 ABPA-Like Syndrome with Other Fungi (Allergic Bronchopulmonary Mycosis)

Allergic bronchopulmonary mycosis is the occurrence of ABPA-like syndrome due to non-*Aspergillus fumigatus* fungal organisms. A variety of fungal agents (Table 5) have been reported to cause this syndrome but the frequency is far less when compared to ABPA [117, 126–140]. This topic is discussed in more detail in the chapter by Dr. Shah, on "How to diagnose ABPA".

Table 5 Fungal agents more commonly implicated in allergic bronchopulmonary mycosis

Aspergillus niger [117]
Aspergillus ochraceus [128]
Aspergillus terreus [130]
Bipolaris spp. [136]
Candida [*Torulopsis*] spp. [132, 134]
Cladosporium spp. [139]
Curvularia spp. [136]
Drechslera spp. [131]
Fusarium spp. [138]
Helminthosporium spp. [126]
Mucor-like spp. [133]
Penicillium spp. [127]
Pseudallescheria spp. [135]
Saccharomyces spp. [140]
Schizophyllum spp. [137]
Stemphylium spp. [129]

11 Conclusions

ABPA is a disorder which has been described from across the globe and is not uncommon in any part of the world. The population prevalence of ABPA in asthma is not clear but specialised chest clinic data estimates the prevalence between 4.5 and 20%. The prevalence of *Aspergillus* hypersensitivity is much higher and varies from 13 to 45%. In CF, the prevalence of ABPA varies from 1 to 15% depending on the criteria used for defining ABPA. A high index of suspicion for ABPA should be maintained in patients with asthma and CF, being aware of its diverse manifestations, especially in patients with CF. Genetic factor plays an important role in the epidemiology of ABPA, and in the next decade the picture is likely to become clearer. Advances in immunological and radiological investigations are likely to facilitate the diagnosis. It is important to make an early diagnosis and initiate therapy to prevent permanent lung damage. Screening patients with asthma for *Aspergillus* sensitivity helps in narrowing the group of asthmatics that are at the highest risk for development of ABPA. Finally ABPA has been shown to complicate diseases other than asthma and CF and ABPA-like syndrome has been described with various other fungi. All these points should be kept in mind while managing patients with this complex disorder.

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ABPA as an Occupational Disease

Kazuo Akiyama

Abstract Allergic bronchopulmonary aspergillosis (ABPA) as an occupational disease has been rarely reported. Seventeen ABPA cases due to *Aspergillus fumigatus* amongst workers in cane sugar mills and six cases due to *Aspergillus oryzae* amongst family members of workers in small factories of soybean products were described. The patients were considered to have been exposed to innumerable spores of the causative fungi scattered in the air around them. Some of the patients showed the causative fungi in cultured sputum, positive immediate and late skin reactions, positive IgE antibodies and precipitating antibodies in their sera. Environmental control to lessen the exposure to the causative fungi is most important to prevent the spread of the disease.

Keywords ABPA · *Aspergillus fumigatus* · *Aspergillus oryzae* · Cane sugar mill · Soybean products

Contents

1 Introduction

There are some reports on occupational allergic diseases caused by *Aspergillus* species. Malt worker's lung is an example of extrinsic allergic alveolitis or hypersensitivity pneumonitis, in which *A. clavatus* growing in mouldy barley on the floor

K. Akiyama (✉)
Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa 228-8522, Japan
e-mail: k-akiyama@sagamihara-hosp.gr.jp

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of breweries has been implicated as the cause of the disease [1–3]. Occupational asthma due to *A. niger* amongst workers of a biotechnology plant in the United Kingdom (UK) was reported [4]. However, there are only a few reports on allergic bronchopulmonary aspergillosis (ABPA) as an occupational disease. Mehta et al. described 17 cases of occupational ABPA due to *A. fumigatus* amongst workers in cane sugar mills [5]. There have been reports of six cases due to *A. oryzae* amongst workers and their family members in small factories that produces soybean products such as soy sauce and soybean paste in Japan [6–11] and one ABPA case due to *A. fumigatus* in a garbage collector from Germany [12].

A. fumigatus is the most important and common species that causes ABPA in conventional settings, but in occupational settings, other *Aspergillus* species such as *A. oryzae* should be considered depending on the characteristics of the occupation.

2 ABPA Related to Cane Sugar Mills

Mehta and Sandhu described 17 ABPA cases amongst workers in cane-sugar mills [5]. An aeromycological study revealed the preponderance of *A. fumigatus* at the bagasse-holding sites in cane-sugar mills located in the Northwest part of the Punjab state of India. *A. fumigatus* represented 44–55% of the total colony counts at the sites. The fungus was frequently isolated from the sputum cultures of the mill workers and a significant number of the workers showed positive reactions when challenged intracutaneously with *A. fumigatus* antigen. Immediate type I and late Arthus-type skin hypersensitivities were observed in 10.6 and 4.4%, respectively, of the 548 workers investigated (Table 1). Skin hypersensitivity was significantly higher amongst the workers who had been employed in the mills for a prolonged period. The incidence of hypersensitivity was particularly high amongst the workers having chronic respiratory problems. During a sample survey of 238 workers having chronic respiratory complaints, the diagnosis of allergic bronchopulmonary aspergillosis based on positive clinical and laboratory findings was made in 17 (7.1%) workers. The precipitating antibodies were detected by both double immunodiffusion and counter-immunoelectrophoresis tests against only *A. fumigatus* in the sera of 17 workers with ABPA. Serologically positive individuals invariably showed dual skin reactions to the intracutaneous test with the *Aspergillus* antigen. ABPA can cause severe discomfort with negative repercussions on the overall efficiency of the workers in sugar mills.

3 ABPA Related to Small Workshops of Soybean Products

The case reports from Japan were on ABPA caused by *A. oryzae,* and all the cases were related to the workers of small factories of soybean products. Soybean products such as soy sauce and soybean paste (*miso*) are two of the most important and popular raw materials in Japanese and Chinese cuisines. *A. oryzae* is a filamentous fungus that ferments soybeans to produce soy sauce and soybean paste (*miso*). The fungus is also used by both cultures to saccharify rice, potatoes and grains for fermentation

Table 1 Summary of laboratory findings and diagnosis of allergic bronchopulmonary mycoses among 548 cane-sugar mill workers (adapted from reference [5])

	Workers with chronic respiratory complaints ($n = 238$)		Apparently health workers ($n = 310$)		Total ($n = 548$)	
Features studied	*n*	%	*n*	%	*n*	%
Skin sensitivity						
A. fumigatus						
Type I	49	20.6	9	2.9	58	10.6
Type III	24	10.1	0	0	24	4.4
C. albicans						
Type I	30	12.6	23	7.4	53	9.7
Type III	7	2.9	0	0	7	1.3
Serum precipitins against						
A. fumigatus	17	7.1	0	0	17	3.1
C. albicans	4	1.7	0	0	4	0.7
Positive sputum culture for						
A. fumigatus	88/136[a]	64.7	14/94[a]	14.9	102/230[a]	44.3
C. albicans	49/136[a]	36.0	24/94[a]	25.5	73/230[a]	31.7
Diagnosis of						
ABPA	17	7.1	0	0	17	3.1
Suspected ABPA	8	3.4	0	0	8	1.5
ABPC	4	1.7	0	0	4	0.7
Suspected ABPC	3	1.3	0	0	3	0.6

[a]Number positive/number investigated.
Legend: ABPA, allergic bronchopulmonary aspergillosis; ABPC, allergic bronchopulmonary candidosis.

in the manufacture of alcoholic beverages such as *sake, awamori* and *shochu* in Japan. The fungus is also used for the production of Japanese rice vinegar. The protease enzymes produced by this species are marketed by a company. The importance of *A. oryzae* has led to its recognition as Japan's national microorganism (*kokkin*), just as the sakura cherry blossom is Japan's national flower. Mycelia of *A. oryzae* grow rapidly on rice and soybean kernels. The fungus secretes amylases (α-amylase and glucoamylase) and carboxypeptidase, which are used for the production of soy sauce, soybean paste and alcoholic beverages [13].

There are more than 2,500 small factories that make soybean paste and more than 10,000 persons are involved in this type of occupation in Japan. Most factories are just next to the home of the workers and their families. Between March and May each year, raw materials are mixed together in a huge barrel to make soybean paste, and then *A. oryzae* is added for fermentation purposes. Innumerable spores of *A. oryzae* are scattered in the air, making the air in the factory and home thick and the

Table 2 Case reports of ABPA caused by *Aspergillus oryzae* in Japan

Author (References)	Patient age/sex	Patient/occupation
Tanizaki [10]	17 years/male	Family member[a]/soy sauce
Kino [7]	16 years/male	Family member/soy sauce, soy bean paste
Kobayashi [8]	25 years/male	Family member/soy bean paste
Akiyama [6]	19 years/female	Family member/soy bean paste
Tochigi [11]	52 years/female	Neighbour/soy sauce, soy bean paste
Kurosawa [9]	15 years/female	Family member/soy sauce, soy bean paste

[a]Most of the affected individuals are family members of workers involved in the soy bean industry. See text for more detail.

surroundings yellow. It is estimated that more than 30,000 persons, including family members, are exposed to this environmental atmosphere during the season.

Most of the patients described were not the workers themselves but their young family members living in the home next to the factory (Table 2). One patient was a neighbour of the factory. The exact role of environmental exposure to *Aspergillus* in the pathogenesis of ABPA is unknown, but exposure to an environment rich in *Aspergillus* spores may play a role in the disease. Although the patients described did not always enter the workplace, it was easy to speculate that they had been exposed to a high concentration of *A. oryzae* spores every spring when the fungus was used for fermentation. By employing *A. oryzae* antigens, a positive skin reaction and precipitating antibodies were shown in all the cases. Positive RAST results were shown in four patients. Furthermore, positive histamine release from the patient's peripheral blood leukocytes caused by *A. oryzae* was shown in some cases (Table 3). *A. oryzae* was cultured in mucoid impaction or sputa of four patients.

Table 3 Results of histamine release from leukocytes of patient and healthy control stimulated with *Aspergillus oryzae* antigens (adapted from reference [6])

		Patient % release	Healthy control % release
Spontaneous release		5.4	5.9
Anti-IgE		51.0	43.0
Medium control	10^{-3}	3.3	7.4
(Czapek)	10^{-4}	2.9	7.7
	10^{-5}	2.8	4.4
	10^{-6}	2.9	7.6
Aspergillus oryzae	10^{-3}	29.0	8.3
(CM)	10^{-4}	3.9	5.9
	10^{-5}	2.8	5.4
	10^{-6}	2.8	5.7
Aspergillus oryzae	10^{-3}	50.0	6.6
(WB)	10^{-4}	5.6	8.8
	10^{-5}	3.9	7.9
	10^{-6}	1.4	9.2

Legend: CM, culture medium; WB, whole body extract.

4 Treatment and Prevention

Oral steroid is the first choice for the treatment of ABPA even as an occupational disease. Some patients were effectively treated by amphotericin B inhalation and bronchial toileting via bronchofiberscope. Environmental control to lessen the exposure to the causative fungi is most important to prevent the spread of the disease.

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Pathogenesis of ABPA

Peter A.B. Wark and Peter Gibson

Abstract *Aspergillus fumigatus* is a ubiquitous environmental fungus, which through its size, reproductive cycle, ability to germinate at body temperature, secretion of proteases and its highly antigenic nature is an important pathogen in pulmonary disease. We will review the pathogenesis of this organism in terms of its ability to cause allergic bronchopulmonary aspergillosis (ABPA). This involves reviewing new data that examines the importance of the innate immune response to infection, especially how this limits airway colonisation and minimises the airway inflammatory response. We will also review the importance of adaptive immune responses to the organism, especially how a T helper-2 lymphocyte response and chemokine signalling predisposes to the development of ABPA and is associated with recurring disease.

Keywords ABPA · Allergic bronchopulmonary aspergillosis · Pathogenesis

Contents

P.A.B. Wark (✉)
Department of Respiratory and Sleep Medicine, Centre for Asthma and Respiratory Disease Hunter Medical Research Institute, John Hunter Hospital, University of Newcastle, Lookout Rd New Lambton, NSW 2305, Australia
e-mail: peter.wark@hnehealth.nsw.gov.au

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1 Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is an uncommon, but serious complication of asthma and somewhat more frequently cystic fibrosis (CF). Its development in both conditions heralds important clinical changes for the worse. When ABPA complicates asthma this leads to a far more destructive process that includes acute eosinophilic pneumonia, mucus impaction of the airways with distal airway collapse, bronchiectasis and eventually severe fibrosis. However ABPA does not occur within the airways of normal subjects despite what is certain to be a similar level of exposure to the fungus, *Aspergillus fumigatus*. These observations provide important to the pathogenesis of this disorder and greater understanding of the mechanisms of airway inflammation in asthma and CF, the biology of the fungus and the immune response to its presence has led to significant advances in the understanding of ABPA that will be reviewed here.

2 *Aspergillus Fumigatus*, Pathogen and Allergen

Aspergillus fumigatus is a saprophytic fungus that is ubiquitous growing within the soil [1]. The fungus grows as branching hyphae, and when mature the organism produces a head full of conidiophores resulting in a high sporulating capacity that can release hundreds of conidia daily [2]. The conidia are 2–3 μm in diameter that allows them to become airborne and respirable, where they are not only inhaled by us all, but are able to reach as far as the alveoli [2]. The fungus thrives at body temperature and if the conidia lodge in a favourable environment they germinate and form mycelium and eventually branching hyphae [1]. Once *A. fumigatus* is in its mycelial form it is also capable of producing a number of proteases that facilitate persistence of the fungus within the airway; these include serine proteases, phosphoplipases, metalloproteases and superoxide dismutases [3]. These proteases are thought to contribute to the following effects; they have the capacity to damage the airway epithelium, along with the lung matrix, they can impair mucociliary clearance, they can impair the phagocytic and killing capacity of phagocytic cells (neutrophils and macrophages) with the net result being persistence of *A. fumigatus* within the airway [3, 4]. These proteases are also in part responsible for eliciting an inflammatory response within the airway, such as the release of interleukin (IL)-8 and IL-6 from airway epithelial cells [4, 5], which contributes to the influx of neutrophils and the activation of both neutrophils and eosinophils [6]. These virulence factors are summarised in Table 1.

Importantly it has also been found that multiple proteins from *A. fumigatus* are also potent allergens, capable of eliciting specific IgE antibodies and possibly skewing the immune response towards a T helper (Th)-2 type lymphocyte response [7, 8].

Table 1 *Aspergillus fumigatus* putative virulence factors (adapted from reference [1])

Class	Molecule	Effect on host
Adhesins	Complement and laminin receptor	Adhesion to host proteins
Cell wall pigments		Inhibits phagocytosis
Toxins	Haemolysin, gliotoxin, RNase	Toxic to epithethelial cells and immune cells
Enzymes	Serine proteases and matrix metalloproteases	*Aspergilus fumigatus* colonisation and lung matrix destruction
	Superoxide dismutase and catalases	Inhibit phagocytosis Damages epithelial cells

The dominant allergen from *A. fumigatus* is the Asp f1 protein but numerous IgE antibodies have been found in individuals sensitised to *A. fumigatus*. In subjects with ABPA, IgE antibodies to Asp f2, Asp f3, Asp f4, Asp f6 and Asp f16 are particularly elevated compared to subjects sensitised to *A. fumigatus* alone and rise with acute flares of ABPA [9]. In subjects predisposed to responding with an allergic or Th-2 immune response, *A. fumigatus* appears to be a highly allergenic stimulus.

3 Clinical and Pathologic Features of ABPA

While exposure to *A. fumigatus* is ubiquitous the development of ABPA occurs in only a few, demonstrating the importance of/ necessity for specific host susceptibility factors for the development of the disease. These may be genetic predisposition and/or the development of impaired airway immune responses.

The diagnosis of ABPA has been standardised and requires the development of a number of immunologic and clinical responses in the host to occur which reflect the development of the disease [10, 11]. The subjects needs to have a pre-existing diagnosis of asthma or CF, they need evidence of an IgE-mediated hypersensitivity response (either from skin prick testing or *A. fumigatus*-specific IgE in serum), a significant elevation of total serum IgE (usually >1,000 IU/ml), evidence of IgG antibodies to *A. fumigatus* and during an acute exacerbation will have a rise in total IgE and peripheral blood eosinophilia. The disease can be associated with serologic evidence of ABPA alone (and is termed ABPA-S), or with the development of central bronchiectasis (ABPA-CB) [11, 12].

Pathologic studies of ABPA-CB describe the presence of dilated bronchiectatic airways filled with inspissated mucus that contains eosinophils, Charcot-Leyden crystals, Curshman spirals and fungal hyphae [13]. In the case of ABPA complicating CF a similar picture is also seen; with bronchocentric infiltration of the airways with lymphocytes and eosinophils, along with evidence of eosinophilic degranulation and IgE antibodies to *A. fumigatus* [14]. A study of five subjects with ABPA using bronchoalveolar lavage (BAL), also demonstrated a mixed inflammatory infiltrate of lymphocytes, eosinophils and neutrophils, along with evidence of elevated Th-2 cytokines (IL-4 and IL-5), but also an increase in Th-1 cytokines (IL-2 and

interferon-γ) [15]. In a larger study of subjects with ABPA using induced sputum we have confirmed that even during clinically stable disease subjects have a intense inflammatory cell infiltrate involving both eosinophils and neutrophils (mixed granulocytic pattern of inflammation), marked elevation in IL-8, and evidence of significant eosinophilic degranulation, with the severity of this inflammation correlating with the degree of radiographic bronchiectasis on high resolution CT of the chest [16]. We have also demonstrated evidence of elevated proteases capable of contributing to airway matrix destruction [17]. These studies demonstrate that ABPA is characterised by an intense bronchocentric inflammatory infiltrate with evidence of both neutrophils and eosinophils, granulocyte activation along with a complicated lymphocytic response with features of both Th-1 and Th-2 activation. Closely associated with this intense and destructive inflammatory process is the presence of *A. fumigatus* within the airways, suggesting that viable fungal persistence is central to driving this inflammatory process which is destructive to the host, but unable to eradicate the organism.

4 Dysfunction of Cystic Fibrosis Transmembrane Regulator, the Airway Epithelium and ABPA

A pre-existing history of asthma or CF is the first requirement for the diagnosis of ABPA. This statement, while somewhat arbitary, does emphasize the clinical observations that the ABPA complex is largely confined to these subjects. Clearly the consequences of theses diseases on the airways and the characteristic(s) of the airway immune response of these subjects to *A. fumigatus* due to either acquired or genetic factors is required for the development of ABPA.

The overrepresentation of ABPA within the CF population raises the possibility that the genetic disorder in CF that leads to dysfunction of the epithelial membrane chloride channel, the cystic fibrosis transmembrane regulator (CFTR) is also involved in the pathogenesis of ABPA, either directly or indirectly. Dysfunction of CFTR leads to dehydration of the airway mucus resulting in impaired mucociliary clearance and predisposes to recurring endobronchial infection [18, 19]. The abnormal mucus and reduced mucociliary clearance that results from dysfunction of CFTR may provide a favourable environment for *A. fumigatus* conidia to adhere, avoid surveillance of the innate immune response and germinate to the mycelial form. In support of this a number of small studies do suggest that subjects with ABPA have a higher carrier rate of CFTR mutations compared to the general population [20–22] and this implicates CFTR dysfunction in predisposing to ABPA. However it is also clear that abnormal CFTR is not a necessary prerequisite for the development of ABPA, since most patients with ABPA do not carry an abnormal CFTR mutation.

The airway epithelium acts not only as a barrier but has come to be recognised as an important contributor to the innate immune response, providing immune surveillance for pathogens and through the release of inflammatory mediators, attracting immune cells. An in vitro cell culture model using A549 cells, demonstrated that *A. fumigatus* bound to epithelial cells and then was internalised, but this lead to at best

only a modest release of IL-6 and IL-8 [23]. This would suggest that the epithelium may not react to *A. fumigatus* as well as it does to other pathogens, though the choice of a cell line such A549 cells with an alveolar cell phenotype may not have been ideal.

5 The Innate Immune Response and the Development of ABPA: The Role of Collectins

The constant exposure of the airways to airborne *A. fumigatus* conidia in healthy individuals fails to lead to either invasive infection or the development of ABPA and perhaps with the exception of extreme exposure no significant inflammatory response. This is a testament to the efficiency of the airway innate immune response that is able to remove *A. fumigatus* or eradicate it from the airways without eliciting a significant inflammatory response.

The airway innate immune response involves a system of a tissues (cells, extracellular matrix) and proteins interacting to recognise and react to foreign pathogens. This system includes the barrier function of airway epithelium, the mucociliary escalator, antimicrobial peptides, dedicated phagocytic cells and finally antigen presenting dendritic cells to elicit a more specific acquired immune response. It is not surprising therefore that dysfunction of the innate immune response in the airways appears to play an important role in the pathogenesis of ABPA.

The collectins are a family of antimicrobial peptides secreted into the airways; these include surfactant protein (SP)-A, SP-D and mannan binding lectin (MBL). They interact with carbohydrate structures on the surface of foreign organisms and facilitate phagocytosis and killing by alveolar macrophages and neutrophils [24]. The role of these collectins in *Aspergillus*-related disease was investigated by Madan et al. [25], who demonstrated that SP-A, SP-D and MBL all bound to *A. fumigatus* conidia and enhanced phagocytosis by neutrophils. They went on to use a mouse model of *A. fumigatus* infection with transgenic knockouts for each of the collectins. SP-A and SP-D knockout mice had increased fungal growth along with lung eosinophilia with elevated Th-2 cytokines (IL-4 and IL-13) and 100% mortality. Replacement of SP-A alone had no effect on mortality but did reduce eosinophilia and IL-4/13 levels along with enhanced levels of the Th-1 cytokines; tumour necrosis factor-alpha (TNF-α) and interferon-γ. Replacement with SP-D had a similar favourable effect on inflammatory response but also demonstrated an improvement in survival to 60%, while replacement of SP-D and MBL improved survival further to 80%. A murine model of ABPA was also used to demonstrate that these collectins down regulated pulmonary eosinophilia and specific IgG and IgE to *A. fumigatus* [25]. These results demonstrate the importance of collectins in defence against *A. fumigatus*, promoting a Th-1 type immune response which is effective in eradicating the organism, while their absence promotes the development of a Th-2, IgE-mediated hypersensitivity response which is clearly ineffective at eradication.

In humans a genetic predisposition to ABPA has been seen in subjects with polymorphisms in the SP-A gene [26]. Subjects with ABPA had an odds ratio of 4.8 for the SP-A2 1660G allele and if a second allele, SP-A2 1649G was present the

odds ratio for ABPA rose to 10.8, they also demonstrated positive correlations with peripheral blood eosinophilia and negative correlations with lung function. This suggests that collectin dysfunction is likely to be an important factor in the pathogenesis of ABPA in humans.

6 The Innate Immune Response and the Development of ABPA: The Role of Phagocytic Cells

Alveolar macrophages are the first cells to encounter *A. fumigatus* conidia and eradicate them by phagocytosis effectively and with minimal inflammatory consequences [27]. Alveolar macrophages detect *A. fumigatus* via Toll like receptor (TLR)-4 which can recognise fungal hyphae and TLR-2 which recognises zymosan and β-glucan on *A. fumigatus* conidia [28]. The phagocytic activity of macrophages is enhanced by the collectins, as well as the inflammatory cytokines TNF-α [29], IL-12 [30] and interferon-γ [31].

If *A. fumigatus* is able to lodge within the airway, germinate and form hyphae then it escapes macrophage surveillance and killing must be performed by neutrophils. However unlike macrophages, the ability of neutrophils to eradicate the fungus is dependent upon the release of proteases and other inflammatory mediators. Neutrophils also use TLR-2/TLR-4 to detect *A. fumigatus.* Signalling via TLR-2 triggers superoxide generation and fungicidal activity [32]. TLR-4 dependent signalling enhances fungicidal activity and also leads to the release of the anti-inflammatory cytokine IL-10 [28]. Neutrophil activity against *A. fumigatus* is enhanced by granulocyte colony stimulating factor [27] as well as TNF-α which increases the oxidative burst as well as degranulation of neutrophils [29].The neutrophil therefore plays a key role in immunity against *A. fumigatu*, and this is particularly emphasised by the fact neutropenic patients are so susceptible to invasive pulmonary aspergillosis. In the case of ABPA neutrophil activation is clearly evident and while this may prevent invasive disease, once *A. fumigatus* is able to persist within the airways and germinate forming hyphae, the inflammatory response required to eradicate it is likely to contribute to the development of airway matrix destruction and bronchiectasis [16].

A recent study showed no difference in the allele frequencies and genotype distribution of the TLR-2 and TLR-4 between ABPA patients and controls [33]. ABPA patients had a significantly higher frequency of allele C for the T-1237C polymorphism in TLR-9 than controls (20.5% vs. 9.4%, respectively; $p = 0.043$). TLR-9 has been associated with anti-allergic activities. Accordingly, polymorphisms in TLR-9 have been associated with an increased risk of asthma and other allergic and chronic inflammatory conditions [34].

Eosinophils are the other major phagocytic cell present in the airways of subjects with ABPA and there is evidence of eosinophilic degranulation [16], though the effect of eosinophils on *A. fumigatus* eradication is not clear. In asthma, eosinophilic airway inflammation plays an important role in pathogenesis. In asthma, IL-10

levels are reduced and this is thought to encourage the development of airway eosinophilia [35]. Using a mouse model of *A. fumigatus* allergic disease, an IL-10 knockout mouse had increased lung eosinophilia, airway hyper responsiveness and mortality, compared to wild type mice [36]. In humans a potential role for IL-10 polymorphisms in the pathogenesis of ABPA was suggested by a study of CF subjects with ABPA which showed a certain allele associated with reduced IL-10 (-1082A) was overrepresented in ABPA [37].

A summary of the role of the innate immune response to *A. fumigatus* and how dysfunction may lead to ABPA is provided in Table 2.

Table 2 Innate immune response to *Aspergilus fumigatus* and its role in allergic bronchopulmonary aspergillosis

Innate immune response	Effect on *Aspergillus fumigatus*	Dysfunction associated with ABPA
Mucocilary clearance	Reduced clearance of *A. fumigatus* conidia and hyphae	Mutations of CFTR*
Collectins, SP-A, SP-D, MBL**	Facilitate phagocytosis and activation of neutrophils and alveolar macrophages	Polymorphisms SP-A2 gene
Neutrophils	Recognised by TLR-2 and TLR-4 Phagocytosis and degranulation of *A. fumigatus* hyphae	Neutropenia and neutrophil dysfunction associated with invasive aspergillosis Role in ABPA?
Alveolar macrophages	Recognised by TLR-2 and TLR-4 Phagocytosis of *A. fumigatus* conidia Recruitment of adaptive immune response	Role in ABPA?

Legend: ABPA, allergic bronchopulmonary aspergillosis;* CFTR, Cystic Fibrosis Transmembrane Regulator;** MBL, mannose binding lectin; SP, surfactant protein; TLR, tool-like receptor.

7 The Adaptive Immune Response and the Development of ABPA

ABPA was thought of as a hypersensitivity response to *A. fumigatus* and early clinical work linked this to a predominant Th-2 immune response [38]. In subjects with ABPA the majority of T cell clones were found to have a Th-2 phenotype (CD4+, IL-4+), the majority of these T cell clones were also found to react to the *A. fumigatus* allergen Asp f1 [39]. Further analysis demonstrated that nearly all of these belonged to the major histocompatibility complex HLA-DR2 or DR5 [39] and this was confirmed in another case-control study of subjects with ABPA,

where they found 16/18 subjects with ABPA had either or both of these alleles [40]. Inspection of the independent genotypes in the DR2/5 groups showed that only the DRB1∗1501 and DRB1∗1503 alleles were associated with ABPA. To determine the importance of these alleles in vivo, the investigators designed transgenic mice with humanised DRB1∗1501 and DRB1∗1503 as well as DRB1∗1502 (known to be associated with reduced allergic inflammation) and then challenged them with *A. fumigatus* [41]. The DRB1∗1501 and DRB1∗1503 were less able to clear *A. fumigatus*, they responded also with the production of more mucus and eosinophilic airway inflammation, while the DRB1∗1502 mice had less intense airway inflammation characterised by an infiltrate of lymphocytes [41]. This now provides strong evidence that the major histocompatibility complex plays a central role in determining the regulation of pulmonary immunity to *A. fumigatus* and these susceptibility genes drive the adaptive response to a Th-2 phenotype. Further demonstrating that a Th-2 response seems to predominate; peripheral blood monocytes from subjects with ABPA show increased sensitivity to IL-4 and a greater proportion of B cells were found to express IgE [42].

Th-2 cells from subjects with ABPA may also be unique and predispose to the disease. When examined the Asp f1 specific T cells from subjects with ABPA, 86% demonstrated vbeta13 gene, compared to T cells from subjects without ABPA who had T cells that expressed vbeta1 [43].

T cells require recruitment to the airways and this is influenced by the chemokines released in response to airway inflammation. Th-2 cells appear to preferentially express the chemokine receptor CCR4 and are attracted by the ligands thymus and activation-regulated chemokine (TARC) and macrophage derived chemokine (MDC) [44]. Using a mouse, sensitised to *A. fumigatus*, exposure lead to pulmonary eosinophilia along with increases in IL-4, IL-5 and IL-13 as well as leading to airway hyper responsiveness [45]. However in transgenic mice without CCR4, these effects were dramatically reduced. Thus far only one case-control study in humans has investigated the role of TARC and MDC in ABPA [46]. The researchers determined that in subjects with ABPA as well as ABPA and CF; serum TARC levels were higher at baseline, correlated with specific IgE antibodies to *A. fumigatus*, rose with acute exacerbations and was a better predictor of the presence of ABPA and exacerbations of ABPA when compared to total serum IgE [46]. These results are important because they suggest that TARC levels may be a valuable marker of disease activity and they also demonstrate that Th-2 immune activation is enhanced in both stable and exacerbating disease. Chemokine receptor expression was compared in a case-control study of subjects with asthma and ABPA though no differences were seen between the two groups in terms of expression of CCR4 at baseline [47]. When CD4 cells from subjects with ABPA were exposed to Asp f antigens they reacted with the release of IL-4, 13 and interferon-γ, but in comparison to subjects with asthma showed downregulation of CCR4 expression. These seemingly contradictory findings certainly point to dysregulation of Th-2 cells and clearly require further investigation to clarify the role of CCR4 and its ligands in the pathogenesis of ABPA. The role of the adaptive immune response in ABPA is summarised in Table 3.

Table 3 Adaptive immune response to *Aspergillus fumigatus* and its role in allergic bronchopulmonary aspergillosis

Th-2 lymphocyte response	Dysfunction associated with ABPA
Th-2 CD4 response release IL-4 and IL-13	Association with asthma and need to demonstrate sensitisation to *A. fumigatus* Increased sensitivity of T cells to IL-4 stimulation CCR4 expression and response to ligands TARC and MDC HLA-DRB 1501 and 1503 Role for reduced IL-10?
IgE antibodies to *Aspergillus fumigatus*	*Aspergillus* allergens Increased sensitivity of T cells to IL-4 stimulation Increased number of B cells express IgE in ABPA
Airway eosinophilia	Association with asthma HLA-DRB 1501 & 1503 Reduced IL-10

Legend: ABPA, allergic bronchopulmonary aspergillosis; HLA, human leukocyte antigen; IgE, immunoglobulin E; IL, interleukin; MDC, macrophage derived chemokine; TARC, thymus and activation-regulated chemokine; Th-2, T helper-2.

8 Conclusions

ABPA is an important complication of both asthma and CF, though not common it seriously impacts on the disease in both conditions leading to irreversible lung disease and a marked increase in treatment to control it. The widespread environmental exposure of the population to *A. fumigatus* and its ability to infect the lung makes it the most important fungal pathogen in respiratory disease to devise better mechanisms of diagnosis and more effective treatment strategies.

Recent advances in the understanding of the innate and adaptive immune response to *A. fumigatus* have led to considerable insight into understanding the pathogenesis of ABPA and the susceptibility of certain individuals to develop it. The innate immune response has now been seen to be vital in early clearance of the organism before it can germinate and colonise, as well as in recruiting immune cells and enhancing their action. The well known association of ABPA with a Th-2 immune response and allergic disease has also been further expanded and specific susceptibility to ABPA seen with HLA DR subtypes.

What is now required is a greater understanding of how defects in the innate immune response and adaptive immune systems interact to predispose to ABPA. It is already evident that this interaction is two-way. This is seen with conditions such as CF, with reduced airway clearance of the organism. While a Th-2 immune response, characterised by release of IL-4/IL-13, will increase *A. fumigatus*-IgE

antibodies to *A. fumigatus* allergens and result in airway eosinophilia and bronchial hyper responsiveness, a response that is inefficient in eliminating airway infection and may predispose to ongoing colonisation and a worsening cycle of airway inflammation and airway destruction. It is possible that other abnormal innate immune responses may also be important, subtle problems with recognition via TLR by alveolar macrophages or inefficient phagocytosis may reduce *A. fumigatus* clearance. These responses may also be impaired in a Th-2 inflammatory airway environment. Similarly high levels of airway colonisation, inefficient phagocytosis or high levels of airway damage through the actions of granulocytes may further influence the adaptive immune response to a Th-2 phenotype. The interaction between innate and adaptive responses in ABPA will require further investigation. In addition the role of Th-17 T cells and regulatory T cells in predisposing to ABPA has so far not received any attention though are both rapidly developing areas of investigation in allergic airways disease and potentially important.

Further investigation of the pathogenesis of ABPA is vital to further understand the disease process in ABPA, improve diagnosis and ultimately lead to novel interventions.

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Clinical Manifestations and Natural History of Allergic Bronchopulmonary Aspergillosis

Ritesh Agarwal and Arunaloke Chakrabarti

Abstract Allergic bronchopulmonary aspergillosis (ABPA) is a pulmonary disorder secondary to a complex hypersensitivity to *Aspergillus* antigens. It is important to recognise this condition because if unrecognised the disease can progress and result in extensive bronchiectasis, pulmonary hypertension and respiratory failure. Recognition of the disease early in its course and appropriate therapy with steroids can prevent the occurrence of central bronchiectasis. In fact, recent data suggests that bronchiectasis is a poor prognostic marker associated with frequent exacerbations. In this chapter we summarise the clinical features and natural history of ABPA. For the purpose of this review we have performed a systematic search of the electronic databases – MEDLINE and EMBASE for relevant studies published from 1987 to 2007 using free text terms: allergic bronchopulmonary aspergillosis. In addition, we reviewed our personal files. A total of 102 articles were reviewed in detail for the purpose of this chapter.

Keywords ABPA · *Aspergillus* · Asthma · IgE · Natural history

Contents

R. Agarwal (✉)
Department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India
e-mail: riteshpgi@gmail.com

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1 Introduction

Aspergillus species are responsible for at least three definite forms of pulmonary hypersensitivity disorders (Table 1) [1]. The first disorder manifests as IgE-dependent asthma and is mediated by mast cell mechanisms, and includes severe asthma with fungal sensitisation (SAFS), which is discussed elsewhere in this book. Hypersensitivity pneumonitis occurs in non-atopic individuals who are heavily exposed to *Aspergillus* or other fungal conidia. The third disorder is allergic bronchopulmonary aspergillosis (ABPA) and generally occurs in patients with asthma. ABPA is a pulmonary disorder caused by a complex hypersensitivity reaction to antigens of *Aspergillus* species, an ubiquitous thermophilic fungi that colonises the bronchial tree. The disorder was first described by Hinson in 1952 from the United Kingdom (UK) [2], and then in 1957 from Australia [3] and in 1958 from the United States of America (USA) [4]. From India, the first case was described in 1971 [5]. In the past two decades there has been an increase in the number of cases possibly due to heightened physician awareness and easy availability of automated serologic assays for the diagnosis of ABPA [6–10]. The disease presents with varied clinical and radiological manifestations ranging from asymptomatic patient with or without pulmonary infiltrates to severe uncontrolled asthma with or without central

Table 1 Main pulmonary disorders associated with *Aspergillus* species[a]

Allergic
IgE-dependent asthma and severe asthma with fungal sensitisation (SAFS)
Hypersensitivity pneumonitis
Allergic bronchopulmonary aspergillosis (ABPA)
Chronic non-invasive
Aspergillus colonisation
Fungal ball (aspergilloma)
Chronic cavitary pulmonary aspergillosis (CCPA)
Invasive
Chronic necrotising pulmonary aspergillosis (CNPA or subacute invasive aspergillosis)
Invasive aspergillosis

[a]Some overlap syndromes may occur (see the chapter by Dr. Soubani in this textbook).

bronchiectasis and pulmonary fibrosis [11–18]. There are reports of mean diagnostic latency between the occurrence of first symptoms and diagnosis of as long as 10 years [19]. However, early diagnosis is not difficult with increased clinical suspicion directed at patients at higher risk for ABPA.

2 Clinical Features of ABPA

There is no gender predilection for the development of ABPA and most patients are diagnosed in the 3rd–4th decades of life. Although the initial descriptions involved adults, an increasing awareness of the disorder by pulmonary and non-pulmonary physicians has led to recognition of the disease in infancy [20], as well as in early childhood [21]. The clinical features of ABPA encountered in four large series published in the last 20 years are summarised in Table 2.

Table 2 Clinical features encountered in four large case series of allergic bronchopulmonary aspergillosis published in the last 20 years

	Behera et al. [6]	Kumar et al. [25]	Chakrabarti et al. [8]	Agarwal et al. [10]
Number of patients	35	32	89	155
Male to female proportion	14:21	14:18	53:35	79:76
Mean age (years)	34.3	34	36.4	33.4
Mean duration of asthma (years)	11.1	12	12.1	8.9
History of asthma	94%	100%	90%	100%
Expectoration of sputum plugs	NA	31%	69%	46.5%
Blood eosinophilia (>500 cells/μl)	43%	100%	100%	76.1%
Fleeting shadows	77%	69%	74%	40%
History of intake of anti-tuberculous drugs	34%	NA	29%	44.5%
Skin test against *Aspergillus*	51%	100%	85%	100%
Type 1	25.7%	100%	16.9%	83.2%
Type 3				
Elevated IgE levels (%)	NA	100%	NA	100%
Aspergillus-specific IgE/IgG	NA	100%	NA	100%
Serum precipitins against *Aspergillus*	77%	70%	71.9%	86.5%
Central bronchiectasis	71%	78%	69%	76.1%

Legend: NA, not available.

3 History

Patients with ABPA can be occasionally completely asymptomatic with the diagnosis being performed on routine screening of asthmatic patients [9, 10, 22]. On the other hand, most patients are symptomatic and the usual clinical clue to the

diagnosis of ABPA is the presence of a poorly-controlled asthma [23, 24]. Clinically symptomatic ABPA may manifest with low grade fever, wheezing, bronchial hyper reactivity, haemoptysis or productive cough. Patients can also present with episodes of systemic illness with fever, weight loss and malaise. Expectoration of brownish black mucus plugs is seen in 31–69% of patients, and is believed to be a characteristic symptom, which can help in the diagnosis [8, 10, 25]. A classic scenario is an asthmatic patient who is poorly controlled with medications despite a good compliance with drugs. In this patient symptoms such as haemoptysis and expectoration of brownish-black mucus plugs, and a history of pulmonary opacities should strongly point towards the diagnosis of ABPA. However, it should be remembered that ABPA can complicate the course of apparently well-controlled asthma and should be suspected in all asthmatics. In a large series of 155 cases of ABPA, 19% of patients with ABPA had well-controlled asthma [10].

4 Physical Examination

There are no characteristic features on physical examination to diagnose ABPA confidently. Physical examination can be normal or reveal findings of asthma with variable degrees of polyphonic wheeze. Clubbing is seen only in 16% of patients, particularly in those with long-standing bronchiectasis. On auscultation, coarse crackles can be heard in 15% of patients [10]. The finding of wheeze on physical examination and pulmonary opacities on chest radiograph may suggest ABPA. Another utility of physical examination is to look for ABPA complications such as pulmonary hypertension (raised jugular venous pressure, left parasternal heave, palpable second heart sound, loud pulmonic component of the second heart sound and pansystolic murmur of tricuspid regurgitation), and/or respiratory failure.

During exacerbations of ABPA localised findings of consolidation (localised crackles, bronchial breath sounds), and atelectasis (diminished breath sounds) can occur. Therefore ABPA should be differentiated from other pulmonary diseases. Clinically it is often difficult to diagnose ABPA based on clinical assessment alone. In these settings, IgE values are of great help in differentiation.

5 Laboratory Findings

5.1 Skin Tests

Clinically once the diagnosis is suspected the first step is to look for *Aspergillus* hypersensitivity which is defined by the presence of immediate cutaneous reactivity to *Aspergillus* antigen as demonstrated by skin tests. This is usually performed by an intradermal injection of *Aspergillus* antigen. The reactions in skin test are classified as type I if a wheal of >3 mm greater than control and erythema develop within 1 min, reaches a maximum after 10–20 min and resolves within 1–2 h. A type III

reaction is read after 6 h and any amount of subcutaneous oedema is considered to be a positive result. The type IV reaction is read after 72 h and an induration of >5 mm is considered positive. The intradermal test is more sensitive than the skin prick test [26]. In many institutes a prick test is first performed and if negative then an intradermal test is done. In a series of 126 patients with ABPA, late cutaneous reactions of the type III and type IV variety was seen in 75 and 8% of patients, respectively. The reader is referred to the chapter by Dr. Shah for more detail on the laboratorial diagnosis of ABPA.

5.2 Immunological Studies

ABPA is primarily a diagnosis which should be suspected in all patients with asthma, and confirmed by immunologic investigations. Hence, immunological tests are the hallmark in the diagnosis of ABPA. Predefined criteria can help in the diagnosis and the Rosenberg-Patterson criteria are the most frequently used for the diagnosis of ABPA [11]. Other chapters in this book review in detail *Aspergillus* allergens and specific IgE testing. However, there is no clear definition of optimal disease-specific cut-off values, and in many instances lack of standardisation of *Aspergillus* antigen or assay method complicates the definitive diagnosis.

Furthermore, the differentiation of patients with ABPA from patients with *Aspergillus* hypersensitivity can be difficult in many instances. The prevalence of *Aspergillus* hypersensitivity (immediate cutaneous hypersensitivity to *Aspergillus* antigens) in patients with asthma ranges from 13 to 45% in different studies [7, 10, 22, 25–40]. Serum precipitins to *A. fumigatus* are present in 69–90% of patients with ABPA [10, 24, 27, 28, 41] and in 9% of patients with asthma [28]. Central bronchiectasis can be seen in patients with asthma without ABPA [42–44]. In many such situations the use of IgE and IgG levels specific to *A. fumigatus* can help in diagnosis as they are significantly raised in patients with ABPA in comparison to asthmatic patients. In fact, values of IgG/IgE more than twice the value of the pooled serum samples from patients with *Aspergillus* hypersensitivity and asthma may greatly help in diagnosis of ABPA [45, 46].

The total IgE levels are elevated in the serum in all patients with ABPA [23]. It is also important to note that 1 kilo unit (KU) per litre is equivalent to 1 international unit (IU) per millilitre and in turn is equal to 2.42 ng/ml of IgE [47, 48]. The total IgE levels reflect disease activity and serial measurements serve as a guide to therapy [49, 50]. However, there is no relation of the severity of immunologic findings with the disease severity [9, 10]. Another problem is the cut-off to be used for classifying the disease, as there is no consensus on this issue. Most studies have used a level equivalent to 1,000 IU/ml in the past [13, 14, 51, 52] and present [9, 10, 53], but many authors use a cut-off value equivalent to 1,000 ng/ml [22, 25, 54, 55]. We believe that a value of 1,000 ng/ml is likely to lead to an over diagnosis of ABPA as it is often encountered in patients with aspergillus hypersensitivity. Hence we currently use a value of 1,000 IU/ml for diagnosis.

While investigating a patient with asthma, we follow an algorithm (Fig. 1). We first perform an *Aspergillus* skin test. Once it is positive then total serum IgE levels

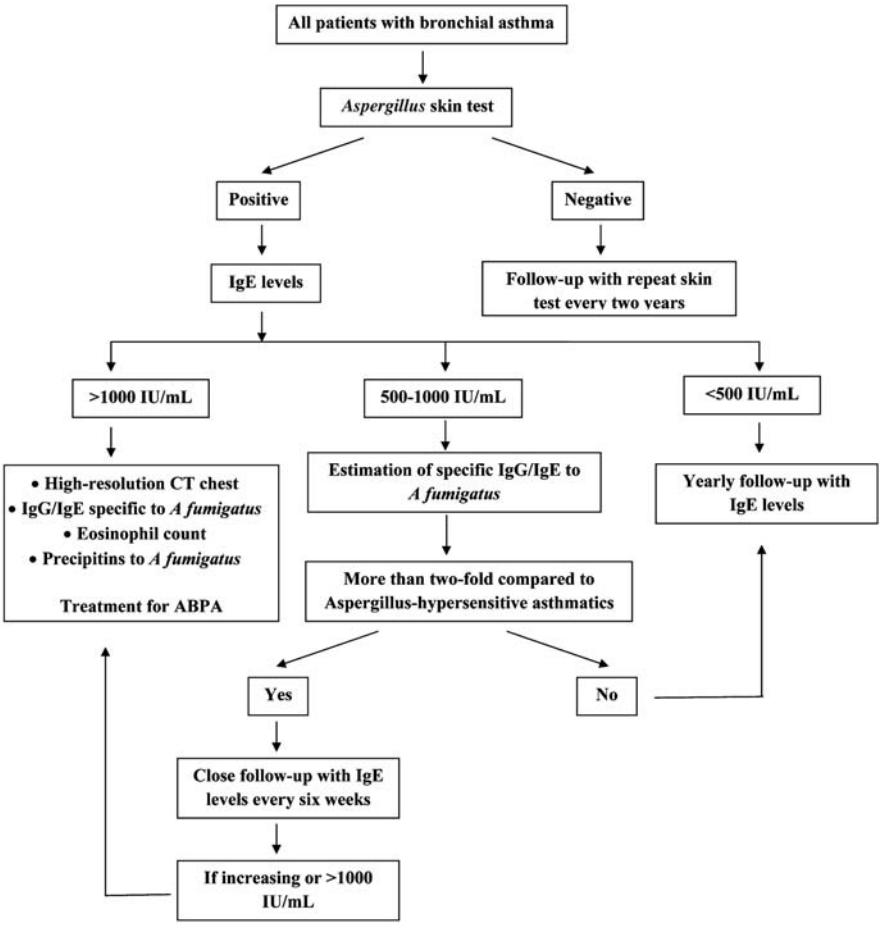

Fig. 1 Proposed algorithm for the diagnosis of allergic bronchopulmonary aspergillosis (ABPA) in patients with asthma. CT, computed tomography

are done. If the value is more than 1,000 IU/ml, then we do the other tests which include a high-resolution computed tomography (HRCT) of the chest, estimation of IgE and IgG specific to *A. fumigatus*, absolute eosinophil count and serum precipitins to *A. fumigatus*. If the value is between 500 and 1,000 IU/ml then the next step is to estimate specific IgE and IgG to *A. fumigatus*. If the levels are raised, then the patients are followed every 6 weeks with total IgE levels. If the absolute value is >1,000 IU/ml or there is a rising trend with clinical/radiological deterioration then antifungal treatment is started. If the value is between 500 and 1,000 IU/ml and specific IgE and IgG to *Aspergillus fumigatus* levels are not raised, then the patient is followed up with a yearly total IgE level.

6 Radiological Investigations

The chest radiographic findings of ABPA include transient or fixed pulmonary opacities (Fig. 2) which are often described as consolidation [56, 57]. Other findings include tramline shadows, finger-in-glove opacities, and tooth paste shadows [57–59]. All these findings are due to mucus impaction of the bronchiectatic cavities [60]. On follow-up of these patients there can be fibrosis and collapse mainly affecting the upper lobes [60]. This may lead to chronic cavitary pulmonary aspergillosis. Although there are no unique roentgenographic findings to define a particular stage [61], central bronchiectasis with peripheral tapering of bronchi highly suggests ABPA [62]. Patients with ABPA who have central bronchiectasis (CB) are classified as "ABPA-CB" and who meet all other criteria but do not have central bronchiectasis are labelled as "serologic ABPA" (ABPA-S). The prevalence of CB in ABPA ranges from 69 to 78% in different studies (Table 2). HRCT (Fig. 3) has replaced bronchography as the imaging modality of choice for the diagnosis of bronchiectasis [63]. Findings on HRCT include central bronchiectasis, mucoid impaction, mosaic attenuation, presence of centrilobular nodules and tree-in-bud opacities [64, 65]. Mucoid impaction in ABPA can be of normal or high density [10]. High attenuation mucus (Fig. 4) is a pathognomonic finding seen in ABPA [10, 66–71]. Although minimal bronchiectasis can be encountered on HRCT in patients with asthma [42, 43], the findings of bronchiectasis affecting three or more lobes, centrilobular nodules, and mucoid impaction on HRCT are highly suggestive of ABPA [44]. The uncommon radiological manifestations include peri-hilar opacities, which simulate hilar lymphadenopathy [21, 57, 72], pleural effusions [73–75] and pulmonary masses [76–81].

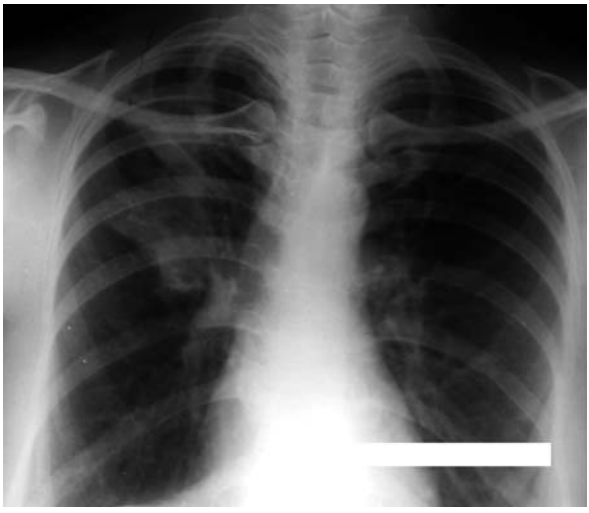

Fig. 2 Chest radiograph showing finger-in-glove opacities in right mid zone

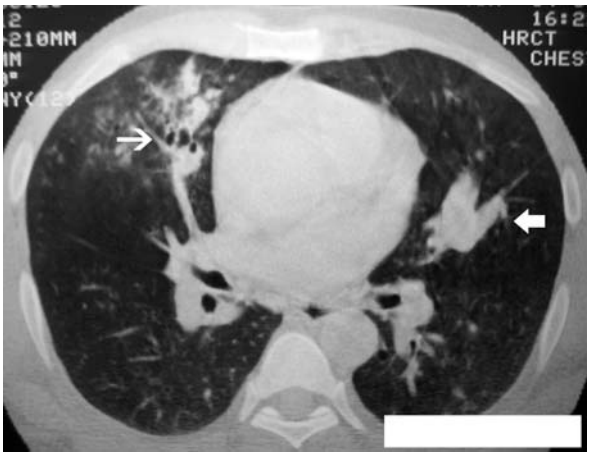

Fig. 3 High-resolution computed tomography scan of the chest showing central bronchiectasis (*thin arrow*) and mucus filled bronchoceles (*thick arrow*)

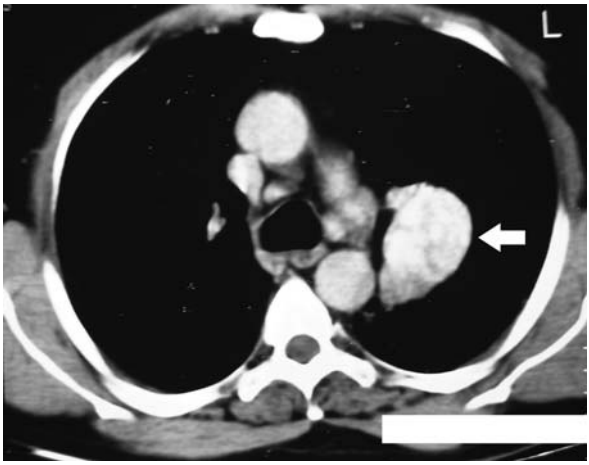

Fig. 4 High-resolution computed tomography scan of the chest showing high-attenuation mucoid impaction in a patient with allergic bronchopulmonary aspergillosis. Note that the density is far higher than the paravertebral skeletal muscles

7 Pulmonary Function Tests

Bronchial challenge with *Aspergillus* antigens have been used in the past, but are not recommended as they can lead to acute bronchospasm. The pulmonary function tests generally show obstructive physiology with varying severity with reduction in diffusion capacity [23, 41, 82]. Since lung function tests may be normal in ABPA, these are not useful tests to screen ABPA [9]. In many centres only patients with demonstrable obstruction on pulmonary function tests or those with poorly controlled asthma are screened for ABPA. It is recommended that all patients with asthma should be screened routinely for ABPA whatever the clinical severity or the lung function in physiological laboratories [16, 18, 45, 54, 55, 83–96].

8 Natural History

The natural history of ABPA is characterised by recurrent episodes of remission and relapse and can be easily understood if two important classification schemes proposed for ABPA is recognized: (i) classification of ABPA into 5 stages as described by Patterson et al. [13]. (Table 3); and, (ii) classification of ABPA into ABPA-S and ABPA-CB as described by Greenberger et al. (Table 4) [17].

Table 3 Stages of allergic bronchopulmonary aspergillosis [1, 93]

Stage	Description	Clinical picture	Radiological findings	Immunologic features
I	Acute phase	Usually symptomatic, fever, weight loss, wheeze	Normal or presence of radiological opacities; often mistaken for pneumonia	IgE >1,000 IU/ml Raised specific IgG/IgE and precipitins to *A. fumigatus*
II	Remission	Asymptomatic	Generally normal or significant resolution of radiological opacities from the acute phase	Usually 30–50% decline in IgE levels by 6–12 weeks. IgE levels do normalise in most patients, even after steroids are stopped
III	Exacerbation	Symptomatic as in acute phase	Transient or fixed pulmonary opacities but in many patients radiology may not be revealing	Doubling of IgE levels from baseline with or without pulmonary opacities
IV	Steroid-dependent ABPA	Symptomatic	Transient or fixed pulmonary opacities	Two groups can be identified: (i) IgE levels do not vary much but require steroid for asthma control (steroid-dependent asthma); (ii) steroids are required to continually suppress high IgE levels (steroid-dependent ABPA)
V	End-stage (fibrotic) ABPA	Symptomatic, findings of fixed airway obstruction, severe pulmonary dysfunction, type II respiratory failure, cor pulmonale, right heart failure	Evidence of bronchiectasis, pulmonary fibrosis, pulmonary hypertension	Serum IgE levels and specific immunoglobulins do not become normal in most patients and even these patients can have frequent exacerbations

Table 4 Radiological classification of allergic bronchopulmonary aspergillosis (ABPA) [17]

Classification	Features
Seropositive ABPA (ABPA-S)	All ABPA features except for central bronchiectasis. Patients with ABPA-S may be classified as Patterson stages I to IV and do not have irreversible fibrosis or end-stage lung disease. These patients may have recurrent pulmonary infiltrates and may also be classified as stage III
ABPA with central bronchiectasis (ABPA-CB)	All the features of ABPA including central bronchiectasis. Patients with ABPA-CB may belong to any of the Patterson stages

ABPA is a low-grade, non-acute respiratory syndrome of varying severity that lingers for years without diagnosis. It is characterised by chronic airway inflammation and airway damage due to persistent colonisation and sensitisation to the fungus *A. fumigatus*. Although inhalation of environmental airborne conidia is the initiating event for most fungal lung diseases, this by itself it is not sufficient to cause lung damage in ABPA. In fact, lung injury in ABPA is mostly due to heightened immune response to *Aspergillus* antigens by the host. In ABPA patients hyphal growth in the mucus of the bronchial tree is followed by release of numerous antigens with resultant stimulation of an immune response predominantly involving Th2 CD4+ T-cells as well as IgE and IgG antibodies that leads to the characteristic pathophysiological changes [97]. The net result is intense airway inflammation leading to tissue damage and remodelling. The course of ABPA is characterised by repeated episodes of remissions and exacerbations. Generally, ABPA is a reversible disease that responds well to systemic steroid therapy. However, if the disease is not recognised, the chronic inflammatory process can progress inexorably and result in irreversible pulmonary dysfunction due to bronchiectasis and/or pulmonary fibrosis, and ultimately respiratory failure and cor pulmonale [17]. Thus, early diagnosis and the initiation of systemic steroids are essential for the treatment of ABPA to prevent pulmonary fibrosis. The natural history of ABPA is currently not well characterised and is often difficult to predict because of the fact that spontaneous improvement of symptoms and pulmonary opacities characterise an important aspect of the disease [14, 52, 97–100]. However, early detection and treatment can minimise the disability from ABPA. In fact, in many centres where most cases of ABPA are diagnosed and initiated on treatment early in the course of the disease, there are reports that progression to fibrotic lung disease has not been seen [101].

ABPA has been classified into five stages [13], however they are not phases of the disease and thus patients do not necessarily progress from one stage to the other in a sequential fashion (Table 3). Patients who present with acute to subacute symptoms are generally in Stage I, and usually present with radiographic infiltrates, elevated total IgE levels (usually in the range of 5,500–7,500 IU/ml) and specific IgG/IgE to *A. fumigatus*. Serum precipitins are also raised in 81–90% of the patients [10]. With

institution of steroids there is clearing of radiographic opacities in majority of the patients with 25–50% decline in IgE levels by 6 weeks. By 6–9 months most patients show ≥50% decline in the total IgE levels. These patients are classified as remission or Stage II. Our personal experience showed that decline in IgE levels is generally less brisk in patients who have IgE levels less than 2,500 IU/ml (unpublished data).

Almost 25–50% of the patients have relapse/exacerbation of the disease (Stage III), which is characterised with doubling of the baseline IgE levels irrespective of the patient's symptoms or the appearance of radiological infiltrates [9, 13, 14]. We have also observed that rising IgE levels with or without clinical symptoms or radiological opacities usually portends an exacerbation. If the patient does not have any additional ABPA exacerbations over the next 3 months after stopping therapy, we give an additional label of "complete remission". Patients who are in complete remission are followed by measuring IgE levels every 6 months for the first year and then generally annually or more frequently depending on the clinical status of the patient. Even in patients who have achieved complete remission the IgE levels are generally raised and in majority of the patients IgE values do not return to normal levels [97]. This is important because the aim of steroid therapy is not achievement of normal IgE levels, but a 30–50% decline by 6–12 weeks [102]. Some patients in stage III may enter into prolonged remission or even a permanent remission. A complete remission, however, does not imply a permanent remission in every case, as exacerbations of the disease has been documented as long as 7 years of remission [99].

Patients in Stage IV (steroid-dependent disease) are of two types. The first group (steroid-dependent asthma) includes patients who require oral steroids for treatment of persistent asthma, but in whom the IgE levels do not vary significantly over time (albeit the IgE levels being generally elevated with values as high as 5,000 IU/ml). In the other group of patients (steroid-dependent ABPA), the IgE levels keep fluctuating once the steroids are tapered and the individual continually requires steroid (10–25 mg of prednisolone daily) to suppress disease activity secondary to ABPA [9, 15]. It is important to remember that only in a minority of patients with stages II and V (<10% of stages II and V) does the IgE level remain <1,000 IU/ml [52, 97].

Patients in Stage V present with widespread bronchiectasis and varying degrees of pulmonary dysfunction on lung function tests, although there are no unequivocal criteria for diagnosis of this stage. Because in our centre a significant number of patients are often referred late [103], and 5–6 lung segments are often involved by bronchiectasis at presentation, we currently define patients in stage V only if they have type II respiratory failure and/or cor pulmonale. Unlike chronic renal failure wherein the primary disease generally burns out at end stage, this is not true of Stage V ABPA, as the disease can be clinically as well as immunologically active [100, 104]. Long-term therapy with steroid may be required for control of asthma and/or disease activity. If symptoms stabilise and the patient's forced expiratory volume in 1 second (FEV1) is >0.8 l then the patient's 5-year survival probability is >80%. We had 8 patients of ABPA with stage V with a follow-up period of 1.5–6.5 years who developed pulmonary hypertension and/or type II respiratory failure [10, 103]. All these patients had severe obstructive pulmonary defect on spirometry (FEV1

values of <40% predicted) without bronchodilator reversibility. One patient recently expired because of end-stage respiratory failure and 7 of these 8 patients are on long-term oxygen therapy and steroids (3 for recurrent exacerbations and 4 for asthma control).

Although progression to stage V disease has never been proven prospectively, there is documentation in literature of a case followed for 7 years in which the authors noted progression through 4 stages: acute, remission, exacerbation, and steroid-dependent asthma [13]. Some minimally symptomatic or totally asymptomatic patients have also been classified in stage V (fibrotic) lung disease [100]. However, it is generally hypothesised (on the logical basis) that earlier the diagnosis is made and treatment started, it is less likely that the patient will develop end-stage fibrotic lung disease. The impact of antifungal therapy on the management of ABPA patients is beyond the scope of this chapter.

ABPA can be further classified as ABPA-S or ABPA-CB depending on the absence or presence of bronchiectasis, respectively (Table 4) [17]. Patients with ABPA-S probably represent the earliest stage of the disorder. It has also been suggested that patients with ABPA-S have a milder clinical course and less severe immunologic findings when compared to ABPA-CB [17, 105, 106]. This observation is supported by three large studies involving ABPA patients that together included 124 patients. However, in the largest of these studies (which included 76 patients and was also the first study suggesting that ABPA-S is less severe immunologically than ABPA-CB), the severity of immunologic findings in ABPA-CB group was restricted only to IgG levels specific to *A. fumigatus*; i.e., only IgG levels were higher in patients with ABPA-CB and all other immunologic parameters were not significantly different between the two groups [17]. Kumar et al. has also classified the disease into three groups: ABPA-S (mild), ABPA-CB (moderate) and ABPA-CB associated with other radiological findings, in a study on 18 patients [106]. In the study published from our centre which included 126 patients of ABPA, we observed that the clinical, spirometric, and immunologic findings were not significantly different regardless of the ABPA classification used (i.e., ABPA-S and ABPA-CB or ABPA-S, ABPA-CB and ABPA-CB associated with other radiological findings) [9]. This conclusion is logical, because in ABPA-S the disease is truly active and the immunologic damage is in progress. Thus it is expected that the immunologic findings in ABPA-S will not be significantly different from those in ABPA-CB and in due course, if untreated, is likely to culminate in formation of bronchiectasis. The current evidence suggests that staging of severity of ABPA based on the presence or absence of CB may not be of any significance. However, it remains prudent to diagnose and treat ABPA early before bronchiectasis (a sign of irreversible lung damage) sets in.

On the other hand, we concur with the suggestion made by Greenberger et al. that the course of patients with ABPA-S is likely to be less severe when compared to those with ABPA-CB. In a multivariate analysis of 155 patients with ABPA, we showed that the severity of bronchiectasis and presence of hyperattenuating mucoid impaction on HRCT predicted ABPA relapses (odds ratio [OR], 1.23; 95% confidence interval [CI], 1.13–1.42; and OR, 3.61; 95% CI, 1.23–10.61, respectively).

However, failure to achieve complete remission was influenced by the severity of bronchiectasis and not by the presence of hyperdense mucus (OR, 1.55; 95% CI, 1.29–1.85; and OR, 3.41; 95% CI, 0.89–13.1, respectively). This is an important observation and suggests that an early diagnosis of ABPA is essential as it prevents permanent lung damage and improves the likelihood of a smoother course of this relapsing-remitting disorder.

9 Conclusions

A high index of suspicion for ABPA should be maintained while managing any patient with asthma whatever the severity or the level of control. The manifestations are dependent upon varying combinations of direct tissue injury by the fungi and endogenous immunologic responses. Host immunologic responses are central to the pathogenesis and are the primary determinants of the clinical, biological, pathological and radiological features of this disorder. ABPA may precede the clinical recognition of the disorder for many years or even decades, and is often misdiagnosed for a variety of pulmonary diseases. In India, it is often mistaken for pulmonary tuberculosis in 29–45% of patients, and patients have even received 3–4 courses of anti-tuberculous drugs before the diagnosis is made. Finally, many patients with ABPA may be minimally symptomatic or asymptomatic. This underscores the need for a better recognition of the disorder and to screen patients with asthma with an Aspergillin skin test. In patients with *Aspergillus* hypersensitivity further immunologic studies are routinely warranted to identify patients in an early stage so as to diagnose ABPA before the development of bronchiectasis, as bronchiectasis is a poor prognostic marker in the natural history of this disease.

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How to Diagnose Allergic Bronchopulmonary Aspergillosis

Ashok Shah

Abstract An immunologically mediated lung disease, allergic bronchopulmonary aspergillosis (ABPA) is predominantly found in patients with asthma and cystic fibrosis (CF). This indolent entity is diagnosed by a set of well established criteria that have unfolded over time. Other than demonstration of central bronchiectasis (CB) with normal tapering bronchi, which is regarded as a key component for the diagnosis of ABPA in patients without CF, we still do not have a single test that could clinch the diagnosis. Of late, ABPA has also been observed in patients without asthma. The Cystic Fibrosis Foundation ABPA Consensus Conference, in 2001, proposed diagnostic as well as screening criteria for ABPA in CF. Radiologic findings have always been crucial to the diagnosis and monitoring of ABPA. Although not pathognomonic, "transient pulmonary infiltrates", also known as "fleeting shadows", are characteristically seen, and often provide the first clue in *Aspergillus* sensitised asthmatics. Identification of CB on computed tomography is recognised as a hallmark and, when present, the patient is labelled as ABPA-CB. Those fulfilling all the serologic diagnostic criteria except presence of CB are categorised as ABPA-S. During the last decade or so, recombinant *Aspergillus fumigatus* allergens have been developed for skin testing and serodiagnosis, especially in those with CF. In essence, this potentially destructive lung disease must be sought for in asthma and CF patients with a positive skin prick test to *Aspergillus* antigens.

Keywords Central bronchiectasis with normal tapering bronchi · Eosinophilia · Precipitating antibodies · Skin allergy test · Total and specific IgE

A. Shah (✉)
Department of Respiratory Medicine,Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110007, India
e-mail: ashokshah99@yahoo.com

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Contents

1 Introduction

The first report on allergic bronchopulmonary aspergillosis (ABPA) was published a little more than a half-a-century ago when Hinson and colleagues [1] documented three cases of "a variety not previously recognised". The clinical course in these patients was characterised by recurrent pyrexial attacks associated with cough and purulent sputum. The acute attacks were characterised by wheezing dyspnoea, chest pain on a few occasions, eosinophilia, pulmonary infiltrates in different areas, and *Aspergillus* mycelia on microscopic examination of the purulent sputum. Saccular bronchiectasis was noted in two cases. The authors argued that these three patients should be classified as a separate entity because of some peculiar features not observed in the general group of patients with pulmonary eosinophilia. These distinguishing features included production of sputum "plugs" and its relation to clearing of radiological infiltrates, a protracted course of illness, bronchoscopic findings of mucosal oedema and bronchial spasm without any obstructing masses. Necropsy findings, in one patient, revealed markedly dilate bronchi that were filled with sticky, tenacious mucus. The histopathologic findings on the biopsied material suggested an allergic response due to *Aspergillus* sensitisation. Moreover, these cases were unlikely to be grouped under "mycetomata" since fungal masses were not observed.

The increasing number of cases that were published from Europe during the 1960s [2, 3] accorded an insight into this immunologically-mediated lung disease. It was not until 1968, that the first case of ABPA in the United States of America (USA) was described [2]. Whilst it was still considered *a North American rarity* at that point in time [3], a large series of 111 patients from the United Kingdom (UK) was described [4]. The authors found ABPA to be the commonest form of asthma and pulmonary eosinophilia. This potentially destructive lung disease is now fairly well-recognised and has been documented from all continents [5].

2 Proposed Diagnostic Criteria

The diagnostic criteria for ABPA have evolved over time. A set of eight major criteria (Table 1), based on a constellation of clinical, radiological and laboratory features, is now well-acknowledged for patients with ABPA [6, 7]. Till date, apart from demonstration of central bronchiectasis (CB) with normal tapering bronchi, there is no single test that establishes the diagnosis. This feature was first described by Scadding [8], and is said to be pathognomonic of ABPA. However, CB may also extend to the periphery in a small number of segments [9]. Three minor criteria (Table 1) have also been described [10, 11]. The lack of a single diagnostic criterion with a standardised test probably reflects the variable prevalence rates of ABPA [6] (the epidemiology of ABPA is presented elsewhere in a distinct chapter by Drs. Agarwal and Chakrabarti).

Amongst the major criteria, all features may not be present in the same patient at all times. Some of them can be identified only during the acute phase (Stage I) or during an exacerbation (Stage III). Furthermore, most of these parameters, barring CB and type 1 hypersensitivity, are affected by therapy with steroids. In light of this, a set of minimum essential criteria has been advocated [7]. This includes: (i) asthma; (ii) immediate cutaneous reactivity to *A. fumigatus*; (iii) total serum IgE >1,000 ng/ml; (iv) elevated specific IgE and IgG to *A. fumigatus*; and (v) CB in

Table 1 Diagnostic criteria for ABPA [8, 9]

Major criteria
(i) Asthma
(ii) Presence of transient pulmonary infiltrates (fleeting shadows)
(iii) Immediate cutaneous reactivity to *A. fumigatus*
(iv) Elevated total serum IgE
(v) Precipitating antibodies against *A. fumigatus*
(vi) Peripheral blood eosinophilia
(vii) Elevated serum IgE and IgG to *A. fumigatus*
(viii) Central/proximal bronchiectasis with normal tapering of distal bronchi
Minor criteria
(i) Expectoration of golden brownish sputum plugs
(ii) Positive sputum culture for *Aspergillus* species
(iii) Late (Arthus-type) skin reactivity to *A. fumigatus*

the absence of distal bronchiectasis. Central/proximal bronchiectasis in the absence of distal bronchiectasis is still considered to be a *sine qua non* for the diagnosis of ABPA [10]. However, it is now recognised that CB may not be present in the early phase of the disease or in a subset of patients with a milder form of the disease. Such serologically positive patients who satisfy the remaining criteria for ABPA are categorised as "ABPA-S" and treatment may be commenced to prevent further lung damage. When CB is present, these patients are categorised as "ABPA-CB" [11].

3 ABPA in Association with Other *Aspergillus*-Related Diseases

The clinical categories of *Aspergillus*-related respiratory disorders usually remain mutually exclusive. In spite of similar immunopathologic responses, it is surprising that concomitant ABPA and allergic *Aspergillus* sinusitis (AAS) occur infrequently [12–14]. However, our review suggested that the occurrence of AAS may not be all that uncommon in patients with ABPA [12]. Coexistent ABPA with fungal balls (aspergillomas), though infrequent, too has been described [13], whilst association of ABPA, AAS and fungal balls in the same patient has been documented only twice so far [14, 15].

3.1 Allergic Aspergillus *sinusitis*

In AAS, mucoid impaction akin to that in ABPA occurs in the paranasal sinuses [16–18]. This is discussed in other chapters by Drs Marple and Ryan. It is likely that, as in ABPA, release of antigenic material from *Aspergillus* species sets into motion a chain of immunologic reactions leading to the development of AAS. To highlight the expression of fungal hypersensitivity in both upper and lower airways, the term "sinobronchial allergic mycosis" (the SAM syndrome) was coined [16]. This description of the co-occurrence of allergic mycosis in the bronchi and in the sinuses was on the basis of three patients, three of whom were earlier reported by us for their unusual presentations [14, 19, 20]. The authors further stated in the addendum that four other cases of concomitant ABPA and AAS reported by us [12] also fulfilled the criteria for SAM syndrome. Since asthma and sinusitis are often seen by two different specialities, the occurrence of AAS in ABPA and of ABPA in AAS may easily be overlooked [12].

3.2 Fungal Balls

Although cavitation is known to occur in ABPA, the coexistence of ABPA with fungal balls is rather uncommon [13, 21]. Even though chronic lung damage appears to provide a favourable milieu for fungal ball formation, aspergillomas are rarely encountered in patients with ABPA. In a background of a cavitary lung disease,

formation of a fungal ball might be accelerated by therapy with steroids [17]. The occurrence of ABPA consequent to fungal ball formation has also been recorded. It has been postulated that a fungal ball may function as a nidus for antigenic stimulation in a genetically predisposed individual, thus leading to ABPA [18]. The reader is referred to a chapter by Dr Soubani for a more detailed discussion on overlap syndromes due to *Aspergillus* species.

4 ABPA and Cystic Fibrosis

In 1965, ABPA was first reported in two children with cystic fibrosis (CF) [19]. This immunologically-mediated lung disease has thence found to complicate up to 15% of patients with CF [21]. Atopy in CF patients is an important risk factor for ABPA [20]. The mucous present within the bronchi provides a favourable environment for the germination of inhaled *Aspergillus* spores.

It is not easy to diagnose ABPA in patients with CF because of the striking similarities between the clinical and radiological features. Patients with CF often develop airway obstruction, pulmonary infiltrates and bronchiectasis. Until a decade ago, uniform diagnostic criteria for ABPA in CF were lacking. In 1999, the criteria adopted by the Epidemiologic Study of Cystic Fibrosis (ESCF) database, conducted in the USA and Canada [22], included the presence of two of the following three: (i) immediate skin reactivity to *A. fumigatus* antigens; (ii) precipitating antibodies to *A. fumigatus* antigens; and (iii) total serum IgE of >1,000 IU/ml. In addition, at least two of the following six features were required: (i) bronchoconstriction; (ii) peripheral blood eosinophilia >1,000/μl; (iii) history of pulmonary infiltrates; (iv) elevated specific IgE/IgG against *A. fumigatus*; (v) *A. fumigatus* in sputum by smear of culture; and (vi) response to steroids. Based on this, the overall reported prevalence of ABPA in CF was found to be 2%. In a subsequent study from Europe [23], based on analysis of the Epidemiologic Registry of Cystic Fibrosis (ERCF) data from nine countries, the overall prevalence of ABPA in CF patients was 7.8%. However, the prevalence was low in children under 6 years of age. Recently, the Cystic Fibrosis Foundation Consensus Conference, held in 2001, proposed diagnostic as well as screening criteria for ABPA in CF (Table 2) [20]. It is recommended that ABPA should be periodically looked for in all patients with CF above 6 years of age [24].

5 Familial Occurrence of ABPA

In spite of familial preponderance of asthma, familial occurrence of ABPA is a rarity. We recently attempted to find the familial occurrence of ABPA and reviewed the literature available on this [25]. Amongst 164 patients with ABPA, diagnosed over a period of 22 years, familial occurrence was detected in four pairs (4.9%), two sets of parent-child and two sets of siblings. Search of the literature revealed only six earlier reports, one of which was published by us [26].

Table 2 Diagnostic criteria for allergic bronchopulmonary aspergillosis (ABPA) in patients with cystic fibrosis (CF) [30]

Classic case
1. Acute or subacute clinical deterioration (cough, wheeze, exercise intolerance, exercise-induced asthma, decline in pulmonary function, increased sputum) not attributable to another aetiology
2. Serum total IgE concentration of >1,000 IU/ml (>2,400 ng/ml), unless patient is receiving systemic steroids (if so, retest when steroid treatment is discontinued)
3. Immediate cutaneous reactivity to *Aspergillus* (prick skin test wheal of >3 mm in diameter with surrounding erythema, whilst the patient is not being treated with systemic anti-histamines) or in vitro presence of serum IgE antibody to *A. fumigatus*
4. Precipitating antibodies to *A. fumigatus* or serum IgG antibody to *A. fumigatus* by an in vitro test
5. New or recent abnormalities on chest radiography (infiltrates or mucus plugging) or chest CT (bronchiectasis) that have not cleared with antibiotics and standard physiotherapy

Minimal diagnostic criteria
1. Acute or subacute clinical deterioration (cough, wheeze, exercise intolerance, exercise-induced asthma, change in pulmonary function, or increased sputum production) not attributable to another aetiology
2. Total serum IgE concentration of >500 IU/ml (>1,200 ng/ml). If ABPA is suspected and the total IgE level is between 200 and 500 IU/ml, repeat testing in 1–3 months is recommended. If patient is taking steroids, repeat when steroid treatment is discontinued
3. Immediate cutaneous reactivity to *Aspergillus* (prick skin test wheal of >3 mm in diameter with surrounding erythema, whilst the patient is not being treated with systemic anti-histamines) or in vitro demonstration of IgE antibody to *A. fumigatus*.
4. One of the following: (a) precipitins to *A. fumigatus* or in vitro demonstration of IgG antibody to *A. fumigatus;* or (b) new or recent abnormalities on chest radiography (infiltrates or mucus plugging) or chest CT (bronchiectasis) that have not cleared with antibiotics and standard physiotherapy

Consensus conference suggestions for screening for ABPA in CF
1. Maintain a high level of suspicion for ABPA in patients >6 years of age.
2. Determine the total serum IgE concentration annually. If the total serum IgE concentration is >500 IU/ml, determine immediate cutaneous reactivity to *A. fumigatus* or use an in vitro test for IgE antibody to *A. fumigatus.* If results are positive, consider diagnosis on the basis of minimal criteria
3. If the total serum IgE concentration is between 200 and 500 IU/ml, repeat the measurement if there is increased suspicion for ABPA, such as by a disease exacerbation, and perform further diagnostic tests (immediate skin test reactivity to *A. fumigatus,* in vitro test for IgE antibody to *A. fumigatus, A. fumigates* precipitins, or serum IgG antibody to *A. fumigatus,* and chest radiography)

Legend: CT, computed tomography.

6 ABPA Without Asthma

This topic is also discussed in the chapter by Drs. Agarwal and Chakrabarti (epidemiology of ABPA). In the absence of CF, ABPA is chiefly a disease of the asthmatics. Nonetheless, it has also been documented in patients without clinical asthma. The first such case was described in 1981 [27], and subsequently approximately a score of patients have been reported [28–32]. A noteworthy aspect of this subset of patients was that more than half were initially worked up for bronchogenic

carcinoma. Furthermore, the remarkable radiological similarity to pulmonary tuberculosis has important clinical implications in high tuberculosis prevalent areas. The patient with ABPA without clinical asthma whom we had reported was referred to us as "multi-drug resistant tuberculosis" for further management. Recently, a patient with ABPA was described from Korea who had associated broncholithiasis but without asthma [33].

7 ABPA and Other Lung Disorders

ABPA has also been reported in patients with chronic obstructive pulmonary disease [34]. The association of ABPA or an overlap condition that resembles ABPA was described in patients in some patients with hyper-IgE syndrome and chronic granulomatous disease [35]. In these congenital immunodeficiency neutrophilic conditions, it is essential to distinguish ABPA from invasive aspergillosis as administration of systemic steroids in such patients may hasten the invasive process thereby resulting in fatality.

8 Allergic Bronchopulmonary Mycosis

Some patients present with bronchial asthma, a history of expectoration of sputum plugs, fleeting shadows on serial chest roentgenograms, peripheral blood eosinophilia and raised total IgE but with negative skin and serologic tests for *Aspergillus*. This syndrome, akin to ABPA, is caused by fungi other than *Aspergillus* and is collectively known as allergic bronchopulmonary mycoses (ABPM). Sputum examination usually provides the first clue to the diagnosis. More than 26 different fungi have been shown to cause ABPM [36] (please check Table 5 in the chapter by Drs. Agarwal and Chakrabarti on "Epidemiology of ABPA"). The common fungi are *Candida albicans, Helminthosporium, Curvularia lunata, Penicillium* spp.*, Dreschlera hawaiiensis, Pseudallescheria boydii, Geotrichum candidum* and *Stemphylium lanuginosum* [37]. It may be difficult, without the facilities of a research laboratory, to prove the fungus responsible for ABPM. When features suggestive of ABPA are present and in particular CB, ABPM should be suspected and such patients initiated on oral steroids to which they respond. This is a prudent approach as ABPM, if untreated, can lead to progressive lung damage [36]. Long-term antifungal therapy might also be considered for these patients.

9 Radiological Findings

Although ABPA is now recognised as a worldwide disease, it is still not diagnosed as frequently and as early as it should be. Since its first description, radiology has been crucial to the diagnosis of ABPA. Not only do imaging techniques help to establish the diagnosis, they also help to monitor the progress of the disease [9, 28].

9.1 Plain Chest Roentgenography

This "picturesque" disease causes a wide spectrum of plain chest radiographic appearances [28–31]. The changes can be either transient (temporary) or permanent (fixed) (Table 3).

9.1.1 Transient Changes

Fleeting Shadows

In the seminal description of ABPA [1], the authors stated that ". . .serial radiographs are essential to show the sequence of incidents of lobar or segmental collapse and consolidation, first in one part, then in another and in either lung". This is a classical description of "fleeting shadows", which Hinson and colleagues [1] recognised as a characteristic feature of ABPA. These opacities, also known as "transient pulmonary infiltrates", usually appear and disappear in different, and sometimes the same sites in the lung over a period of time, and affect a segment, a lobe or the whole lung. These changes reflect disease activity, and are usually seen in either the acute or the exacerbation stage of the disease (Figs. 1, 2 and 3). This is due to mucoid impaction caused by secretions in the damaged bronchi, and may clear with or without therapy. We reviewed 1,340 chest roentgenograms in 113 patients with ABPA and observed fleeting shadows in 89% [32]. Although no area of the lung remains unaffected, the upper lobes are predominantly involved [9]. The radiological appearances can closely resemble those seen in tuberculosis, but serial roentgenograms in ABPA may reveal the transient nature of these migratory infiltrates [38]. Though characteristic, "fleeting shadows" are not pathognomonic of ABPA. When these opacities keep recurring at the same sites, they are described as "recurrent fixed shadows" [38].

Table 3 Radiological changes in allergic bronchopulmonary aspergillosis (ABPA) [50]

Transient changes
Perihilar infiltrates simulating adenopathy
Air-fluid levels from dilated central bronchi filled with fluid and debris
Massive consolidation – unilateral or bilateral (see Figs. 1, 2 and 3)
Radiological infiltrates
"Toothpaste" shadows due to mucoid impaction in damaged bronchi
"Gloved finger" shadows from distally occluded bronchi filled with secretions
"Tramline" shadows representing oedema of the bronchial walls
Collapses – lobar or segmental
Permanent changes
Central bronchiectasis with normal peripheral bronchi
Parallel-line shadows representing bronchial widening
Ring-shadows 1–2 cm in diameter representing dilated bronchi *en face*
Pulmonary fibrosis
Late changes – cavitation, contracted upper lobes and localised emphysema

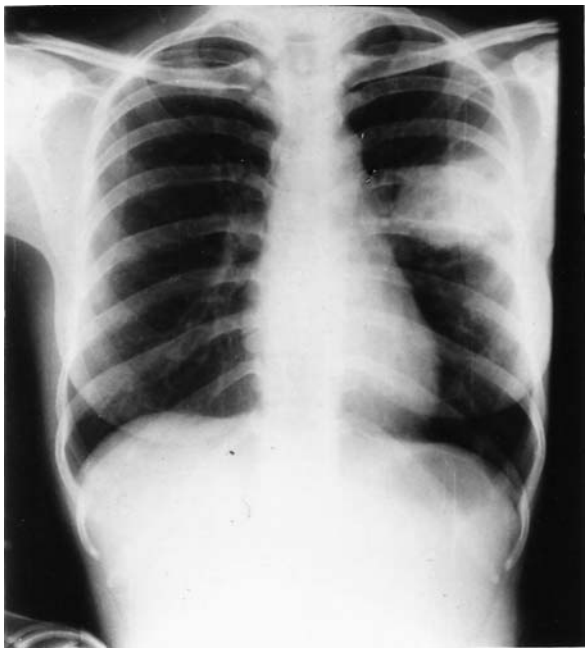

Fig. 1 Plain chest roentgenogram showing consolidation predominantly in the left middle zone

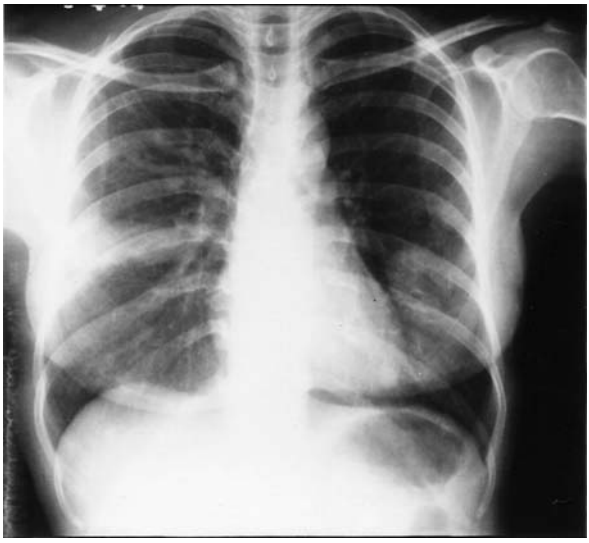

Fig. 2 Plain chest roentgenogram of the same patient from Fig. 1 taken 5 months later, showing a non-homogeneous consolidation in the left lower zone. Another area of right middle and lower zone consolidation is seen. The left-sided consolidation, seen in Fig. 1, has disappeared

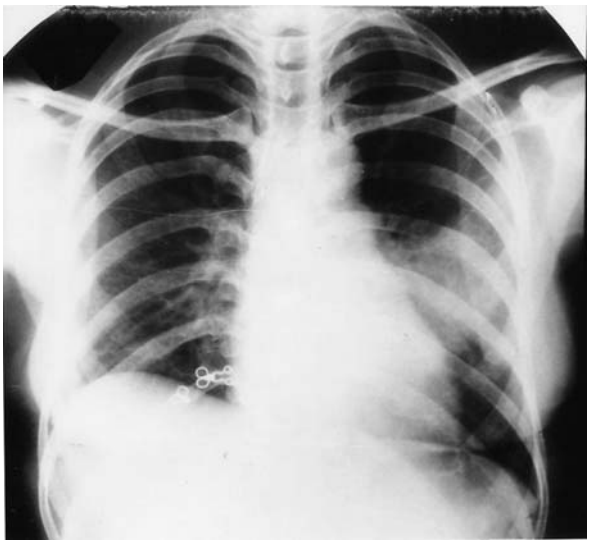

Fig. 3 Plain chest roentgenogram of the same patient from Figs. 1 and 2, taken 2 years after the second roentgenogram (Fig. 2). It shows re-emergence of a large consolidation in the left middle and lower zones. The right middle and lower zone consolidation seen previously in Fig. 2 has disappeared. A right-sided parahilar opacity is also seen. Figures 1, 2 and 3 reveal "transient pulmonary infiltrates" or "fleeting shadows", which are characteristic of allergic bronchopulmonary aspergillosis

Parenchymal Opacities

The most commonly observed pattern is consolidation or non-homogeneous infiltration (Figs. 2 and 3), described in up to 91% of patients with ABPA [30–33]. As described above, these infiltrates may typically migrate from one area to another. These opacities often clear with therapy, but may disappear spontaneously.

Perihilar or "pseudohilar" opacities may simulate hilar adenopathy in ABPA. These shadows were initially described by Mintzer and colleagues [31] in 40% of their patients. In our series, "pseudohilar" opacities were observed in 77% [32]. These are seen surrounding the dilated, central bronchi, which are filled with secretions. True hilar adenopathy, which resolved on therapy, was also reported in ABPA [28, 39]. Recently, we reported the first paediatric case of ABPA with true hilar adenopathy in a 42-month-old boy [40].

Bronchial Abnormalities

The "tramline" sign [10], which represents bronchial wall oedema, is seen when there is thickening of the wall of a bronchus whose diameter is normal. Tramline shadows were initially described as "two parallel hair-line shadows extending out from the hilum in the direction of the bronchi, the width of the transradiant zone between the lines being that of a normal bronchus at this level [30]", and are observed in 45–92% of patients [29–31]. This transient change is not specific for ABPA as it can also be seen in patients with asthma, CF and acute left heart failure.

Mucoid impaction and retention of respiratory secretions in the distorted bronchi give rise to band-like shadows, also known as "toothpaste" shadows [30]. These

are seen in 24–65% of patients [30, 31, 38, 40]. The ends of the distal bronchi, when occluded, become expanded and rounded. These cast shadows that resemble "gloved fingers" [30], and may disappear after coughing or with treatment. "Gloved fingers" shadows are seen for 11–23% of patients [30–33], and when present, should raise the possibility of ABPA. In the upper lobes bronchi, mucoid impaction may lead to "V-shaped"/"wine glass" shadows [38]. These shadows, highly suggestive of ABPA, are seen in 27% of patients [32].

Air-fluid levels in the central bronchi occur when the dilated central bronchi are filled with fluid secretions and debris. These have been described in up to 20% of patients [31, 38]. Lobar or segmental collapse is not uncommon in these patients. Lobar collapse is more frequently seen because of proximal occlusion of the bronchi. Atelectasis are observed in 14–39% of patients [29, 31–33]. We have earlier described a patient with concomitant ABPA and AAS presenting as a middle lobe syndrome [41].

Pleural Involvement

Ipsilateral pleural effusion due to the mechanical effect of lung collapse was also reported by us in a patient with concomitant ABPA, AAS and an operated fungal ball [15]. Such an association of three different clinical categories of *Aspergillus*-related respiratory disorders has been documented only twice so far [18, 19]. The effusion cleared with re-expansion of the lobe after therapy with steroids. Pleural effusions previously reported in two patients with ABPA could also have been attributed to the mechanical effect of lung collapse [42]. A bilateral pleural effusion, larger on the right, was reported in a young adult male [43]. The authors postulated that the pleural effusions resulted ". . . from an inflammatory pleural reaction adjacent to inflamed lung tissue . . .". Spontaneous pneumothorax was reported in a patient with ABPA with chronic fibrotic lung disease [44], whilst development of bronchopleural fistula along with spontaneous pneumothorax was also described in another patient [45].

9.1.2 Permanent Changes

Permanent opacities reflect the irreversible, fibrotic changes in the bronchial walls and parenchyma. Unlike transient changes, they tend to persist throughout life even when the patient is in remission. Amongst the permanent changes, the most characteristic is the occurrence of CB with normal peripheral bronchi. This is recognised as a hallmark of the disease [8].

Other permanent changes include parenchymal fibrosis, cavitation, contracted upper lobes, and localised emphysema [28]. Parenchymal fibrosis is seen in the form of linear scars, reticulonodular markings or honeycombing. McCarthy and colleagues [30] described honeycomb appearances in 27 (24%) of their 111 patients with ABPA. Cavitation, though not a common feature of ABPA, can occur along with the fibrotic stage wherein it may be difficult to distinguish the disease from fibrocavitary pulmonary tuberculosis [13]. Most patients however suffering from

cavitary pulmonary tuberculosis will have positive sputum smears for acid-fast bacilli – in addition, endobrochial dissemiantion is common in patients with tuberculosis. Cavities are reported in 3–14% of ABPA patients [32, 38], and localised emphysema in up to 27% [32, 38]. "Local emphysema" as evidenced by "an area of increased transradiancy with either no vessels or narrow vessels", is noted in the initial roentgenograms (on presentation) in 10% of patients [30]. This figure increased to 25% on reviewing the current or "final" radiographs. Parallel lines or ring opacities were visualised in the same area in all the cases, thereby suggesting that the emphysema was secondary to bronchial damage. None of these patients had widespread emphysema.

9.2 Demonstration of Central Bronchiectasis

It is believed that bronchiectasis occurs in areas with previous radiological lesions and may result in fibrosis [46]. On plain chest roentgenograms, CB is seen either as parallel-line opacities representing widening of the bronchi, or as ring opacities, 1–2 cm in diameter, representing dilated bronchi *en face*. Parallel line shadows are observed in 65–70% of patients with ABPA [41, 43] and ring shadows in 45–68% [42–44]. Central bronchiectasis can be demonstrated by bronchography, linear tomography or computed tomography (CT).

Bronchography, once regarded as the reference standard for the demonstration of CB, gave a one-time complete picture of the entire tracheobronchial tree [47]. In ABPA, the classical lesion is a localised area of varicose bronchiectasis affecting the medium-sized bronchi proximally with normal tapering bronchi distally. However, CB may extend to the periphery as well in a few segments [9]. Even in the same patient, there may be marked variations in the extent, type and site of bronchiectasis [48]. Earlier, linear tomography was employed to document CB in view of the fact that it was non-invasive in nature [49]. Currently, CT of the thorax has emerged as the investigation of choice for the demonstration of bronchiectasis [9, 44]. We have already shown that CT, in comparison to bronchography, a procedure thought to be unsafe in asthma, has a sensitivity of 83% and a specificity of 92% in detecting CB in patients with ABPA [47]. CT scans also enabled us to rapidly and safely establish the diagnosis in children with ABPA who presented with acute severe asthma [50].

On CT bronchiectasis is characterised by the "string of pearls" and the "signet ring" (Fig. 4) appearances [51]. In ABPA, the central bronchiectasis tends to be more common in the upper lobes [9, 49]. This is in contrast to the "usual" bronchiectasis that is predominantly seen in the lower lobes. In our study [9], CB was identified in all patients, affecting 114 (85%) of the 134 lobes and 210 (52%) of the 406 segments studied. Extension of CB to the periphery was noted in 30% of the lobes and 21% of the segments. However, demonstration of CB with normal peripheral bronchi, which occurred in the majority of segments, should continue to be regarded a highly suggestive finding for the diagnosis of ABPA in patients without cystic fibrosis [9]. Studies have also shown that mild CB may be seen in asthma and this does not necessarily indicate the presence of ABPA [52–54].

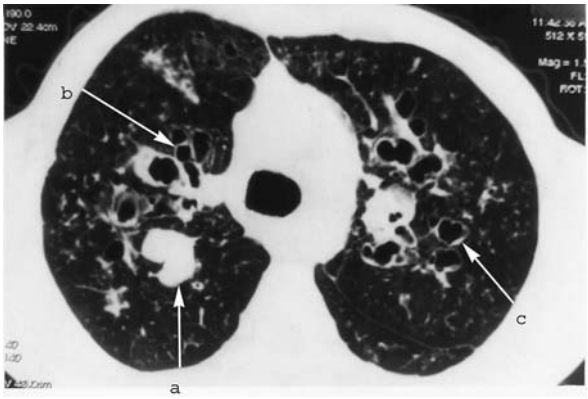

Fig. 4 High resolution chest computed tomography scan (lung window) showing (**a**) mucus-filled dilated bronchi (*arrow*) and extensive bilateral central bronchiectasis characterised by (**b**) "string of pearls" and (**c**) "signet ring" appearances

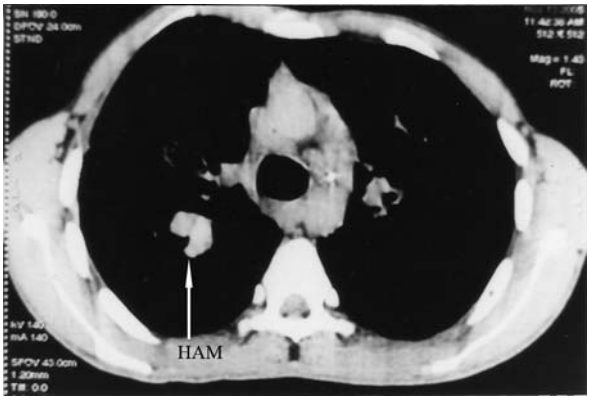

Fig. 5 The corresponding section on the mediastinal window shows high attenuation mucous (HAM; *arrow*) impaction in a patient with allergic bronchopulmonary aspergillosis

9.3 Other Computed Tomography Appearances

Apart from bronchiectasis, the other bronchial abnormalities observed on CT in our experience [9] includes dilated and totally occluded bronchi (48%) as evidenced by beaded, tubular opacities and dense, circular opacities, air-fluid levels within dilated bronchi (22%), bronchial wall thickening (43%) and parallel-line opacities extending to the periphery (30%). On high resolution CT, high-attenuation mucous plugs (Figs. 4 and 5) were also reported in 28% of patients with ABPA [55]. Atelectasis, due to proximal mucoid impaction, can sometimes be a presenting feature. The atelectasis may be segmental, lobar or may involve the entire lung [56].

Common parenchymal abnormalities seen in our study [9] included consolidation (43%), non-homogeneous patchy consolidation (67%) and parenchymal scarring of varying extent (83%). Segmental or lobar collapse (17%), cavities (13%) and emphysematous bullae (4%) were also observed. Cavitation in the fibrotic stage of ABPA may be difficult to distinguish from fibrocavitary pulmonary tuberculosis [15, 23]. Parenchymal lesions extending up to the pleura were seen in 43% of our

patients [9]. In another study, pleural thickening was also observed in 82% of the patients with ABPA [53]. Pleural involvement in ABPA is yet to receive recognition, but this may not be of major clinical significance. Our study [9] showed that CT of the thorax can provide a sensitive method for the assessment of bronchial, parenchymal and pleural abnormalities in patients with ABPA, and should constitute a part of the diagnostic work-up of the disease along with plain chest roentgenograms.

10 Sputum Examination

Aspergillus species are ubiquitous organisms, which spores are easily dispersed by wind in the atmosphere. Inhalation is the primary route of exposure in the vast majority of aspergillosis forms including ABPA. *A. fumigatus* was cultured in the sputa of all the three patients in the first reported cases of ABPA [1]. Moreover, two of the three patients reported "plugs" in the sputum that coincided with febrile episodes. Thereafter, expectoration of golden-brownish sputum plugs has been described in 54% of patients with ABPA [4].

The amount of sputum expectorated by patients with ABPA varies widely from minimal to a half cupful per day. More often than not, sputum eosinophilia is noted. Patients with ABPA-S – who do not have bronchiectasis – may not produce any sputum at all. During the acute (Stage I) or an exacerbation (Stage III), the volume and purulence of sputum may be increased. In our analysis of 113 patients with ABPA, sputum production was recorded in 98% and sputum plugs were expectorated by 37% [32].

Since the presence of *Aspergillus* species in the sputum can either be colonisation or contamination, presence of golden-brownish sputum plugs and positive sputum culture for *Aspergillus* spp. are enlisted as minor criteria for the diagnosis of ABPA. Repeated positive cultures of *Aspergillus* should be considered significant.

Sputum analysis may be useful in monitoring the disease severity and the course of the disease. Eosinophils and neutrophils in induced sputum were found to be higher in patients with ABPA-CB than in those with ABPA-S [57]. Sputum examination is also helpful in assessing cytokines that mediate immune responses in ABPA. In a study comprising 29 patients with ABPA, 9 asthmatics without ABPA and 21 healthy controls, induced sputum interleukin (IL)-8 protein was significantly higher in ABPA [58]. The authors further found sputum IL-8 gene expression and protein release to correlate well with sputum neutrophilia and the degree of airway obstruction.

11 Blood Tests

Serological tests are integral to the diagnosis of ABPA. The blood tests incorporated in the diagnostic criteria include eosinophilia, total IgE, IgE- and IgG-specific to *A. fumigatus*, and precipitating antibodies against *A. fumigatus*. Elevated total serum

IgE and elevated specific IgE/IgG to *Aspergillus* are two of the five minimal essential criteria required for the diagnosis of ABPA [7].

11.1 Eosinophilia

Peripheral blood eosinophilia can almost always be demonstrated, although this finding is often absent in patients on steroids. During an exacerbation, this can range between 1,000 and 3,000 cells/μl. Eosinophilia was present in 83% of our 113 patients with ABPA [32].

11.2 Precipitating Antibodies Against A. Fumigatus

The utility of this test is discussed in the chapter by Dr. Barton along this book. Using the double gel immunodiffusion technique [59], precipitating antibodies against *A. fumigatus* can be detected in the unconcentrated sera of 70% of ABPA patients [60]. When serum is concentrated, these antibodies can be detected in 92% of patients with a radiological infiltrate. We detected precipitating antibodies to *A. fumigatus* in 86% of our patients [32]. However, these precipitating antibodies are not highly specific for ABPA as they may also be present in 10% of asthmatics who do not have ABPA [58]. Importantly, some patients with long-standing ABPA do not maintain detectable precipitins in serum (Drs. Pasqualotto and Denning, unpublished data).

11.3 Serum IgE

The association between elevated total serum IgE and ABPA was first recognised in 1972 [16]. The total serum IgE levels are generally >417 kU/l (>1,000 ng/ml) in patients with ABPA. In acute cases, who have not previously received systemic steroids, the levels may be as high as 20,000 kU/l, or even more. This test is a useful monitor of therapy as a fall of 35% in 8 weeks is suggestive of effective treatment [61]. On the other hand, doubling of remission values indicates an exacerbation in an asymptomatic patient [7].

11.3.1 Specific IgE/IgG to *A. Fumigatus*

Specific IgE and IgG to *A. fumigatus* were initially measured by enzyme-linked immunosorbent assay (ELISA). Since the ELISA technique was not sufficiently sensitive to detect low levels of specific IgE against *A. fumigatus*, radioimmunoassay (RIA) was used [62]. However, the drawbacks of the RIA method were the short shelf life of the radioisotope, unnecessary exposure to radioactivity and the long time period needed for the testing. Subsequently, the biotin-avidin-linked

immunosorbent assay (BALISA) was employed [63]. Using this method in 13 patients with ABPA, 12 with fungal balls, 9 with *Aspergillus*-sensitised asthma, and 9 control subjects without asthma, significantly higher levels of specific IgE against *A. fumigatus* levels were present, even with 1:1,000 dilutions, in patients with ABPA as compared to the other three groups. The authors postulated that this study provided evidence for a polyclonal antibody response to *Aspergillus* antigens in patients with ABPA and not in those with *Aspergillus* sensitised asthma. Specific IgE testing (RAST) against *Aspergillus* species are presented elsewhere in this book.

11.4 Recombinant Aspergillus fumigatus *Allergens*

During the last 10–15 years, recombinant *A. fumigatus* allergens, having similar functional characteristics to commercial fungal extracts, have been cloned, purified, and standardised for serologic testing [64, 65]. Since specific IgE and/or IgG against *A. fumigatus* can be detected in the sera of some patients with *Aspergillus*-sensitive asthma [66], it was thought that these two clinical entities could possibly be distinguished by evaluating responses to the different recombinant allergens of *A. fumigatus*. Unsurprisingly, initial studies with recombinant *A. fumigatus* allergen I/a were unable to differentiate between *Aspergillus*-sensitised asthma and ABPA [64, 65]. Subsequent assessment of immunologic responses to four recombinant allergens viz. rAsp f 1, rAsp f 3, rAsp f 4, and rAsp f 6 in 60 patients with ABPA, 40 patients with *Aspergillus* sensitised asthma and 20 healthy controls revealed that, whilst rAsp f 1 and rAsp f 3 were recognised by serum IgE of all the 100 test subjects, rAsp f 4 and rAsp f 6 specifically bound to IgE in the sera of patients with ABPA only [65]. The investigators found that diagnosing ABPA serologically with rAsp f 4 and rAsp f 6 had a sensitivity of 90% and specificity of 100%. Studies have also demonstrated exclusivity of rAsp f 2 for the serodiagnosis of ABPA [63, 66]. An overview of *Aspergillus* allergens is provided elsewhere in this book by Dr Crameri and colleagues.

11.4.1 Diagnosing ABPA in CF Using Recombinant Allergens

Patients with CF develop an immune response to *Aspergillus* at an early stage. As mentioned earlier, they are at a higher risk of developing ABPA. Serological studies with rAsp f allergens have shown to differentiate/distinguish ABPA from *Aspergillus* sensitisation in CF. One of the initial studies using rAsp f I/a showed a 10-fold increase in rAsp f I/a-specific IgE, a 5-fold increase in rAsp f I/a-specific IgG1, and a 4-fold increase in rAsp f I/a-specific IgG4 antibodies in patients with ABPA when compared to those with sensitisation to *Aspergillus* but without ABPA, as well as CF controls [67]. Serologic studies with the different rAsp f allergens confirmed that rAsp f 4 and rAsp f 6 were singularly detected by IgE from sera of CF patients with ABPA only and not of those without ABPA [68]. A study using seven recombinant purified allergens (rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, rAsp f 6, rAsp f 12, rAsp f 16) in patients with CF demonstrated/detected heightened IgE

reactivity to rAsp f 2, rAsp f 3, rAsp f 4, rAsp f 6 and rAsp f 16 in those having coexisting ABPA when compared with the non-ABPA CF patients [69]. Best sensitivity and specificity were achieved with IgE reactivity to rAsp f 3 and rAsp f 4. In addition, significant positive IgE responses to rAsp f 1, rAsp f 2, rAsp f 3, and rAsp f 6 were noted during exacerbations of ABPA.

Since recombinant allergens are in a purified form, and thereby more consistent than uncharacterised fungal extracts, and also given the fact that they can be produced in large quantities, these cloned allergens could be developed as "standards" for the diagnosis of ABPA. Moreover, they could possibly be used for serial evaluation of IgE reactivity in order to assess disease severity [69]. Further studies are warranted on this score and also for investigating the status of IgE reactivity to various recombinant allergens in the different stages of ABPA.

12 Skin Testing

Although Hinson and colleagues [1] pointed out that ABPA occurs due to an allergic response to *Aspergillus* antigens, skin tests with fungal extracts were "inconstant and unreliable" in their three patients. Subsequently, different workers were able to demonstrate *Aspergillus* sensitisation in almost all patients with ABPA. It is believed that repeated inhalation of *Aspergillus* spores leads to airway colonisation in susceptible hosts, which evokes an immune response. Amongst the more than 150 species of the ubiquitous spore-forming *Aspergilli*, *A. fumigatus* accounts for a majority of *Aspergillus*-related human illness. The other pathogenic species include *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*.

Both type I (immediate) and type III (delayed) skin sensitivity can be elicited by either prick or intradermal testing with different *Aspergillus* antigens. In the series of 111 patients with ABPA from the UK, type I skin prick sensitivity with *A. fumigatus* was present in all patients [4]. In addition, a positive immediate hypersensitivity to *A. terreus* was observed in 92 (83%). Following prick testing, type III reactions to the *A. fumigatus* extract were noted in 16% of the cases. When intradermal testing with *A. fumigatus* protein extract fraction was performed in 80 patients, immediate hypersensitivity was positive in all and type III reaction was seen in 97%. Furthermore, the authors found that therapy with steroids completely suppressed the type III response but had little or no effect on the type I reaction. We identified an 11-amino acid synthetic peptide epitope of Asp f 1 for its potential use in intradermal testing and development of a standardised immunodiagnostic test for ABPA [62].

12.1 Skin Testing with Recombinant Allergens in Cystic Fibrosis

Since sensitisation to *A. fumigatus* is demonstrated in up to 56% of patients with CF [70], it is important to ascertain the presence of ABPA in these patients. This would help in promptly initiating oral steroid therapy, thereby reducing morbidity. The utility of recombinant *Aspergillus* allergens for the serological diagnosis of ABPA in CF has been detailed above. Skin testing using these recombinant allergens has

also been performed, mainly to differentiate ABPA from *Aspergillus* sensitisation in patients with CF. Initial studies on skin testing with recombinant *A. fumigatus* allergen I/a (rAsp f I/a) showed positive reactions in all *A. fumigatus* sensitised patients irrespective of whether they had ABPA or not [71]. However, 3 out of 6 patients with ABPA did not demonstrate hypersensitivity to rAsp f I/a. Subsequently rAsp f 3, rAsp f 4 and rAsp f 6 were cloned. Skin testing with rAsp f 1, rAsp f 3, rAsp f 4 and rAsp f 6 in 50 patients with CF (12 with ABPA, 17 with *Aspergillus* sensitisation without ABPA, and 21 not sensitised to *A. fumigatus*) revealed that the 38 patients without ABPA did not demonstrate skin test positivity to 1:100 or higher dilutions of rAsp f 4 and rAsp f 6 [67]. The authors found these two recombinant allergens to be reliable markers for ABPA in CF.

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The Treatment of ABPA

Sandra K. Gilley*, Mark R. Goldblatt*, and Marc A. Judson

Abstract The treatment of allergic bronchopulmonary *aspergillosis* (ABPA) focuses on controlling the inflammatory host response to *Aspergillus* antigens. For this reason, systemic steroids are the cornerstone of therapy. As acute ABPA is a form of asthma, standard asthma care should be also be used. An attempt should be made to taper patients off steroids after acute exacerbations. However, this is often problematic and cannot be achieved. Azoles show promise in this instance as steroid-sparing agents. Cystic fibrosis patients are at high risk of developing ABPA. Such patients present management dilemmas as it is often challenging to distinguish cystic fibrosis from ABPA exacerbations.

Keywords Allergic bronchopulmonary *aspergillosis* · *Aspergillus* · Asthma · Azoles · Corticosteroids · Treatment

Contents

M.A. Judson (✉)
Division of Pulmonary and Critical Care Medicine, Medical University of South Carolina, Charleston, SC 29425, USA

*They are co-first authors.

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1 General Principles of Treatment of Allergic Bronchopulmonary *Aspergillosis*

The goals of treatment of allergic bronchopulmonary aspergillosis (ABPA) are to preserve lung function through suppression of the immunologic response to the *Aspergillus* antigens and the inflammatory response of asthma [1]. The natural history and progression of ABPA is not known, but limiting exacerbations is critical to preventing airway destruction. Studies have suggested that early treatment of ABPA may prevent progression to fibrotic lung disease [2]. However, long-term treatment with systemic steroids should be avoided because of long-term systemic effects of the drugs. Exacerbations of ABPA have been reported during systemic steroid therapy which suggests that factors in addition to steroid-responsive bronchial inflammation are involved in the disease [3]. Therefore, treatment of ABPA should be aimed at limiting exacerbations, eradicating colonisation and proliferation of *Aspergillus* in the airway, managing asthma, and preventing pulmonary fibrosis (Fig. 1).

2 Steroid Therapy

Although there is a lack of double-blind controlled clinical trials of steroid therapy in ABPA, systemic steroids have become the mainstay treatment of choice. All patients with asthma (particularly those with steroid-dependent disease) should be evaluated for ABPA with skin testing, blood eosinophil count, serum *Aspergillus*-specific IgG and IgE levels, and total serum IgE. Patients in whom the criteria for diagnosis of ABPA are fulfilled should be treated with oral steroids to prevent progressive lung destruction. ABPA diagnosis and clinical manifestations are discussed elsewhere in this book.

In the acute stage of ABPA, treatment with steroids lowers serum IgE levels, reduces peripheral blood eosinophilia, clears pulmonary infiltrates and decreases bronchospasm. Various dosing regimens have been described [3, 4]. For acute exacerbations, prednisone 0.5 mg/kg/day for 2 weeks, followed by 0.5 mg/kg every other day for 6–8 weeks has been recommended [4]. The dose of prednisone is then be decreased by 5–10 mg every 2 weeks, as symptoms dictate. As the dose of prednisone is decreased, mild asthmatic symptoms may be return and can be controlled with inhaled bronchodilators and/or inhaled steroids. Although a study conducted in India [5] suggests that an initial dose of prednisone 0.75 mg/kg/day may improve outcomes, this higher dosing regimen has not yet been proven to be of any additional therapeutic benefit and increases the risk of adverse drug effects. The dosing of oral steroids and duration of therapy should be tailored to the individual patient according to the severity of asthma symptoms and frequency of exacerbations [6].

After prednisone is discontinued, prolonged remissions have been reported. Maintenance doses of systemic steroids are not routinely indicated during remission.

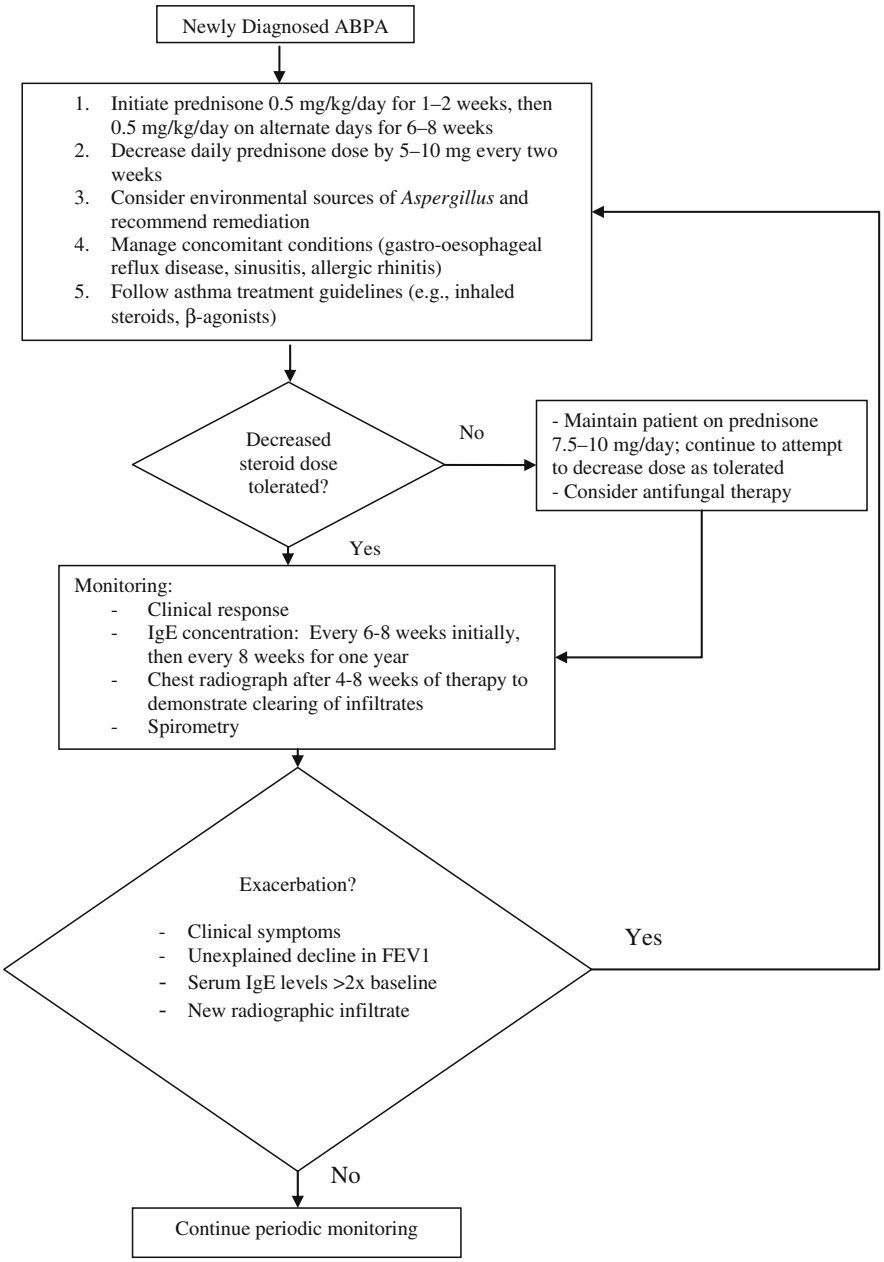

Fig. 1 Initial treatment in ABPA. Adapted from reference [2]
Legend: ABPA, allergic bronchopulmonary aspergillosis; FEV1, forced expiratory volume in the first second

However many ABPA patients are steroid-dependent and cannot be completely weaned. The use of inhaled steroids in these patients may prevent steroid dependence and avoid recurrent exacerbations [7]. Monitoring of clinical symptoms, chest roentgenograms, *Aspergillus* precipitins, and serum IgE levels should be done periodically to detect exacerbations. Guidelines for monitoring therapy are discussed further below.

Many patients with ABPA develop systemic steroid-dependent asthma symptoms. Often prednisone doses of 7.5–10 mg/day may be required to prevent new pulmonary infiltrates [8, 9]. An observational study of patients requiring long-term steroids to avoid the recurrence of pulmonary infiltrates suggests that such therapy may prevent declines in pulmonary function [10]. However, exacerbations may still occur, even in patients already taking systemic steroids. Because of the complications associated with prolonged steroid therapy, every effort should be make to lower the steroid dose as much as possible.

Methods to reduce steroid dependence include environmental manipulation to lessen exposure to *Aspergillus* spores, eradication of *Aspergillus* species from the airways, treatment of coexistent chronic airway infections, and management of concommitant conditions such as gastro-oesophageal reflux disease, allergic rhinitis, or sinusitis [2]. While there have been no studies demonstrating that environmental control reduces the number of ABPA exacerbations, it has been noted that ABPA exacerbations correlate with higher atmospheric mould counts [11, 12]. Thus, attempts at reducing the systemic steroid dose may be more successful during months when atmospheric mould counts are lower. Although *Aspergillus* species are ubiquitous organism and impossible to completely avoid, it is reasonable for patients with ABPA to avoid exposure to materials containing high fungal concentrations such as compost heaps and stored grain [13].

Inhaled beclomethasone has been shown to improve ventilatory function and control of asthma, but did not affect the frequency of pulmonary eosinophilic infiltrates [14]. However, there is anecdotal evidence suggesting higher doses of inhaled steroids may be beneficial [15]. Antifungal therapy, which is discussed below, may have an important role as an adjunct to steroid therapy, allowing for a reduction in steroid dose [16]. Other treatment modalities, such as sputum clearance techniques or antibiotic therapy may be useful in fibrocavitary ABPA.

Patients with fibrocavitary ABPA usually have extensive bronchiectasis and obstructive pulmonary disease that cannot be reversed by systemic steroid therapy. This represents advanced disease that generally has a poor prognosis. If a reversible obstructive component is present on spirometry, moderate to high doses of prednisone are usually required for control of asthma. Pulmonary infiltrates in these patients may be the result of lung infections from *Staphylococcus aureus, Pseudomonas aeruginosa* or other organisms that have colonised the airways rather than from mucoid impaction. A decline in respiratory status in these patients may be due to asthma, viral or bacterial infections, or a combination of these, making management decisions problematic [17].

3 Antifungal Therapy

Eradication of *Aspergillus* species from the airways is another approach to the treatment of ABPA. Although *Aspergillus* species are ubiquitous and are inhaled in large quantities by humans on a daily basis [18], antifungal therapy may lower the fungal antigen load in airways. The azoles (e.g., ketoconazole, itraconazole, voriconazole, and posaconazole) have known efficacy against *A. fumigatus* through inhibition of ergosterol synthesis in the fungal cell membrane, thereby inhibiting fungal growth. Ketoconazole and itraconazole have also been shown to reduce eosinophilic airway inflammation. Although studies using ketoconazole have shown reductions in serum *A. fumigatus*-specific IgG and IgE antibody, total serum IgE, and in some cases improvement in symptoms, no change in lung function has been demonstrated [19, 20]. The severe side effects of ketoconazole, including hepatic toxicity, suppression of testosterone synthesis, and suppression of adrenal steroid synthesis limit its usefulness [21].

The use of itraconazole in ABPA and other *Aspergillus*-related pulmonary diseases has been evaluated in several retrospective studies or case series, all of which have shown positive results [22–29]. A randomised, double-blind trial of treatment with either 200 mg of itraconazole twice daily or placebo for 16 weeks for steroid-dependent ABPA showed statistically significant differences in one or more of the following outcomes: (a) a reduction of at least 50% in the steroid dose; (b) a decrease of at least 25% in the serum IgE concentration; and (c) an improvement of at least 25% in exercise tolerance or pulmonary-function tests or resolution or absence of pulmonary infiltrates [30]. The rate of adverse events was similar in the two groups. In another double-blind trial involving stable patients with ABPA [29], patients were randomised to receive itraconazole 400 mg daily or placebo for 16 weeks. Therapy with itraconazole was associated with improvement of immunologic parameters such as eosinophilia, total IgE, and *Aspergillus*-specific IgG suggestive of a potential anti-inflammatory and immunological effect. Neither of these randomised trials of itraconazole included patients with cystic fibrosis, but published case series have demonstrated small improvements in pulmonary function, symptoms, or a reduction in steroid usage in that population [27]. Based on these data, itraconazole may be useful as adjunctive therapy in patients with ABPA, allowing for a reduction in steroid dose in those who are steroid-dependent. Since itraconazole interferes with steroid metabolism, steroid dose has to be reduced once itraconazole is started for these patients.

The newer azole antifungal agents, voriconazole and posaconazole have potent in vitro activity against *Aspergillus* and have a more favourable safety profile than itraconazole. These drugs are promising new agents for the eradication of *Aspergillus* species from the airways, though there is little experience with these drugs in patients with ABPA thus far [31]. Other antifungal therapies such as inhaled natamycin and oral clotrimazole have been ineffective in ABPA [32, 33]. The role of amphotericin B in ABPA is limited by its toxicity, cost, and its limited ability

to reach the airway in high concentrations. In a recent case report of a cystic fibrosis patient with ABPA who had undergone lung transplantation and not responded to itraconazole, nebulised amphotericin was successfully used in conjunction with intravenous amphotericin [34].

In summary, although the data are limited, azole therapy may be a useful adjunct in ABPA in patients with and without cystic fibrosis. Some authors would recommend treating all ABPA patients with itraconazole, regardless of the need of steroids. In our view, azoles should be reserved for ABPA patients who are steroid-dependent where they may function as a steroid-sparing agent. Azoles also may be particularly useful in ABPA patients with frequent asthmatic exacerbations. Itraconazole is the most studied azole for the treatment of ABPA. Long-term therapy with oral antifungal agents is particularly limited by toxicity and costs. Given the in vitro data of the activity of voriconazole and posaconazole against *Aspergillus* species, it is likely that these agents would also be effective for ABPA. Since secondary resistance may occur in ABPA patients treated with itraconazole (particularly in the presence of sub-therapeutic itraconazole blood levels) [35], there is a limited role for voriconazole or posaconazole as second-line therapies in patients failing itraconazole, since cross-resistance amongst azoles is a well-known phenomenon. For these patients, lack of alternative oral antifungal drugs for long-term treatment is problematic. Table 1 outlines the recommended indications, and dosing of antifungal agents for ABPA.

Table 1 Antifungal dosing in allergic bronchopulmonary aspergillosis

Drug	Dose	Evidence for Efficacy	Comments
Ketoconazole	400 mg/day	Open studies with limited number of patients	Inconsistent results and serious drug toxicities
Itraconazole	200 mg twice daily	Case reports, case series, two randomised placebo-controlled trials	Itraconazole levels and liver function should be routinely monitored. Better levels but more gastrointestinal toxicity with itraconazole solution. Infrequently has negative inotropic effects
Voriconazole	150–200 mg twice daily	Rare case reports and case series in cystic fibrosis	Liver function should be routinely monitored. Photosensitivity with chronic use. Therapeutic drug monitoring probably also needed
Posaconazole	400 mg twice daily	No clinical data have been published	Liver function should be routinely monitored. Less drug interactions than other azoles. Cross-resistance may occur amongst azoles.

4 Other Adjunctive Anti-asthma Therapies for ABPA Exacerbations

In addition to systemic steroid therapy, ABPA exacerbations should be managed according to general asthma guidelines including avoidance of asthma triggers and the use of β-agonists and other non-steroidal anti-asthmatic medications. Patients with ABPA should receive instructions on how to monitor their asthma symptoms and pulmonary function and notify their physicians whenever there is a decline. As with non-ABPA asthma exacerbations, patients with a forced expiratory volume in the first second (FEV1) or peak expiratory flow (PEF) of <40% should be managed in the emergency department or as an inpatient. In addition to systemic steroids, patients with exacerbations causing severe asthma should receive supplemental oxygen and repeated doses or continuous short-acting β-agonist therapy. Intravenous magnesium sulfate and heliox have been used in asthma exacerbations that are unresponsive to initial therapies [36], but have not been evaluated in acute ABPA exacerbation.

5 Monitoring Therapy

Following the diagnosis and treatment of ABPA it is essential to closely monitor the patient so that progression of the disease can be prevented. Patients with serologically limited disease tend to fair better than those who have developed radiographic changes such as bronchiectasis and fibrosis [37]. Although no consensus statements or formal guidelines exist concerning the monitoring of ABPA, we propose the following recommendations.

The monitoring of pulmonary symptoms should not be overlooked in the management of ABPA. The symptoms include not only typical asthma symptoms such as wheezing, dyspnoea, cough, chest tightness and congestion but also symptoms that are unusual for an asthma exacerbation such as fever and expectoration of yellow to brown flecks or plugs. The development of such symptoms should prompt further testing for the possibility of an ABPA exacerbation which should include obtaining a serum IgE level at a minimum.

ABPA patients may have asymptomatic exacerbations regardless of the radiographic findings. Therefore monitoring serum IgE levels following the initial diagnosis is important. This should be done on a monthly to bimonthly basis for at least 1 year after the diagnosis [38]. Radiographic improvement and resolution of clinical symptoms has been associated with a 35% reduction in serum IgE levels [39]. When the serum IgE level demonstrates a 2-fold increase in asymptomatic patients, radiographic imaging is recommended [38]. If such patients do not demonstrate new radiographic infiltrates, have stable pulmonary function tests, and are without pulmonary symptoms they may continue to be monitored closely without adjusting their therapy. However, if new infiltrates worsening respiratory symptoms, or worsening pulmonary function develops, therapy should be resumed or intensified [40].

Table 2 Monitoring of allergic bronchopulmonary aspergillosis (ABPA)

Methods to monitor ABPA	Frequency
Symptoms (cough, asthmatic symptoms, fever, expectoration of yellow/brown plugs)	As they occur
Serum IgE levels	Every 4–8 weeks for 1 year
Spirometry	Once per year, and also with exacerbations
Chest radiograph	4–8 weeks after initiation of treatment and with significant changes in symptoms
Chest computed tomography	Not presently indicated for monitoring (see text)

Some investigators have suggested that chest computed tomography (CT) should be used for monitoring ABPA [41]. New bronchiectatic areas or hyperattenuated mucoid impaction on CT suggests potentially destructive disease that warrants therapy. However, we are unaware of specific evidence that chest CT scanning is superior to chest radiography for monitoring ABPA. Furthermore serial CT scanning has been demonstrated to pose significant radiation risk [42]. We therefore do not recommend routine chest CT scan monitoring of ABPA patients. Follow up imaging either by chest radiograph or CT should be performed 4–8 weeks after initiation of therapy to confirm resolution or improvement of prior infiltrates [2]. The utility of CT scan in the diagnosis of ABPA has already been discussed in the chapter by Dr. Shah.

Monitoring with yearly spirometry may be useful as an unanticipated decline in functional vital capacity by 15% or more may as well be indicative of an ABPA flare. Evidence of worsening airflow obstruction with a decline in FEV1 or reduction of midflows would also suggest worsening of ABPA and should prompt further evaluation.

Table 2 outlines our suggested approach to monitoring of ABPA.

6 Differentiating an Exacerbation of Cystic Fibrosis (CF) from ABPA in Patients with Both Diseases

The first case of ABPA in CF was reported in England in 1952 [43]. Since then ABPA has been found to be present in up to 15% of CF patients [44]. The diagnosis of ABPA in CF is problematic because the diseases share several clinical manifestations including bronchiectasis and airflow obstruction. Even when it has been determined that a patient has concomitant ABPA and CF, it is problematic to determine which disease is exacerbating when worsening pulmonary signs or symptoms develop. This differentiation is important because steroid therapy for an ABPA exacerbation might be harmful if the patient truly has a bacterial exacerbation of CF.

Unfortunately there is no reliable method to differentiate an exacerbation of CF from ABPA. Patients with a history of multiple recurrent CF exacerbations might be thought to be of higher risk of a CF exacerbation than one caused by ABPA. However, such patients usually have been treated with multiple antibiotics that may increase *Aspergillus* colonisation and therefore increase the likelihood of an ABPA exacerbation. In support of this contention, studies have confirmed that patients colonised with *Pseudomonas aeruginosa* have a higher incidence of ABPA [39]. Interestingly the presence of *Aspergillus* species in sputum culture is not a predictor for an ABPA [2].

The CF consensus conference addressed differentiating ABPA from CF exacerbations [40]. A summary of their findings follows:

Classical criteria for an ABPA exacerbation in a CF patient include:

1. Acute or subacute clinical deterioration with as worsening cough, new or</subtitle> worsening asthmatic symptoms, decline in spirometry and/or increased sputum production that could not be attributed to a CF exacerbation or aetiology other than ABPA. This determination might require initial empiric treatment of a CF exacerbation in which there was an inadequate clinical response;
2. A serum IgE concentration of >1,000 IU/ml (>2,400 ng/ml) in the absence of systemic steroid use;
3. Immediate cutaneous reactivity to *Aspergillus* skin prick testing (wheal >3 mm) or elevated levels of IgE or IgG to *A. fumigatus* while antihistamines are withheld;
4. The presence of *Aspergillus* precipitating antibodies to *Aspergillus*;
5. New or recent radiographic abnormalities on chest roentography or computed tomography showing new or worsening central bronchiectasis or hyperattenuated mucoiod impaction that fails to resolve with antibiotics or chest physiotherapy.

Minimal diagnostic criteria:

1. Acute or subacute clinical deterioration to include worsening cough,</subtitle> wheezing, decreased exercise tolerance, worsening lung function, increased sputum production that is not attributable to any other cause;
2. A serum IgE concentration >500 IU/ml. In the cases where the serum IgE concentration is <500 IU/ml while the patients are receiving steroids it is recommended withdrawing steroids if possible for 1–3 months and then repeating the serum IgE level;
3. An immediate cutaneous reactivity to *Aspergillus* skin prick testing (wheal >3 mm) or elevated levels of IgE or IgG *to A. fumigatus* while antihistamines are withheld;
4. The presence of one of the following: *Aspergillus* precipitating antibodies, IgG antibodies to *A. fumigatus*,or new/recent radiographic abnormalities on

Table 3 Treatment decisions for patients with allergic bronchopulmonary aspergillosis and concomitant cystic fibrosis

Pulmonary symptoms	Spirometry	Radiographic changes[a]	IgE	Treat for
Present	Decreased	Present	2-fold increase	ABPA[b]
Absent	Unchanged	Absent	2-fold increase	Neither
Absent	Unchanged	Present	2-fold increase	Cystic fibrosis[c]
Present	Decreased	Absent	2-fold increase	ABPA and cystic fibrosis
Present	Decreased	Absent	Unchanged	Cystic fibrosis
Present	Unchanged	Present	Unchanged	Cystic fibrosis

[a]New mucoid impaction or bronchiectasis.
[b]If no new radiographic response, treat for CF.
[c]If no radiographic response, treat for ABPA.
Legend: ABPA, allergic bronchopulmonary aspergillosis.

chest roentography or computed tomography showing new or worsening central bronchiectasis or hyperattenuated mucoiod impaction that fails to resolve with antibiotics or chest physiotherapy.

Table 3 outlines our suggested treatment approach in various clinical scenarios with patients with proven CF and ABPA.

7 Potential Therapies

In 2007 Van der Ent and colleagues published a case report of utilizing anti-IgE antibody (omalizumab) in a 12 year old girl with CF and ABPA who had experienced the complete spectrum of steroid-related adverse events [45]. The mechanism of this improvement is unclear as the dose was inadequate to neutralize the high levels of serum IgE seen in this patient and most others with ABPA. We believe that IgE antibody therapy may have a potential role in the treatment of ABPA but presently remains unsubstantiated.

Other than omalizumab, there are no data on the use of immune modulators in the treatment of ABPA. Leukotriene D4 antagonists have been shown to have in vitro anti-eosinophilic activity and therefore may be of potential benefit in the treatment of ABPA [46]. The monoclononal anti-IL-5 antibody, mepolizumab may have a role in the treatment of ABPA. IL-5 plays a role in eosinophilic maturation and proliferation, and by inhibiting this process ABPA may potentially be ameliorated. Mepolizumab has been successfully used in refractory cases of primary hyper-eosinophilic syndrome. However, it has failed to show any benefit in allergic asthma or atopic dermatitis [47, 48]. Other agents that may be potentially beneficial in the treatment of ABPA include chemokine receptor 3 antagonists which block eosinophilic cell activation and immunotherapy directed specifically at *Aspergillus* antigens. However, at present these agents have not been examined.

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Severe Asthma with Fungal Sensitisation (SAFS)

Curig Prys-Picard and Robert Niven

Abstract Asthma is an increasingly common disease. Those patients with the most severe disease consume a disproportionately high percentage of the healthcare budget. Sensitisation to a number of fungi including *Aspergillus* spp. is commonly found in atopic individuals. Sensitisation to fungi is associated with increased asthma medication usage, measured asthma severity, asthma admissions, intensive care admissions for asthma, respiratory arrest and asthma deaths. A number of mechanisms have been proposed for the ability of fungi to induce an allergic response. These include the wide spectrum of allergenic molecules released by fungi, especially those with inherent proteolytic activity. Volatile organic compounds have also been implicated but the evidence for this is less strong. Allergic bronchopulmonary aspergillosis (ABPA) is the archetypal allergic fungal disease whereby *Aspergillus fumigatus* colonises the airway in a sensitised individual, and is associated with the formation of *Aspergillus*-specific IgE and IgG. Severe asthma with fungal sensitivity (SAFS) describes a group of asthma patients with refractory asthma symptoms, sensitisation to one or more common fungi, but lacking the diagnostic criteria for ABPA (such as *Aspergillus*-specific IgG). The importance of this empirical categorization lies in the response to anti-fungal therapy. The FAST trial showed that such patients had both clinically and statistically significant improvements in quality of life, rhinitis scores, and reductions in serum IgE in response to itraconazole when compared to placebo.

Keywords ABPA · Asthma · Fungal allergy · Itraconazole · SAFS · Steroids

C. Prys-Picard (✉)
PACC Division, The University of Texas Medical Branch, TX, USA
e-mail: copryspi@utmb.edu

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Contents

1 Introduction

Asthma is a common disease and is becoming more so; in the United States of America (USA), between 1980 and 1994 the self reported incidence of asthma rose by 75% [1]. In the United Kingdom (UK) it is responsible for 70,000 hospital admissions per year, 1,200 deaths and the cost of health care is estimated at 1.2 billion pounds.

The pathophysiology of asthma is increasingly recognised as being relatively heterogeneous with both well established variations such as allergic and non-allergic asthma, or brittle asthma [2], and newer discoveries such as polymorphisms of the β_2 adrenergic receptor [3]. As a result "asthma" is better described as a collection of symptoms or syndrome, rather than being a diagnosis per se. The group of patients with the most severe asthma has the most symptoms, are more likely to be taking treatment that does not control their symptoms are more likely to suffer iatrogenic side-effects from such medication and are more likely to suffer from life-threatening events. The cost of asthma health care is not evenly distributed between patients. For instance, it has been estimated that 10% of asthma patients with the most severe disease account for 50% of asthma costs in Canada [4]. With the advent of the "era of the monoclonal antibody", the cost of treating these patients is likely to escalate. In the face of such pressures it is vital that the clinical phenotype of asthma is determined for patients with severe asthma so that appropriate therapy can be prescribed and inefficacious medication avoided [5]. Asthma associated with fungal sensitivity is one such phenotype and offers the opportunity for tailored therapy.

2 Association of Fungal Sensitisation and Severe Asthma

Sensitisation to a number of fungi including *Aspergillus* spp. is commonly found in atopic individuals. A number of studies have demonstrated an association between fungal sensitivity and asthma severity. Whilst there are geographical variations in the sensitising mould, the association with severe asthma appears to be a global phenomenon. O'Hollaren et al. found *Alternaria alternata* sensitisation in the USA to be a significant risk factor for respiratory arrest due to asthma [6]. The study of Dales et al. showed a temporal relationship between airborne mould levels and increased asthma admissions in Canada [7]. Vesper et al. demonstrated higher levels of environmental mould in the houses of children requiring daily asthma medication, as opposed to intermittent or no medication [8]. Black et al. in New Zealand observed that a greater proportion of patients admitted to the intensive care unit with life-threatening asthma were skin test-positive to mould compared, to those asthmatics admitted to the general wards or not admitted at all [9]. The European Community Respiratory Health Survey examined 1,132 adults aged 20–44 years with current asthma. Sensitisation to *Alternaria alternata*, *Cladosporium herbarum* or both by skin prick testing was associated with an increased risk of severe asthma versus mild asthma, with an odds ratio of 2.34 (95% confidence interval 1.56–3.52) [10]. O'Driscoll et al. [11] in the UK recruited asthma patients and grouped them according to number of lifetime admissions (none, one and multiple). Subjects were skin tested to five common mould allergens and four aeroallergens. Skin reactivity to all allergens was greatest in the multiple admissions group, but this relationship was strongest for mould and dog allergens. Seventy six percent of multiple admission-patients were positive for mould, compared to 16–19% for the other groups. Similarly multiple mould skin test positivity was significantly more common in the group with multiple admissions. A further positive correlation was found between the size of mould skin test reaction and number of admissions [11].

Asthma symptoms have been shown to be increased when spore counts of *Cladosporium* spp. and *Alternaria* spp. are high [12]. Emergency department attendances for asthma also increase with airborne spore but not pollen counts [7, 13]. Asthma deaths in the UK are highest in the months of July to September. This is the peak outdoor air levels of fungal spores [14, 15]. A similar association has have been demonstrated in Chicago [16]. The phenomenon of "thunderstorm asthma" and its association with fungal spores is now well described [6, 17–20]. It is postulated that higher humidity and winds triggers spore formation and release into the atmosphere [21].

3 Fungi as Allergens

Fungi produce a vast array of molecular, chemical and biologically active material. Not all the effect is primarily allergic in nature. The cell wall and matrix of fungi contain glucans. These chemicals have activity akin to those of endotoxins in the cell wall of Gram negative bacteria, with a variety of potential beneficial or harmful effects, though they are not as dramatically pro-inflammatory as endotoxin.

There is a wide range of fungal elements that have been identified as human allergens, and these can be divided into the products secreted by mould species such as proteases, glycosidases, and the enzymes involved in fungal metabolism such as gluconeogenesis or the pentose phosphate shunt. The germination of spores involves fungal growth and manipulation of the local milieu. As a result the host may be exposed to a mixture of these potential allergens either simultaneously or consecutively.

Some allergens such as chicken egg albumin (ovalbumin), if applied only by inhalation, are only capable of inducing limited airway inflammation (airway hyper-responsiveness) [22]. Ovalbumin given only via the airway induces a state of relative tolerance in which IgE is not induced, only IgG1 and IgG2a [23] and the Th2 response is not induced. Only when the organism is primed, usually with an adjuvant at a remote site, is the full inflammatory response achieved (i.e., airway eosinophilia and mucus hyper-secretion) [24–26]. Fungal allergens derived from *Aspergillus* species are capable of eliciting a full allergic response without pre-treatment at a remote site [27, 28]. As such various allergens differ in their ability to induce either tolerance or a full Th2 allergic response. Kheradmand et al. [29] have shown that inherent protease activity was required in *Aspergillus* allergens in order to evoke a Th2 allergic response and that this could be attenuated by addition of protease inhibitors. Similarly Wan et al. have shown that β-D-glucans can also potentiate the production if IgE in mice in response to ovalbumin stimulation [30]. A deeper discussion on *Aspergillus* allergens is given elsewhere in this book.

4 Protease-Induced Airway Inflammation

Over 40 antigens have been characterised from *Aspergillus fumigatus.* Many of these possess protease activity (Asp f5, Asp f10, Asp f13, Asp f15 and Asp f18) which appears to promote the development of allergy.

The mechanisms by which fungal allergens with protease activity induce an immune reaction have not yet been fully elucidated. Proteases in the airway can alter the integrity of the respiratory epithelium. Wan et al. [31] have shown that protease activity of derP1 facilitates allergen translocation across respiratory epithelium by disruption of tight junctions. Tai et al. [32] showed that a fungal protease (Pen ch 13) can cleave occludin (a tight junction protein) in the extracellular domain. Winter et al. [33] found that protease activated receptor-2 (PAR-2), in conjunction with allergen sensitisation, enhances airway inflammation. Activated PAR-2 receptor has been shown to decrease the transepithelial resistance of human airway epithelium by ∼50%. This was associated with an interruption of E-cadherin adhesion and compromised epithelial barrier [33]. Su et al. [34] showed that PAR-2 activating peptide (but not PAR1-AP or PAR4-AP) caused an acute increase in epithelial permeability to protein and water and an increase in airway tone in a mouse model. In addition this was associated with a 12-fold increase in neutrophils. This effect was markedly decreased by the ablation of sensory neurons with capsaicin, suggesting a neurogenic mechanism.

5 Volatile Organic Compounds (VOCs)

VOCs are small organic compounds that can easily vaporise and hence be inhaled. They are known respiratory irritants and include methane, industrial solvents and formaldehyde. It has been estimated that indoor air can contain VOCs in sufficient concentrations to mirror those required to produce airway irritation under experimental conditions [35]. Combinations of VOCs can produce irritant effects at lower individual concentrations than a single compound [36]. Those VOCs derived from microbial sources are called "microbial volatile organic compounds" (MVOCs). Moulds can produce a large number of MVOCs such as terpenes, but the mould species and the substrate on which the mould is growing may determine which are produced [37].

Indoor exposure to VOCs has been associated with an increased risk of asthma [38, 39]. It is uncertain however whether they can cause asthma or are acting as irritants to pre-existing asthmatic airways. These relatively low molecular weight compounds themselves are too small to act as allergens directly. They may act as haptens in much the same way as some chemicals can cause occupational asthma via a non-allergic mechanism. No direct evidence of asthma causation has been demonstrated, and MVOCs may simply be bystander markers of larger allergenic fungal particles.

6 Survivor Effect

Those patients who are aware of what allergens exacerbate their asthma symptoms, tend to avoid them (although it is notoriously difficult to separate asthmatic owners from their cats, dogs or horses – whatever the cost in terms of asthma symptoms). For those individuals that are sensitised to moulds avoidance may be harder. Firstly the vast majority of them will be unaware of their mould sensitivity, and even if they were, limiting exposure is difficult. Obvious sources of moisture or damp within the home that lead to overgrowth of *Aspergillus* spp. (such as leaking bathroom fixtures) can be remedied. There is evidence that increased damp is associated with an increased allergen load including fungi [40]. Lower socioeconomic groups are more likely to live in damp housing and will be least able to improve the quality of their homes or move to better homes. Moving or improving housing may not be sufficient. The cotton industry was founded in Lancashire (North West England) in the 1800s due to the high local moisture content of the air. This was necessary as the high humidity prevented the cotton threads from breaking during spinning. This regional dampness may contribute to higher environmental airborne mould allergen levels. Patients with severe asthma commonly experience a marked and rapid relief of their symptoms whilst holidaying in warmer drier climates than the North West of England. It is a common anecdote that symptoms return whilst picking up luggage from the conveyor belt at the local airport on their return. It is also an aggravating factor in some healthcare systems that the poor are least able to gain access to healthcare or afford appropriate medications.

7 Allergic Bronchopulmonary *Aspergillosis's* (ABPA): A Historical Perspective

There is wide variation in both the fungi involved and also the severity of asthma. Different pathophysiological mechanisms underlie some of the different clinical presentations. The role of *A. fumigatus* in allergic bronchopulmonary aspergillosis (ABPA) is well-established. Fungi other than *A. fumigatus* have been implicated in similar conditions termed allergic bronchopulmonary mycosis. These include (but are not restricted to) species of *Candida* [41], *Saccharomyces* [42] and *Schizophyllum* [43].

The definition of ABPA has evolved over time. The first description of *Aspergillus* as a human lung pathogen is ascribed to Bennett in a letter read at the Royal Society of Edinburgh 1842 [44]. Hinson et al. in their 1952 case series of eight patients with bronchopulmonary aspergillosis, described three patients with wheeze, mucus production, peripheral blood eosinophilia, pulmonary infiltrates, all who grew *A. fumigatus* in sputum [45]. A raised immunoglobulin E level was later associated with the allergic form of pulmonary aspergillosis [46]. The diagnostic threshold for total IgE is empiric and depends on the definition chosen [47]. The fundamental concept of the disease is that in susceptible patients with pre-existing asthma or cystic fibrosis, the airways become colonised with *A. fumigatus*. A Th1 response is elicited, resulting in the formation of *Aspergillus*-specific IgA and IgG antibodies (detected as precipitins). A Th2 response also occurs, with the formation of an allergic sensitisation. The combination of this colonisation with sensitisation to the fungus results in a clinical picture of mucus plugging, worsening airway inflammation and ultimately bronchiectasis. It is now accepted that central bronchiectasis is not a pre-requisite and patients without high resolution computed tomography (HRCT) evidence of bronchiectasis are denoted seropositive ABPA (ABPA-S), and presumably represent an earlier form of the same disease process [48, 49]. The progression of ABPA from being seropositive only to having confirmed bronchiectasis was described as early as 1968 [50]. This already highlights the potential for a spectrum of disease for which ABPA is the extreme presentation.

8 Severe Asthma with Fungal Sensitisation (SAFS)

SAFS describes a theoretically simpler scenario whereby sensitisation occurs with or without colonisation, but without the formation of IgG antibodies. The source of fungal allergens is less clear and probably varies. Fungal spores are aeroallergens [7] and as such gain access to the immune system mainly via the lungs. Initial sensitisation presumably occurs via the inhalation of spores. Propagation of the allergic response requires continued exposure, which may occur by repeated inhalation. There are case reports of fungi other than *A. fumigatus* causing allergic bronchopulmonary syndromes [41, 42, 51, 52]. It is unclear whether fungi such as *Alternaria*

Table 1 Comparison of features of severe asthma with fungal sensitisation (SAFS) and allergic bronchopulmonary aspergillosis (ABPA)

	SAFS	ABPA
Aspergillus-specific IgE	May be present	Always present
Aspergillus-specific IgG	Not essential	Always present
Other fungal specific IgE	Common	Rare as primary allergen, may be raised as part of polyclonal rise in IgE
Total IgE	Moderately raised (<1,000 IU/ml)	IgE >1,000 IU/ml. Absolute value depends on definition used
Bronchiectasis	May be present, generally mild	Present in ABPA with central bronchiectasis
Mucus plugging	May be present, generally mild	Extensive mucus plugging with difficult expectoration

spp. or *Trichophyton* spp. can colonise the lungs. For some fungi, colonisation of either the gastrointestinal tract (*Candida* spp.) or skin (*Candida* spp. or *Trichophyton* spp.) may provide haematogenous delivery of fungal allergens to the lungs. There are case reports of treatment of chronic fungal skin infections leading to a significant improvement of asthma control [53]. This mechanism is likely to be limited to those fungi that will grow at body temperature (which includes many species in the *Aspergillus* genus).

As will be discussed below, many of the other features of ABPA can also be seen as a sequelae of severe asthma, whether fungal related or not. Table 1 shows a comparison of ABPA and SAFS.

9 Diagnosis of SAFS

SAFS is more of a concept than a discrete entity with strict diagnostic criteria. As can be seen later there is no single element in the original description that is not open to interpretation and as such the specific criteria are likely to develop over time with the advent of knowledge based on prospective evaluation of change of airway calibre, immunological and physiological changes, with tighter observation of severe asthma cohorts. Certainly patients with SAFS form one end of spectrum of fungal allergen related airway diseases of which ABPA is the best recognised.

The definition of SAFS as proposed by Denning et al. [54] has three main criteria: (i) severe or poorly controlled asthma; (ii) evidence of fungal sensitisation as defined by positive prick testing, or fungus or fungal antigen specific IgE; and (iii) insufficient criteria to meet a diagnosis of ABPA. These are discussed in more detail as follows.

9.1 Severe Asthma

Different grading systems for the severity of asthma exist [55–57] and are mostly used as research tools. Gradings are based on treatment level, lung physiology, symptoms or a combination of these. It is beyond the scope of this book to go into a discussion of the relative merits of each grading scale. As a rule of thumb, however, asthma that is not controlled (remains symptomatic or at risk of life threatening events) on high dose inhaled steroids and long-acting beta agonists, or requires frequent courses or continuous systemic use of steroids, is generally considered as being severe.

9.2 Fungal Sensitisation

Any evidence of fungal specific IgE is currently deemed sufficient for the diagnosis of fungal sensitisation. As such either a positive skin prick test or serological evidence (raised specific IgE to fungus) can be used. The inclusion criteria for the FAST study (see below) included a positive result on one or other of these tests of sensitisation.

9.3 Distinction Between SAFS and ABPA

The diagnosis of ABPA warrants its own section (see earlier chapter by Dr Shah). There will be overlap between ABPA and SAFS depending on the criteria used. The more severe SAFS patients will be close in the spectrum to the less severe ABPA patients. Indeed it is plausible that the two conditions represent different stages of the same disease process, i.e. early fungal sensitisation and high IgE with severe asthma (SAFS) progresses with increased fungal colonisation and higher IgE titres (seropositive ABPA) and then to bronchiectasis with prolonged disease (ABPA with central bronchiectasis). The term SAFS has potential clinical usefulness and was developed to describe those patients with fungal sensitisation and severe asthma that nevertheless do not fulfil the criteria for ABPA, but still may benefit from antifungal therapy (see treatment below).

In a study evaluating polymorphisms in tol-like receptors in patients with different forms of aspergillosis, no particular genotype was associated with SAFS [58]. In contrast, susceptibility to ABPA was associated with allele C on T-1237C (tol-like receptor 9) ($p = 0.043$). Bowyer et al. [59] showed patients with ABPA and SAFS seem to react to different *Aspergillus* allergens. Both ABPA and SAFS patients reacted with Asp f1 and Asp f2, and in no case did 100% of patients with any condition react with a single allergen. Although SAFS patients manifested raised RAST responses to total *A. fumigatus* protein mixture, extremely low reactivity was observed amongst these patients to the *Aspergillus* allergens Asp f1, Asp f2 and Asp f6. No SAFS patient showed reactivity with Asp f4.

9.4 Imaging

Plain chest radiographs are generally not helpful in the diagnosis of SAFS. HRCT however does have a role. Whist central bronchiectasis is part of the earlier descriptions of ABPA, as discussed earlier, it is not a pre-requisite for SAFS. Conversely bronchiectatic changes (bronchial wall thickening with bronchial lumen dilation) have been repeatedly been shown to be present in approximately 25% of unselected patients with severe asthma [60–63]. As a result bronchiectasis is neither confirmatory nor exclusionary for SAFS but contributes to the clinical spectrum of fungal-related airway disease. There are no features of SAFS on HRCT that distinguish it from severe asthma of any other cause.

10 Treatment of SAFS

10.1 Antifungal Azoles – The Fast Study

In the light of definitive evidence of the benefit of azoles in ABPA [64], and evidence of the effectiveness of other antifungals agents in asthma patients, pilot work in patients with severe asthma and evidence of sensitisation to *Aspergillus fumigatus* was undertaken and supported a potential role for this therapy [65]. On the basis of this the FAST trial was designed, and results of this study have just been published [66].

The FAST trial was the first to directly investigate the potential therapeutic benefit of treating patients with SAFS with anti-fungal therapy. Fifty eight patients with severe asthma requiring therapy consistent with step 4 or higher of the British Thoracic Society guidelines [56] were enrolled. Entry criteria included sensitisation to at least one of the following fungi: *Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum, Candida albicans, Trichophyton mentagrophytes, Saccharomyces cerevisiae* or *Botrytis cinerea*. Sensitisation was defined either as skin prick test positivity or positive specific serum IgE (RAST). Exclusion criteria were total IgE >1,000 IU/ml or positive results for *Aspergillus* IgG precipitins (to exclude those patients with ABPA) [47]. The primary endpoint was the Juniper Asthma Quality of Life Questionnaire (AQLQ) score with other traditional asthma research study measures as secondary endpoints.

Subjects were randomised to receive either itraconazole 200 mg twice daily or matching placebo for a total of 32 weeks, with final follow-up occurring at 48 weeks. In addition, the subjects at one of the four recruiting sites also had assessments made of plasma cortisol, plasma adrenocorticotropic hormone (ACTH) and 24 h urinary cortisol excretion.

At baseline the mean forced expiratory volume in the first second (FEV1) in both groups was 71% predicted, despite a mean inhaled steroid dose of 1,500 μg beclomethasone equivalent. Mean total IgE was 228 IU/l, and *A. fumigatus* and *C. albicans* were the most common sensitisers.

AQLQ increased by 0.85 in the itraconazole treated group as compared to no change in the placebo group ($p = 0.014$) in the intention to treat analysis. This is a clinically significant improvement in AQLQ. This compares to a rise in AQLQ of 0.42 observed in studies evaluating anti-IgE therapy [67, 68] and 0.55 with long-acting beta agonists [69, 70]. In those subjects who completed 32 weeks of therapy, the increase in AQLQ was even higher at 1.18. In addition, there was a clinically and statistically significant improvement in rhinitis score. Associated with this was a mean drop in total IgE of 51 IU/l (27%), with no significant change in lung function. A subgroup analysis failed to demonstrate a mean difference in adrenal suppression over placebo although some patients on itraconazole did experience profound adrenal suppression and one subject withdrew due to Cushingoid symptoms.

The preliminary pilot work, completed pragmatically, and the study have demonstrated that treatment is associated with a clinically meaningful reduction in total IgE and specific RAST to the implicated fungal agents. The mechanism of benefit remains obscure. It is suggested, though far from proven, that treatment reduces airway colonisation and prevents subsequent IgE mediated allergic inflammation from proceeding. The well-demonstrated interaction between itraconazole and certain inhaled steroids may mean the benefit is purely or in part induced by an augmentation of steroid effect. It is clear that further work is required to either confirm or refute the presence of airway fungal elements to determine the potential mechanism of action and also the safety of long term itraconazole and itraconazole withdrawal, to ensure there is no serious or persistent adrenal suppression.

The benefit of antifungal therapy was also confirmed in a retrospective cohort study involving 33 adult patients who fulfilled criteria for either SAFS ($n = 22$) or ABPA ($n = 11$). All patients received antifungal therapy for at least 6 months. In this study, only patients with SAFS related to *Aspergillus* species were included. Total IgE values decreased after 6 months of antifungal therapy by an average 24.5% in SAFS patients ($p = 0.004$) and by 36.8% in ABPA patients ($p = 0.005$). RAST for *A. fumigatus* decreased by an average 14.8% in the overall population studied ($p = 0.005$), and by 34.1% in SAFS patients ($p = 0.019$). Similarly, a significant decrease occurred in the number of eosinophils in the peripheral blood. Lung function improved for all patients (median FEV1 increased by 190 ml; $p = 0.016$), with a trend towards an improvement in both the subgroups (SAFS, 170 ml increment, $p = 0.075$; ABPA, 230 ml increment, $p = 0.080$). For the 10 patients who entered the study taking oral steroids continuously, 4 (40%) were off oral steroids after 6 months of antifungal therapy. No overt Cushing's syndrome was reported (Pasqualotto AC, personal communication).

10.2 Anti-IgE Therapy

SAFS remains an allergy-mediated asthma phenotype and hence potential therapeutic benefit of anti-IgE immunoglobulin (omalizumab) exists. Single case report [71] and abstract presentations have described the off-label use of anti-IgE in ABPA, where the total IgE exceeds the recommended upper limit for anti-IgE efficacy. In

ABPA the high total IgE is polyclonal, with only a numerically moderate contribution coming from *Aspergillus*-specific IgE. It is well documented that IgE specific to different allergens are clinically significant at different doses. Anti-nut or latex IgE is significant a range approximately one to two orders of magnitude than animal danders such as cat, dog or horse. Fungi appear to be relevant at the lower limits of detection of specific IgE. Since by definition, the total IgE in SAFS is lower, it is reasonable to expect that anti-IgE will be similarly useful in this population. In ABPA, the *Aspergillus* antigen appears to be the "driving" allergen of the allergic process, hence anti-IgE may be able to reduce the quantity of *Aspergillus*-specific IgE to a low enough level to reduce the total allergenic process despite not reducing the total IgE to a level previously considered to be adequate for a therapeutic response.

11 Summary

SAFS remains a description of a clinical phenomenon with ill-defined diagnostic criteria. Denning et al. estimate that between 730,000 and 2.2 million people in the USA will fulfil the diagnostic criteria for SAFS [54].

The importance of considering fungal sensitisation is that it provides an additional therapeutic option. Fungal sensitivity may be equally important in the aetiology of mild as opposed to severe asthma. In the former however, current standardised therapeutic strategies are efficacious (by definition), well-tolerated, have few side effects and are cheap. It is the group of patients whose symptoms remain refractory to high dose conventional asthma therapy that suffer from impaired quality of life, potentially have life threatening events and are subject to iatrogenic morbidity from steroid side effects. A new window of therapeutic opportunities is therefore open for these patients.

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PART V
ASPERGILLUS SINUSITIS

Aspergillus Sinusitis

Matthew W. Ryan and Bradley F. Marple

Abstract *Aspergillus* sinusitis has several distinct clinical manifestations, which are determined by the presence or absence of tissue invasion as well as the host's response to the fungus. Most, but not all fungal sinusitis is caused by *Aspergillus* organisms. This section on *Aspergillus* sinusitis utilizes the currently accepted classification scheme for categorising fungal rhinosinusitis. An aspergilloma (fungal ball) is a non-invasive mycelial mass of *Aspergillus* that accumulates in a paranasal sinus. Surgery is usually curative and no adjunctive antifungal therapy is required. Allergic *Aspergillus* sinusitis is a common form of *Aspergillus* sinusitis. The disease is pathologically similar to allergic bronchopulmonary aspergillosis, and usually develops in young atopic adults. Chronic invasive fungal sinusitis is the most poorly characterised form of fungal sinusitis. This condition may mimic a variety of inflammatory and neoplastic conditions that affect the nose and sinuses. Critical to diagnosis is the identification of tissue-invasive fungal hyphae. Acute invasive fungal sinusitis is the most severe manifestation of fungal sinusitis. Affected individuals are usually immunocompromised, and there is a high mortality rate despite medical and surgical therapy.

Keywords Allergic sinusitis · Aspergilloma · Invasivesinusitis · Rhinosinusitis · Sinusitis · Steroids

Contents

M.W. Ryan (✉)
Department of Otolaryngology, University of Texas Southwestern Medical Center, Dallas, TX, 75390-9035, USA
e-mail: matthew.ryan@utsouthwestern.edu

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1 Introduction

Fungal sinusitis continues to increase in importance. Changing terminology and emerging theories of pathogenesis have also made the subject of fungal sinusitis a source of confusion and controversy. Whilst our understanding of fungal sinus disease continues to evolve, it is now clear that fungal organisms can cause a variety of clinical conditions affecting the nose and paranasal sinuses. However, because fungal sinusitis is still relatively rare, there is a paucity of reports that address fungal sinusitis in a systematic fashion. Additionally, the terminology used to describe fungal sinusitis includes vague, non-specific terms, and the medical literature is replete with examples of inconsistent, confusing terminology. The purpose of this section is to describe the clinical features and treatment of fungal sinusitis caused by *Aspergillus* species. Whilst a variety of fungi can cause rhinosinusitis, this focus

on *Aspergillus* is appropriate because the *Aspergillus* species are responsible for the majority of cases of fungal sinusitis [1].

The first reports of fungal or *Aspergillus* sinusitis were made in the eighteenth and nineteenth centuries, and descriptions of fungal sinus disease began to proliferate in the nineteenth century. These descriptions began drawing distinctions amongst the various fungi; the pathogenic fungus, accordingly, became the touchstone for disease classification. During this period the terms "aspergillosis", "mucormycosis," "phaeohyphomycosis," and "zygomycosis" emerged, terms which persist to this day, and are used commonly in the general medical literature to describe fungal disease at other body sites. Baker's term "rhinocerebral mucormycosis" also survives in the literature [2]. However, these terms should no longer be used to describe fungal sinus disease. The use of the term "sinus aspergillosis" is problematic as this nomenclature is non-specific and may be confusing. For example, the term "sinus aspergillosis" has been used to describe invasive, non-invasive, and allergic forms of *Aspergillus* sinusitis [3–6].

Whilst the focus of this chapter will be on the role of *Aspergillus* species in the various manifestations of fungal sinusitis, fungal sinus disease is no longer defined by the responsible fungus. Rather, the currently accepted definitions of fungal sinusitis incorporate analyses of tissue invasion as well as host immunological response. In 1965 Hora [4] drew the clinical and histological distinction between non-invasive and invasive fungal sinus disease. The non-invasive form of "sinus aspergillosis" which Hora described was characterised by thick, dark, greasy material in the sinuses. This condition was notable because in general, patients with the condition were found to have a good prognosis. Hora also described a rarer form of "invasive aspergillosis" that was characterised by fungal tissue invasion, associated pain, and a soft tissue mass that mimicked the clinical features of malignancy [4]. Whilst many authors continue to use the term "aspergillosis" to describe fungal sinus disease without regard to tissue invasion [5, 7], the importance of distinguishing between invasive and non-invasive disease has become increasingly clear. The importance of this invasive/non-invasive distinction cannot be overemphasised, particularly with regard to treatment. It is apparent from the medical literature that in the past, cases of non-invasive fungal sinusitis were frequently confused with invasive fungal disease and treated with aggressive surgery and toxic antifungal agents. Reviewing reports of these cases, the modern reader will recognise the clinical and histologic characteristics that Millar [8] and Katzenstein [9] recognised as identical to allergic bronchopulmonary aspergillosis (ABPA) when they described "allergic *Aspergillus* sinusitis". The characterisation of allergic *Aspergillus* sinusitis highlighted the importance of the host immunologic response in determining the clinical manifestations of fungal sinus disease.

The subtypes of *Aspergillus* sinusitis can be broken down into three tissue-invasive and two non-invasive forms. These distinct clinical entities are also separated based on the host's immune response to the fungus. The two non-invasive forms of *Aspergillus* sinusitis are allergic *Aspergillus* sinusitis and the fungal ball (also known as an aspergilloma). A fungal ball is a dense mat of fungal hyphae – thick, dark, cheesy debris filling an obstructed sinus, with minimal

mucosal inflammation. Allergic *Aspergillus* sinusitis (also known as allergic fungal sinusitis – AFS) develops in atopic patients, involves multiple sinuses, and is associated with brisk mucosal inflammation and nasal polyposis. The sinuses are filled with thick debris similar to a fungal ball, but histologically the material resembles the pulmonary concretions of ABPA. Invasive fungal sinusitis is divided into acute and chronic forms that correlate with the host's immune competence. Patients with acute invasive *Aspergillus* sinusitis (also known as acute invasive fungal sinusitis – AIFS) are usually immunocompromised, and they will die acutely without proper treatment. AIFS develops over less than 4 weeks, but the disease may take a more chronic course with fluctuating immunocompromise or effective treatment [10]. Chronic invasive *Aspergillus* sinusitis (also known as chronic invasive fungal sinusitis – CIFS), as its name implies, develops over a more prolonged time frame. Patients are usually immunocompetent, and the disease progresses over months or years. Like the acute form, chronic disease may be fatal. Granulomatous and non-granulomatous forms of CIFS have been described. The granulomatous form of chronic invasive *Aspergillus* sinusitis has sometimes been referred to as "primary paranasal *Aspergillus* granuloma" [11].

2 Incidence

The incidence of fungal sinus disease seems to be increasing. Possible explanations include increased practitioner awareness and improved imaging that is uncovering a previously missed diagnosis. There are increasing numbers of immunocompromised individuals as a result of organ transplantation, haematopoietic stem cell transplantation (HSCT) and the spread of HIV. Chemotherapy for haematological malignancy is increasingly successful in generating clinical remission. However, patients must endure repeated cycles of immunosuppression with each treatment course. Antibacterial antibiotics are over-prescribed for viral rhinosinusitis. This inappropriate use may alter the normal nasal microbiota, facilitating fungal growth within the nose and sinuses. Finally, we see an increasing incidence of atopic disease in the developed world and this could influence the prevalence of allergic *Aspergillus* sinusitis. The importance of any of these individual factors is unknown, but fungal sinus disease certainly seems more common compared to just a few decades ago.

Fungal sinusitis is still rare, despite the fact that its incidence seems to be increasing. Non-invasive fungal sinusitis is more common than invasive fungal sinusitis. It is estimated that 4–7% of surgical cases for chronic inflammatory sinonasal disease are for allergic fungal sinusitis [12, 13]. A review by Ferreiro et al. [13] from 1984 to 1994 showed that 3.7% of surgical cases for inflammatory sinus disease were for sinus fungal balls. CIFS is exceedingly rare in the United States of America (USA) with only isolated case reports and a few small series [14–16]. CIFS is much more common in arid regions, and the largest series have come from the Sudan and India [11, 17–21]. Acute invasive fungal sinusitis, though rare, seems to be more common with the advent of AIDS and increasing utilisation of chemotherapy and HSCT. Incidence rates for patients with cancer, leukaemia, or HSCT recipients

are between 1 and 2% [22, 23]. Talbot et al. [24] found a 3.4% annual incidence amongst their population of patients in the hospital for leukaemia. The incidence of all forms of fungal sinusitis will likely continue to increase, and this problem is sufficiently common that fungal sinusitis should be considered a possibility in any patient with sinonasal complaints.

Identification of the fungi responsible for sinonasal disease is important to direct appropriate antifungal therapy. Histopathology is a helpful first step in this process. The Zygomycetes can frequently be distinguished from the hyaline moulds based upon hyphal morphology on frozen section biopsy, but in vivo growth conditions or tissue processing may distort the features and confuse diagnosis [25, 26]. For example, the dematiaceous fungi common in AFS were mistaken in the past for *Aspergillus* spp. because of their histological resemblance [25]. *Pseudoallescheria boydii* may be easily confused with *Aspergillus* species, for example, and this distinction is important to direct appropriate antifungal therapy.

Obtaining histological identification of fungus in tissue sections can be difficult and requires special staining techniques (Fig. 1). A KOH preparation with calcofluour white staining, silver stains or periodic acid-Schiff (PAS) stain may be required to identify fungal hyphae in sinonasal tissues. One or more of these special stains should be used by the pathologist to rule out the presence of tissue invasive fungus in suspicious cases. The Fontana-Mason stain picks up melanin and is useful to identify dematiaceous hyphae, which may grow as hyaline moulds in tissue [25]. The Fontana-Mason stain is important in cases of suspected AFS to document the presence of fungi within the "allergic mucin" of AFS. Whilst the appearance of stained fungal hyphae in tissue can suggest an organism, the definitive identification requires culture and extensive testing [25, 26]. Whilst identification is considered to be important to direct antifungal therapy, in practice, because of delays in obtaining results, empiric treatment is often begun based on the likely organisms

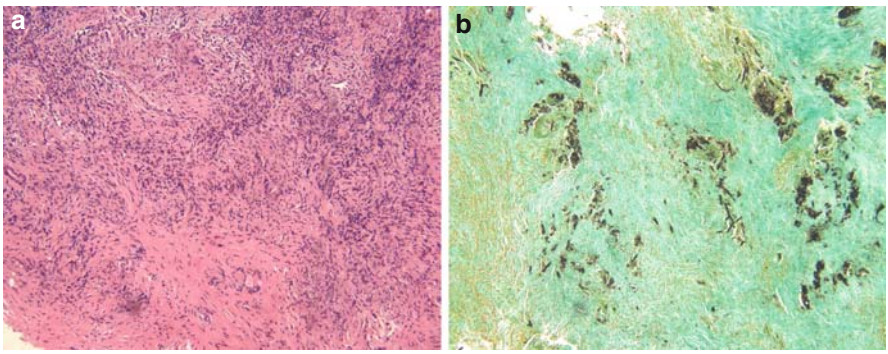

Fig. 1 **a**. Photomicrograph of sinus tissue specimen with Haematoxilin & Eosin (H&E) stain shows mixed chronic and granulomatous inflammation. Fungal hyphae are not seen (original magnification ×100). **b**. Photomicrograph of the same tissue shown in Fig. 1a, but stained with Gomori methenamine silver (GMS) stain. There are clusters of necrotic and viable fungal hyphae. This patient had chronic invasive fungal sinusitis (original magnification ×100)

involved. Further discussion regarding microbiology laboratory analysis is provided elsewhere in this text.

Certain fungi tend to be associated with particular manifestations of fungal sinus disease. Overall the most common fungi are of the genus *Aspergillus* [27]. Acute invasive fungal sinusitis is usually caused by the *Mucoraceae* or *Aspergillus* [6], but the *Aspergillus* species are more common [28]. Likewise chronic invasive fungal sinusitis is usually due to various *Aspergillus* species [4, 19, 20, 29]. Allergic fungal sinusitis was initially attributed to *Aspergillus* species, though it is now clear that other dematiaceous moulds also play an important role in this disease [30–32]. Fungal balls are almost exclusively due to *Aspergillus* and the largest series in the English language literature yielded only *A. fumigatus* [33]. Despite the fact that *Aspergillus* is the leading cause of fungal sinusitis, it should be pointed out that a variety of fungi have been described in these conditions.

3 Immunology and Host-Pathogen Interactions in Fungal Sinus Disease

The pathophysiologic mechanisms of fungal sinus disease are poorly understood. Most humans will suffer from viral or bacterial rhinosinusitis during their lifetimes. But only a small number of individuals suffer from fungal sinusitis. The inoculation of fungal spores into the nose is a daily event – large numbers of fungal spores are inhaled every day, suggesting that fungal sinusitis should be more common than is currently recognised. Using sensitive isolation techniques, multiple studies have shown that fungi are almost always present within the nose [34, 35]. In one study of normal volunteers, *Aspergillus* species were cultured from the nasal lavage samples of 65% of healthy noses [35]. The pathogenesis of fungal sinusitis must therefore involve rare events which are not completely understood.

Local factors are probably very important in the development of fungal sinusitis. Once fungi are inhaled into the nose or paranasal sinuses, they must attach and replicate to cause disease. The number and size of inhaled spores will largely determine the concentration and location of spores filtered out of inspired air. Also, anatomic factors such as septal deviation and enlarged or paradoxical turbinates may play a role. Normal mucociliary clearance is very effective at removing these potential pathogens from the mucosal surface, so general mucosal health and host immune factors are important to prevent disease. But mucociliary clearance can be disrupted by a variety of insults. Fungi may release mycotoxins including proteases and elastases which damage the epithelium and disrupt ciliary function. Also, fungal antigen exposure may trigger local inflammation with a similar effect on ciliary function and mucosal integrity. Finally, inflammation from allergic, bacterial, or viral rhinosinusitis may cause sinus ostial obstruction and trap fungi within the sinuses. Fungal spores that escape elimination from the nose or sinuses may germinate and persist within the lumen or go on to invade the mucosa.

Current evidence suggests that host immune factors determine the manifestations of fungal sinus disease. Fungi seem to elaborate various virulence factors,

but the importance of these factors is poorly understood. The fact that *Aspergillus* species can cause a variety of types of fungal sinusitis argues in favour of a host driven process. Additionally, it does not appear that any particular fungi carry a worse prognosis than any other. *Aspergillus* and the mucoracea cause most invasive fungal sinusitis, but not exclusively so, and there is no evidence that *Aspergillus* has a worse prognosis than the mucoracea and vice-versa. The fact that AFS tends to develop in atopic patients and acute invasive fungal sinusitis tends to develop in the immunocompromised, has led to the theory that fungal sinusitis is a spectrum of disease, the manifestations of which depend upon the host's response to the fungus [10].

4 Fungal Ball (Aspergilloma)

4.1 Introduction

The terms aspergilloma and mycetoma have been used to describe a fungal ball in the sinuses. A true mycetoma is a suppurative and granulomatous subcutaneous infection with draining sinus tracts. And the term "aspergilloma" has been used in the past to describe multiple manifestations of fungal sinusitis. For example, Hartwick and Batsakis [36] used the term "aspergilloma" to describe a non-invasive maxillary sinus fungal ball, but Milosev et al. [20] and Sandison et al. [19] used the term "aspergilloma" to describe chronic granulomatous invasive fungal sinusitis. Other terms have been used to describe sinus fungal balls. In the past, this condition may have been called simply "sinus aspergillosis" [37]. In this chapter, the terms fungal ball or aspergilloma will be used preferentially to describe a non-invasive mycelial mass in a paranasal sinus.

The diagnosis of sinus aspergilloma is usually stumbled upon when treating patients for chronic rhinosinusitis. Fungal balls of the paranasal sinuses usually develop in older (60+) patients and cause non-specific chronic rhinosinusitis symptoms such as nasal obstruction, facial pressure, and post-nasal drainage [33]. Some patients may be asymptomatic, and the diagnosis made only after imaging for some other condition [38]. Typically, patients will give a history of symptoms refractory to common medical treatments such as antibiotics, antihistamines, and nasal steroids. Other elements in the history are not particularly helpful in the diagnosis. There is no evidence of immunocompromise in these patients and no increased incidence of allergic rhinitis to explain the development of this condition. Physical examination may be helpful if findings point to involvement of a single sinus – up to 40% of patients have purulent discharge from the involved sinus and 10% have polyps [33]. The remainder of patients, though, have a generally normal physical examination. Fungal balls usually involve a single sinus. Single sinus involvement and absence of polyps in the majority of cases help to distinguish this condition from AFS. The great majority involve a solitary maxillary sinus or sphenoid sinus, however frontal and ethmoid fungal balls have been described and occasionally 2 contiguous sinuses will be involved. The diagnosis is only suggested by a history of

rhinosinusitis refractory to standard medical therapy and physical exam findings of sinusitis. Characteristic imaging findings and histopathologic examination confirm the diagnosis.

Very limited data is available regarding galactomannan testing in individuals with sinus fungal balls. In a study evaluating test performance in the crushed material removed from the sinus cavity from 23 immunocompetent individuals [39], galactomannan testing was associated with a sensitivity and specificity of 87 and 88%, respectively. In all cases in which the ELISA galactomannan assay showed negative results, fungi were not detected. Cross-reaction was suspected to have occurred in one patient with sinusitis caused by *Scedosporium apiospermum*. Histology compared favourably with antigen detection, while direct microscopic examination and culture were less sensitive. The cut-off used for galactomannan testing in this study was not specified.

4.2 Diagnostic Criteria

The diagnosis of a sinus aspergilloma may be suspected preoperatively based on the clinical features and distinctive radiologic findings, but the diagnosis of fungal ball is best made histologically. At operation, thick inspissated debris forms a mass which partially or completely fills the sinus cavity. The debris found in fungal balls microscopically consists of dense tangles of hyphae with calcifications and oxalate crystals [13]. These may form a centrifugal pattern of thin variably stained hyphae (Fig. 2). By definition there is no fungal tissue invasion and whilst acute or chronic inflammatory infiltrate may be present in the adjacent mucosa, granulomas typically are absent [13, 40]. The usual pathogen is one of the *Aspergillus* species. Despite

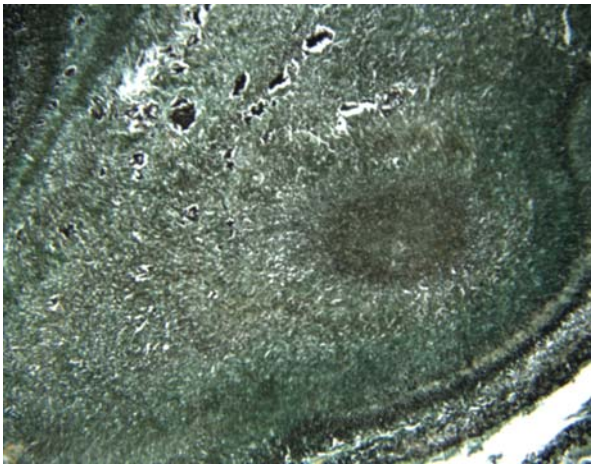

Fig. 2 Photomicrograph of a sinus fungal ball. Silver stain shows central core of variably stained hyphae with peripheral pallisading (original magnification ×100)

the large mass of fungal hyphae, fungal cultures are usually negative. In Klossek's series [33] only 30% of cases grew fungus and all of these were *A. fumigatus*.

4.3 Pathophysiology and Natural Course

For a sinus fungal ball to form, fungal spores or hyphae must become trapped within a sinus and conditions must support their growth. This may develop after some other insult (acute or chronic rhinitis or rhinosinusitis) disrupts normal mucociliary clearance or sinus outflow tract obstruction develops. Zinc may be a growth factor which facilitates the survival of fungi within the maxillary sinus, and in one study 50% of patients with a fungal ball had a history of prior endodontic treatment. It is possible that the zinc within root canal fillings may stimulate fungal growth within the maxillary sinus [33, 41]. In another study, 89% of patients with maxillary sinus fungal balls had a history of endodontic treatment vs. 37% in a matched control group [42]. Presumably, in patients with sinus fungal balls, host immunity and innate mucosal defenses are effective at retarding fungal invasion. Jiang and Hsu examined a series of patients with aspergillomas and found no evidence of humoral immunodeficiency [43]. Whilst immune function appears normal in these patients, there is not the brisk inflammatory response seen in allergic fungal sinusitis.

Aspergillomas follow a slow, benign course. The signs and symptoms of sinus aspergilloma are slow to appear and chronic in nature. Patients may have symptoms for months or years before reaching diagnosis [37]. Severe complications do not seem to develop in these patients, despite years of growth. Bone erosion has been demonstrated by computed tomography (CT) scanning, and conceivably the process could deform the sinonasal anatomy, precipitating secondary bacterial sinusitis or mucocele formation. Treatment of fungal balls is indicated for symptomatic relief, rather than the avoidance of future complications.

4.4 Radiologic Features

Sinus fungal balls have characteristic imaging findings. In over 90% of cases a single sinus is involved [33]. This can be demonstrated within the maxillary and sphenoid sinus using plain films, but CT scanning is a preferable modality to fully evaluate the sinuses for disease. Fungal balls characteristically cause sinus opacification. Centrally within the involved sinus are areas of hyperattenuation which correspond to fungal debris and calcifications (Fig. 3). There is usually minimal or no sinus expansion, but there may be thick osteitic bone reaction from the chronic disease process. Bone erosion is possible, but less common [37]. With magnetic resonance imaging (MRI) the dense sinus contents are hypointense on T1 and T2 weighted images and the mucosa may be hyperintense on T2 or contrasted images [44]. But performing MRI is not usually necessary, since the CT appearance of a single opacified sinus with a central area of high attenuation is highly suggestive of a fungal ball.

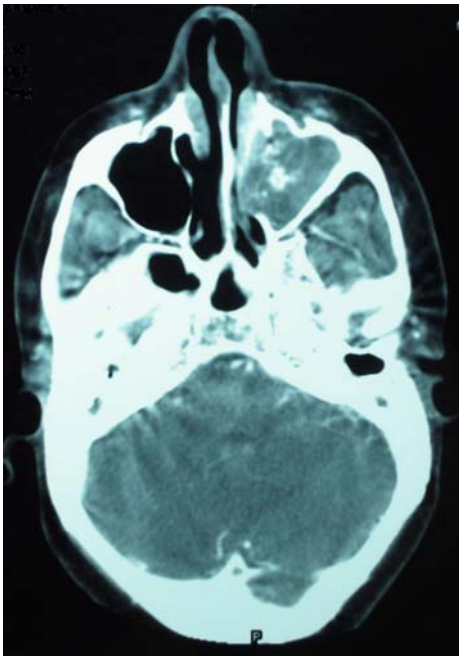

Fig. 3 Axial non-contrasted computed tomography scan in soft tissue windows. This image shows a fungal ball in the left maxillary sinus with complete sinus opacification and multiple central hyperdensities

4.5 Treatment

As a paranasal sinus aspergilloma is non-invasive and non-life-threatening condition, conservative treatment is appropriate. The primary goal of treatment is to remove the hyphal mass and a secondary goal is to establish lasting drainage from the involved sinus. For most cases of aspergilloma this can be accomplished with an endoscopic middle meatus antrostomy or sphenoidotomy. The thick tenacious fungal debris may be difficult to remove and require a combination of curreting and repeated irrigation. Post-operatively treatment with saline irrigations is appropriate until complete healing. No further treatment is required. The widely aerated sinus will quickly return to normal and recurrence is extremely uncommon. In Klossek's series [33], < 4% of patients developed recurrence after a mean follow-up of 29 months. Ferreiro et al. [13] reported a slightly higher rate approaching 7%. These lesions are easily cured with conservative endoscopic surgery, and recurrences are uncommon.

5 Allergic *Aspergillus* Sinusitis

5.1 Epidemiology

AFS is the most common form of fungal sinusitis, and *Aspergillus* species have been implicated since the description of the disease [3, 27, 36]. Anti-*Aspergillus*

IgE and positive cultures for *Aspergillus* species are commonly found in this condition [45, 46]. AFS accounts for about 4–7 % of cases of chronic rhinosinusitis taken to surgery in the USA [9, 12, 47–49]. AFS tends to develop in young adults, though children are also affected. The average age ranges from 23 to 26 [30, 31]. Some series have shown a slight male predominance [31]. About 60% of patients will give a history of allergic rhinitis, and of those who undergo allergy testing, 70–90% show evidence of atopy [30, 50]. Approximately 50% of patients have asthma. As the incidence of atopic disease increases, the incidence of AFS may also increase.

5.2 Clinical Features

The onset of AFS is difficult to pinpoint. Symptoms are slowly progressive. Patients with AFS present with a history of rhinosinusitis symptoms lasting months or years, with nasal congestion and obstruction, anosmia, facial pain or pressure, and postnasal drainage. Some patients will report blowing dark chunks from their nose. This thick mucus is the same "allergic mucin" that is found at surgery. Frequently patients have received prolonged medical therapy including repeated courses of antibiotics without improvement. They may have a history of multiple previous sinus procedures without lasting benefit, with the diagnosis previously overlooked. In rare cases patients may complain of diplopia or visual loss. Unfortunately, disease is usually well advanced before the disease is diagnosed.

The physical exam findings in AFS often reflect the advanced nature of disease at presentation. On physical examination up to 20% of patients will have proptosis or telecanthus due to sinus expansion and fungal mucocele formation [51, 52]. Intranasal examination will reveal polyposis that is unilateral in up to 50% of cases. The polyposis is often massive and presentation with polyps out the nares is not uncommon. Inspissated yellowish debris (allergic mucin) may be seen within the nasal cavities.

Laboratory testing may be important to establish evidence of atopy, but is not usually required for diagnosis. Possible laboratory abnormalities in AFS patients include peripheral eosinophilia and elevated total IgE levels. Total IgE can exceed 1000 IU/ml (normal values, < 180). Skin testing or in vitro testing will demonstrate IgE mediated hypersensitivity to multiple fungal and non-fungal antigens.

The use of newer diagnostic tools such as galactomannan and real-time polymerase chain reaction (PCR) testing has recently been evaluated in sinus mucus samples from 25 patients with chronic rhinosinusitis with nasal polyposis [53]. All fungal rhinosinusitis patients were negative in the galactomannan ELISA, though many false-positive reactions were observed with this test. PCR did not increase the accuracy of fungal rhinosinusitis diagnosis, but led to a faster detection in comparison to conventional methods such as direct microscopy, culture and histopathology. PCR has shown to increase considerably the rate of fungal detection in nasal lavage samples from patients with chronic sinusitis [54]. The issues of PCR testing in the diagnosis of aspergillosis are discussed elsewhere in this book.

5.3 Diagnostic Criteria

There is no universally recognised set of diagnostic criteria for AFS, though there is general agreement about what constitutes AFS. Perhaps the fundamental criterion is the presence of "allergic mucin". Grossly, this thick yellow, brown, or green debris fills the involved sinuses. Whilst grossly the "allergic mucin" found in allergic fungal sinusitis may appear similar to a fungal ball, microscopically the two are quite different. This material is identical to the mucus plugs in allergic bronchopulmonary aspergillosis. Within the "allergic mucin" of AFS, "onionskin laminations" or clusters of necrotic and degranulating eosinophils are prominent (Fig. 4). There may also be areas of scattered necrotic epithelial cells and polymorphonuclear cells. Small hexagonal and bipyramidal crystals can be found scattered within this mucin. These crystals are the result of eosinophil degranulation and consist of lysophospholipase. Fungal hyphae are present, but scarce, and special fungal stains may be needed for identification. Adjacent mucosa and polyps demonstrate a prominent eosinophilic inflammatory infiltrate. These fungal hyphae are present within the mucin but do not invade the mucosa. In some cases, granuloma formation or tissue invasion by fungal hyphae have been noted [55]. When invasion is detected in patients with AFS, there is a higher probability of complications such as orbital involvement ($p = 0.024$), and spread beyond paranasal sinuses (e.g., intraorbital or intracranial spread) ($p = 0.003$). These findings suggest that AFS may exist along a pathologic continuum with chronic invasive fungal sinusitis. The overlap of these conditions is a subject that has not been completely explored. The salient point is that histological features are critical to secure an accurate diagnosis.

The most commonly referenced diagnostic criteria for AFS were proposed by Bent and Kuhn [56]. These authors reviewed a series of 15 patients with AFS and

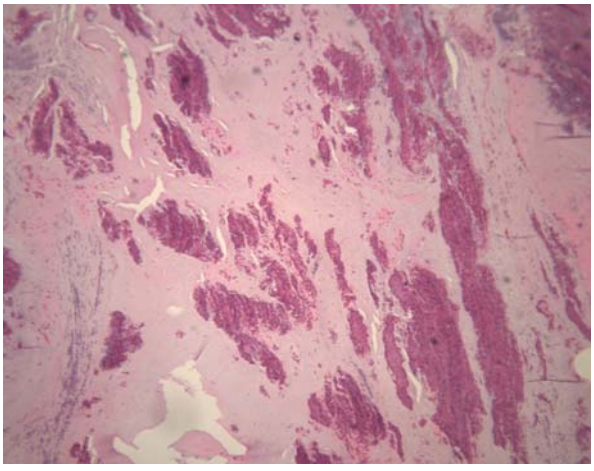

Fig. 4 Photomicrograph of allergic mucin with clusters of eosinophils in a mucinous background (original magnification ×40)

found common features amongst their patients. They proposed 5 criteria, present in all their cases, for the diagnosis of AFS: allergy (via history of inhalant mould allergies, elevated total IgE or positive skin test or RAST to fungal antigens), nasal polyps, characteristic CT findings, eosinophilic mucus, and a positive fungal smear but no evidence of tissue invasion on histopathology. These are the most widely accepted criteria for AFS, but others have developed their own diagnostic criteria. For example, deShazo and Swain [30] reviewed 7 cases of AFS and proposed the following criteria: eosinophilic mucin, positive fungal stain or culture, absence of immunodeficiency or diabetes mellitus, radiographic sinusitis and lack of fungal invasion. These criteria are very similar but have important differences. The Bent and Kuhn criteria [56] demand some demonstration of allergy, and the deShazo and Swain criteria [30] exclude AFS in diabetics. The inconsistencies and lack of both sensitivity and specificity of these criteria have stimulated attempts at a more exact diagnosis.

Ponikau et al. proposed a very different way of diagnosing AFS to deal with these problems [34]. From their review of the literature they concluded that the only unrefuted diagnostic criteria of AFS were chronic sinusitis, eosinophils and their byproducts, and the presence of fungal organisms within mucin by histology and/or culture. They used a sensitive technique for isolating fungi and examining nasal washings in a consecutive series of 210 patients with chronic rhinosinusitis. Of 101 patients taken to surgery, 94 met these criteria for AFS. The mucosal disease in these patients ranged from minimal polypoid changes to massive polyposis and no further description of the gross disease encountered at surgery is mentioned in their report. It is uncertain then, how many of these patients would have had the characteristic CT findings described by Bent and Kuhn [56]. Additionally, they found a much lower incidence of allergy by skin testing and lower IgE levels than that reported from other series of AFS patients. From this they concluded that type I hypersensitivity was probably not important in the pathophysiology of AFS. But it is likely that their inclusion criteria pool patients with a variety of disease states, not just classical AFS. An interesting finding from their work is that fungi could be cultured from 100% of their control patients' noses. The high prevalence of "AFS" in their surgical cases and finding that fungi are universally present in the nose suggest that the Ponikau criteria [34] are much too sensitive to be useful in distinguishing AFS from other forms of rhinosinusitis.

In contrast to Ponikau et al. [34] who suggest that AFS is grossly under-diagnosed, others worry that it is over-diagnosed. Allphin was the first to recognize that in some cases of presumed AFS, no fungi could be seen in fungal smears or isolated in culture [57]. Several authors reported similar cases of this "AFS-like syndrome" [49, 58] and this condition has been labelled eosinophilic mucin rhinosinusitis (EMRS) by Ferguson [59]. Ferguson suggests that EMRS develops from a systemic dysregulation of IgE mediated mechanisms, independent of any fungi. It appears that both fungi and "eosinophilic mucin" can be isolated from the noses of individuals with a variety of conditions. Their use as diagnostic criteria for a particular disease is therefore suspect. The controversies over diagnosis of AFS

suggest that current diagnostic criteria are inadequate and must be further refined with more knowledge about the pathophysiology of chronic sinonasal inflammatory disease.

5.4 Pathophysiology and Natural Course

An immune hypersensitivity to fungus is believed to underlie the pathogenesis of AFS. The nature of this hypersensitivity to fungus is today a matter of great debate. The predominant theory to explain the pathogenesis of AFS was adopted from the theory of ABPA pathogenesis and further developed and refined over a period of years. AFS develops in those patients with a convergence of local, environmental and genetic factors. Fungi enter the nose and sinuses and trigger a Gell and Coombs type I and III response. This inflammation produces "allergic mucin", stasis of secretions, and obstruction of sinus ostia. The trapped fungi continue to stimulate the adaptive immune system in a vicious cycle. Over time massive polyposis develops and fungal mucoceles distort the sinonasal anatomy [52]. There are many lines of support for this theory. AFS tends to be unilateral, suggesting an anatomic basis for its development. The disease also seems to more common in temperate climes where perennial exposure to fungi is a problem. Finally, most AFS patients are atopic by history or testing [31]. Manning and Holman showed powerful evidence for this theory in a series of *Bipolaris* AFS cases. They demonstrated elevated specific IgE and IgG by RAST and ELISA testing as well as positive immediate skin reactivity to *Bipolaris* extract in these patients [31]. Others have also shown elevations of specific IgE and IgG to lend support to the hypothesis that Gell and Coombs types I and III hypersensitivity are involved in the pathophysiology of the disease [48, 50, 60]. For example, McCann et al. showed that most patients with AFS have detectable *A. fumigatus* IgE and that serologic responses to recombinant *Aspergillus* allergens rASO\P f 2, rAs f4, and rAsp f6 are specific for the diagnosis of AFS [61]. They suggest that despite the fact that dematiacious fungi are commonly cultured from allergic mucin, serological evidence as well as histological examination of allergic mucin supports an important role for *Aspergillus* species in the pathogenesis of the disease [61]. The current working model of the pathogenesis of AFS explains many of the observations in patients with AFS, but awaits further confirmation.

The natural course of AFS is characterised by frequent recurrence. After AFS was distinguished as a clinicopathological entity, clinical experience soon revealed that recurrence of this disease following surgical treatment was extremely common. Kupferberg et al. [62] noted universal recurrence in patients treated surgically without vigorous post-operative medical treatment. They also noted that even with medical treatment recurrence was common. Reported recurrence rates ranged from 10 to 100%. Regardless of how they are treated patients are prone to recurrence for many years [52]. Marple et al. [63] published follow-up on 17 patients who were seen on average 82 months after their diagnosis. These patients had been treated with surgery (average 2), systemic or topical steroids, and immunotherapy. Twenty-four percent had polypoid mucosal changes on endoscopy at last follow-up, and the

average total IgE level in this group was 552. Despite this objective evidence of continued disease in some patients, 94% considered themselves free of the clinical manifestations of AFS. This study showed that years after initial treatment a significant number of patients still have objective evidence of inflammatory sinus disease. And this group of patients still required up to 3 courses of oral steroids per year [63]. The chronic and recurring nature of this condition warrants extended follow-up.

5.5 Radiologic Features

AFS produces characteristic features on CT and MRI. CT is the initial study of choice for evaluating these patients. It functions as an important roadmap should surgery be necessary, and the diagnosis may be strongly suggested by imaging findings alone. MRI is not usually required, but may be indicated with CNS or orbital complications. CT imaging reveals multiple opacified sinuses which may be unilateral or bilateral, but with a unilateral predominance. The sinuses are expanded, and bone erosion into the orbit, cranium, or soft tissues of the face may be noted (Fig. 5). Within the sinuses, focal areas of increased attenuation suggest the presence of fungal "allergic mucin." Usually these are irregular, speckled, or serpiginous and correspond to areas of thick "allergic mucin" noted surgically (Fig. 6). On MRI, these sinus contents have a low T2 and isointense or hypointense T1 signal. The peripheral mucosa is hyperintense on both T1 and T2 images consistent with inflamed mucosa. The characteristic CT and MRI findings for allergic mucin may be due to calcium, magnesium, or iron within the mucin or to high protein and low water content. Whilst definitive diagnosis requires histologic verification, the imaging findings facilitate pre-operative planning and patient counselling.

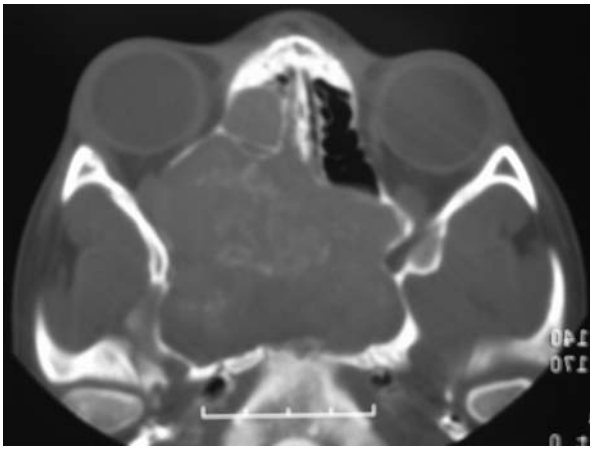

Fig. 5 Axial computed tomography scan of the sinuses in a patient with allergic fungal sinusitis. There is dramatic expansion of the sphenoid sinus and hyperdensities within the sinus contents

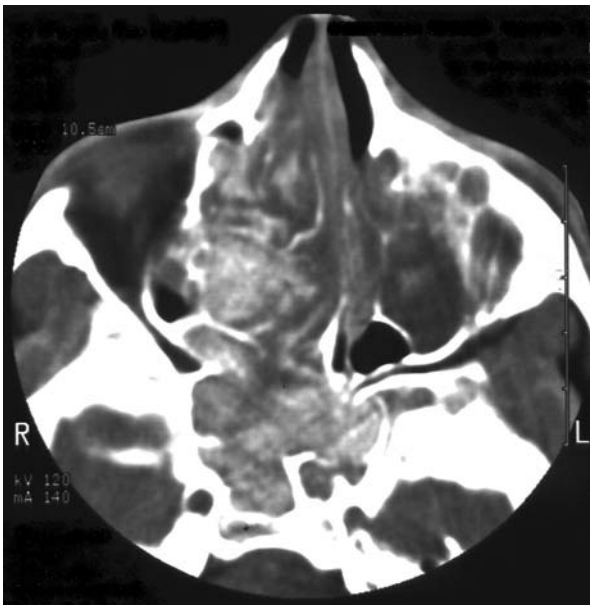

Fig. 6 Axial computed tomography scan of the sinuses in soft tissue windows. This shows multiple opacified sinuses bilaterally and hyperdense sinus contents within the right ethmoid and sphenoid sinuses

5.6 Treatment

5.6.1 Surgical Treatment

Surgery is required in almost all cases of AFS. The goals of surgery are to remove the inciting fungal mucin and to widely marsupialize the involved sinuses [52]. External surgeries (external frontoethmoidectomy, midfacial degloving, craniofacial resection, lateral rhinotomy, open antrostomies with mucosa excision) were replaced by more conservative procedures once the non-invasive nature of the disease was understood. Today, endoscopic surgery is widely employed with excellent results. An external antrostomy approach or frontal sinus trephination may be necessary to clear the thick tenacious mucin from the maxillary and frontal sinuses, however. The sinonasal expansion from massive polyposis and fungal mucoceles actually facilitates surgery by improving surgical access – the nasal cavity, middle meatus and frontal recess are frequently enlarged by the disease process. However, disease may distort the normal intranasal landmarks and erode the important bony barriers to the eye or brain [52]. The integrity of these structures should be carefully scrutinised on CT imaging prior to undertaking surgery. Surgery alone is not sufficient treatment for AFS, but is the crucial first step in management. Patients with a total peripheral eosinophil count of $\geq$ 520 cells/μl and those with asthma are more likely to experience recurrence of chronic rhinosinusitis within 5 years after surgery [64]. Long term follow-up is required for these patients.

For treatment of recurrence, surgery may not be necessary if the recurrence is recognised at an early stage. Surgical treatment for recurrences is indicated when intense medical management fails to clear an exacerbation. Allergic mucin may sometimes be suctioned in the office, and intense medical therapy can reduce polyp volume, but massive polyposis and outflow tract obstruction may not respond to medical management if there is a significant antigenic load within the sinuses. The goals of surgical treatment for recurrence is the same factors as for primary surgery-nasal polyps and other sinus obstructions should be removed and "allergic mucin" thoroughly extirpated from the sinonasal cavities.

Newer surgical technologies facilitate safe and complete surgery for AFS. Powered instrumentation may be very helpful to efficiently remove polyps whilst sparing sinus and nasal mucosa. The powered instrument may also serve as an effective suction to clear blood from the operative field and improve visualisation during surgery. The risks of surgery are increased in cases with dehiscence of the lamina papyracea or skull base. Computer image guidance may be helpful or even necessary in some circumstances where intranasal anatomy is significantly distorted. Complete surgery to remove allergic mucin is critical to reduce the risk of recurrence, and isolated cells high along the skull base or in the sphenoethmoid region may be missed by the surgeon disoriented without normal landmarks and concerned about violating dura or periorbita [52]. The surgeon should take advantage of these new technologies to facilitate surgery in these challenging cases.

5.6.2 Medical Treatment

Medical treatment for AFS usually begins immediately after surgery. The ultimate goal of medical therapy for AFS is to improve the patients' quality of life and prevent the need for further surgery. Medical therapy should be directed toward suppressing polypoid inflammation, preventing reaccumulation of the antigens, and maintaining sinus drainage pathways. Saline irrigations facilitate mucosal healing after surgery, clear crusts, wash away fungal antigens and improve mucus clearance. This treatment should be continued daily until complete mucosal healing and may be an effective long-term treatment to remove antigens from the mucosal surface and improve mucociliary clearance. Topical steroid sprays or aerosols are helpful in the post-operative patient to decrease local inflammation. Nasal steroids have a minimal side effect profile, and are effective at decreasing sinonasal inflammation or even shrinking nasal polyps. Nasal steroids should be continued indefinitely in an effort to suppress inflammation and decrease recurrence, Some authors have recommended that nasal steroid sprays be used at up to 3 times the usual dosage to boost their efficacy [47]. Local treatments are unfortunately often not sufficient to dampen the brisk inflammatory reaction of AFS and prevent recurrence, however.

Systemic anti-inflammatory agents are usually required in the treatment of AFS. Some surgeons will use a brief course of pre-operative systemic steroids to shrink polyps and decrease bleeding during surgery. Systemic steroids may be given intra-operatively and are begun in the immediate post-operative period. These are considered essential to extinguish mucosal inflammation and reduce the early recurrence

of disease. Leukotriene antagonists may be employed, but these have not been systematically studied in AFS. Unfortunately there is no regimen of systemic anti-inflammatory medication that has proven superior to another for improving patient outcome or reducing the need for revision surgery.

Prolonged courses of systemic steroids may delay or obviate any future surgery as long as they are taken. Kuhn and Javer [47] have described a treatment protocol which includes prolonged use of systemic steroids – after surgery patients take oral prednisone 0.4 mg/kg/day for 4 days, then 0.3 mg/kg/day for 4 days, followed by 0.2 mg/kg/day or 20 mg/day, whichever is greater, for up to 5 months, then decreasing to 0.1 mg/kg/day for 2 months. Repeated endoscopy is used to evaluate the mucosa and adjust steroid doses, and the goal is to have normal mucosa for 6 months before stopping prednisone. Some patients treated with this protocol were on prednisone for > 2 years. Prolonged systemic steroid treatment can induce disease remission as long as the steroids are used, and remission can be sustained after these steroids are stopped [47]. Different regimens may also be effective. In a retrospective study, Schubert and Goetz [65] showed that treatment with at least 2 months of oral steroids decreased the risk of recurrence requiring surgery. Prolonged treatment with systemic steroids may abrogate the vicious cycle of mucosal inflammation in AFS, but the ideal dosing and treatment course have yet to be defined.

Briefer courses of oral steroid treatment may be indicated. The prolonged use of oral steroids must be tempered by significant toxicities including hyperglycaemia, glaucoma, psychiatric disturbances, oedema, hypertension, osteoporosis, cataract formation, gastrointestinal ulcers, and aseptic necrosis of the hip. These risks must be balanced against the risk of recurrent disease requiring revision surgery. In paediatric patients, steroids should be tapered more rapidly, even if faced with the alternative of earlier revision surgery because of the significant growth retardation associated with prolonged treatment. In summary, the ideal length of treatment with oral steroids is unknown and independent clinical decisions must be made based on a patient's age, concomitant medical conditions, and response to treatment.

Antifungal therapy for AFS has been explored in an attempt to decrease the reliance on systemic steroids. However, antifungal therapy has not been widely adopted because of a lack of objective evidence that it adds benefit beyond that achieved with steroids, or that it decreases reliance on systemic steroids. Proponents of antifungal therapy quote a study by Denning et al. which showed that treatment of allergic bronchopulmonary aspergillosis (APBA) with itraconazole resulted in lower IgE levels and lower prednisone requirements [66]. Itraconazole in particular, may be a useful antifungal therapy for this condition because of its associated anti-inflammatory activity. However, there are significant differences between the two diseases. The most important is that the bulk of the fungal antigen load in AFS may be surgically removed. The fungi in AFS are in most instances not invasive and are present in scant numbers. The growth-retarding effect of antifungal agents may not be very helpful in a disease where the organisms are rare and difficult even to identify histologically. Antifungal drugs have many potential serious toxicities that limit their usefulness in non-invasive fungal sinusitis. Clearly, more investigation is appropriate to define the appropriate use of antifungal agents in AFS.

Should antifungal therapy be employed, topical delivery seems more promising because of the lower risk of systemic side effects and the benefit of delivering higher doses directly to the site of disease. Topical antifungal therapy was proposed as a useful adjunct to surgery by Bent and Kuhn [67], who performed in vitro susceptibility studies of fungal isolates from their AFS patients. They found that amphotericin B, itraconazole, and ketoconazole were most effective for the organisms they isolated, and they recommended a ketoconazole solution for topical therapy in the post-operative period. The results from this form of treatment have not been published. Agents like amphotericin B which have excellent activity against *Aspergillus* species (and is much less expensive than itraconazole) may be administered topically without the significant toxicities associated with systemic administration. However, these topical therapies also need further investigation to establish their efficacy.

Immunotherapy is another treatment modality that has been proposed to decrease the reliance on systemic steroids in the treatment of AFS. The rationale for immunotherapy presupposes that AFS is an IgE mediated process. Immunotherapy for AFS was once avoided because of reports that it exacerbated ABPA. The concern was that the generation of fungal-specific IgG antibodies would exacerbate the type III hypersensitivity component of the disease. Again, however, the two conditions are not identical, as surgery can remove the bulk of the antigenic load in patients with AFS. Concerns about immunotherapy were quelled by early reports of benefit. In 1994 Goldstein et al. [68] reported on 4 patients with AFS treated with immunotherapy, and Quinn et al. reported a compelling case where a patient requiring multiple surgical procedures and repeated courses of steroids attained clinical resolution after specific immunotherapy for *Bipolaris* species [69]. Immunotherapy for AFS starts about 1 month after surgery and dosing is increased to a maximally tolerated dose via weekly injections for a 3–5 year period. Mabry and co-workers [70] reported their experience with this protocol in 11 patients. They felt there was no adverse effect of immunotherapy and they reported decreased dependence on steroid treatment. Subsequently a formal comparison was published between these 11 patients and 11 controls who were treated similarly without immunotherapy [71]. After an average 33 months of follow-up, they showed that the immunotherapy group had better endoscopic mucosal appearance, had lower chronic sinusitis survey scores, required fewer courses of oral steroids (2 vs. 0), and showed less reliance on nasal steroids (73% vs. 27%). Whilst this was not a randomised double blind study, these results suggest an important role for immunotherapy in the management of cases of AFS.

In summary, commonly employed post-operative medical treatment for AFS includes nasal saline irrigations, a combination of topical and systemic steroids, immunotherapy directed toward all sensitive antigens, and systemic or topical antifungal agents. Specific leukotriene inhibitors may be used to control nasal polyposis, but they have not been systematically studied in AFS. Currently no treatment approach has proven superior to another so clinical decisions must be based upon the characteristics of the patient at hand and the individual clinician's experience.

5.7 Follow-Up

AFS is notorious in its propensity for recurrence. Recurrent disease may silently progress and allergic mucin may re-accumulate without causing pronounced symptoms. Patients may not seek medical attention until massive intranasal polyposis again creates significant nasal obstruction. If discovered at this point, revision surgery may be required. Kupferberg et al. [62] noted that patient symptoms do not correlate with extent of disease, and that endoscopic evidence of disease recurrence precedes a flare-up of patients' symptoms. They recommended following patients by endoscopy every 4–6 weeks and developed an endoscopic staging system to better track the progression of recurrent disease in their patients. This follow-up protocol was then refined to include monthly visits for 6 months after prednisone is stopped, then every 2 months for 2 years, then every 3 months for 3 more years. It is generally agreed that endoscopy is the best way to follow the activity of disease, but some have found IgE levels helpful in monitoring patients for recurrence [65].

Total serum IgE levels and nasal endoscopy are complementary tools in the regular follow-up that all patients should receive regardless of their symptoms. Marple et al. [63] showed disease quiescence in the majority of patients after several years of follow-up, but that some patients continue to develop exacerbations disease many years after their original diagnosis. This underscores that AFS is a chronic disease that must be managed for years.

6 Chronic Invasive Fungal Sinusitis

6.1 Diagnosis

CIFS is the manifestation of fungal sinusitis about which we know the least. Though cases have been rare, a typical clinical picture can be described. Usually CIFS develops in healthy adults, though some have diabetes mellitus. Most patients seem to be immunocompetent [72]. Washburn extensively tested his series of patients with CIFS and was unable to find any evidence of immunocompromise [15]. Large numbers of patients have not been subjected to immunologic evaluation, but most patients reported in the literature have been free of diagnosed immunodeficiency. Patients will usually present with rhinosinusitis symptoms, "allergies", or nasal polyposis that have been refractory to standard medical treatments [14]. These signs and symptoms may indicate pre-existing sinonasal disease that predisposes the patient to subsequent fungal sinusitis, or it may simply be the result of delayed diagnosis. The most common presenting feature is unilateral proptosis, but some patients will develop other ocular symptoms such as eye irritation, diplopia, or decreased visual acuity [11, 14]. Chronic headache has rarely been reported [73]. Cranial nerve deficits, cavernous sinus syndrome, orbital apex syndrome, seizures or mental status changes may develop before diagnosis [14]. The non-specific nature

of the initial symptoms probably explains why diagnosis is often delayed until extra-sinus manifestations of the disease are present. Physical exam findings suggest an ominous diagnosis. Physical examination frequently reveals cranial nerve deficits, palatal erosions, or an intranasal mass [74]. Like the patient's history, the endoscopic nasal examination may be non-specific. Severe congestion, an intranasal mass of variable colour, polypoid mucosa, or fungal debris are common findings. Taken together, the history and physical exam may suggest a wide variety of possible inflammatory and neoplastic conditions. Other differential diagnoses for this presentation include AFS, lymphoma, syphilis, tuberculosis, inflammatory pseudotumour, Wegener's granulomatosis, pituitary tumours, or a benign or malignant neoplasm. Frequently this disease is mistaken for a sinonasal tumour [75]. The clinical presentation of CIFS is non-specific, and the diagnosis may only be revealed after a surgical biopsy is obtained.

6.2 Diagnostic Criteria

The diagnosis of CIFS is made by a combination of the clinical presentation and histopathologic examination. The diagnosis of CIFS is made in patients with a prolonged clinical course (symptoms > 4 weeks at presentation), radiologic evidence of sinusitis, and the presence of hyphal forms within tissue [10, 14]. In CIFS fungal hyphae invade tissue, but vascular invasion is rarely, if ever, present. There is usually a prominent inflammatory response with periarterial inflammation and occasionally a mature plasmacytic infiltrate [40]. A foreign body reaction may be seen with Langerhan's type giant cells. Necrosis may be present but is not as prominent as in acute invasive fungal sinusitis. Veress et al. [11] described proliferative, exudative, necrotising and mixed variants and deShazo made the distinction between granulomatous and non-granulomatous forms [76]. In deShazo's series, non-granulomatous CIFS developed in patients with diabetes mellitus, and these patients had a worse prognosis than those with granulomatous inflammation. The clinical significance of the histologic diversity of CIFS cases is unknown, because published series are too small to yield generalisable results. The important diagnostic features are tissue invasive fungus with an indolent clinical course.

6.3 Pathophysiology and Natural Course

The basis for fungal invasion and incomplete host resistance in CIFS disease is incompletely understood. Most cases of CIFS are due to *Aspergillus* species (though a wide variety of fungi have been reported in this disease) [14]. A possible explanation for the pathogenesis of CIFS is that pre-existing sinus disease renders the patient susceptible to fungal invasion through altered innate immunity. Most cases of CIFS have been reported from arid regions such as the Sudan, and local climactic factors

(causing mucosal disease which leads to fungus trapping) may be extremely important [19]. Also, certain fungal species, like *A. fumigatus* make toxins which inhibit macrophage phagocytosis and complement activation. These toxins may modulate the host's immune response and explain some breaches of host defense in seemingly immunocompetent individuals. Alternatively, it is possible these patients have subtle immune deficits which are not currently detectable. Most likely, a combination of local tissue factors, host systemic factors, and fungal virulence factors interact in the pathogenesis of CIFS.

In contrast to AIFS, patients with CIFS will not usually die for lack of acute treatment, and the disease does not require emergent treatment. CIFS appears to develop over a period of many months to years. But the disease can be fatal, and there is currently no reliable means to assess prognosis. deShazo et al. [76] divided CIFS into granulomatous and non-granulomatous forms, and based on his small series concluded that non-granulomatous cases in patients with diabetes mellitus have a worse prognosis and should be treated more aggressively than non-diabetics. Others refute this [14]. There is certainly a diversity of clinical behaviour in CIFS. Some patients eventually die despite aggressive treatment, whilst others are easily cured. But because of the rarity of this disease, definite prognostic factors have not been identified. To further complicate matters, the clinical distinction between acute and chronic invasive fungal disease becomes murky in patients who are undergoing intermittent immunosuppressive treatments (e.g. chemotherapy cycles). Whilst immunocompromised, the invasive fungal disease may progress in a rapid and dramatic fashion only to become quiescent with the return of normal immune function [10]. The more protracted clinical course of these CIFS cases in immunocompetent individuals facilitates the use of empiric, conservative therapies that can be adjusted based on clinical response.

6.4 Radiologic Features

Just as the clinical presentation of CIFS may not suggest the correct diagnosis, imaging findings in CIFS may also be non-specific. CT is the best initial evaluation of patients with CIFS. A scan obtained for chronic rhinosinusitis may show other concerning findings in CIFS. An intranasal soft tissue mass is frequently noted and may be mistaken for a tumour. Maxillary teeth involved in the process may become displaced and appear as "floating teeth" [18]. These findings raise the spectre of a malignancy. The CT scan may also show areas of mucosal thickening, sinus opacification with areas of hyperattenuation, bone erosion, or sinus expansion (Fig. 7). These findings may suggest fungal sinusitis (particularly AFS), but cannot distinguish an invasive process. MRI scanning is useful in cases with suspected intracranial extension, and to assess for dural involvement, or intracranial extent [15]. The addition of MRI may also help refine an extremely broad radiologic differential diagnosis that includes a variety of inflammatory and neoplastic processes. This ultimate distinction between CIFS and other disease processes is made histologically [75].

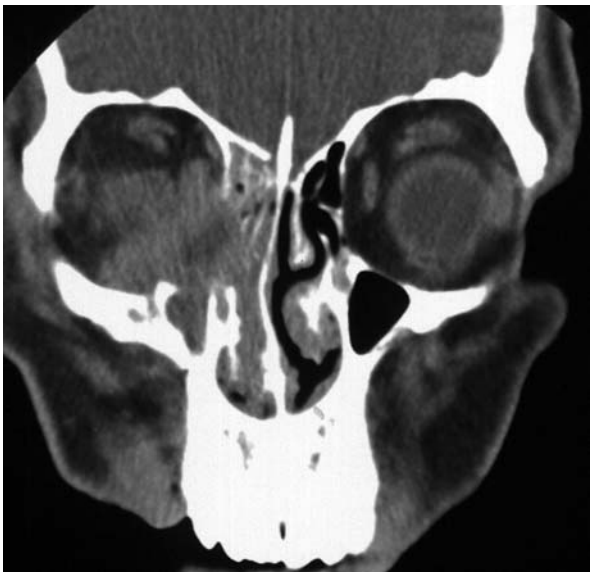

Fig. 7 Coronal computed tomography scan of the sinuses that shows a soft tissue mass in the right ethmoid region with erosion of the lamina papyracea and intra-orbital extension. This patient had chronic invasive fungal sinusitis

6.5 Treatment

6.5.1 Surgical Treatment

There have been no systematic evaluations of therapy for CIFS. Because of the rarity of the condition, the results of small case series and the extrapolation of data from AIFS are relied upon to make management decisions. Most cases have been treated with a combination of surgery and antifungal agents. Unfortunately, treatment recommendations made based on the experience of case reports and small series is not helpful in defining ideal treatment. There seems to be general agreement that surgery is an important part of the treatment of CIFS.

The first goal of surgery for suspected CIFS is to secure a diagnosis. As mentioned, the differential diagnosis generated by a patient with CIFS is extremely broad. At endoscopy congested nasal mucosa, polyps, soft tissue masses, and areas of ulceration, purulence, or fungal debris may be noted. Surgical endoscopy permits a thorough intranasal examination, and biopsy alone may be the initial procedure. Cultures of tissue and exudate should also be obtained to facilitate antifungal therapy, should it be warranted. The diagnosis is ultimately made histologically.

There is no general agreement regarding the extent of surgery required for CIFS. Options described in the literature range from endoscopic biopsy to radical exenteration [14, 19, 72, 77]. It is also uncertain if the granulomatous form should be treated differently from the granulomatous form. deShazo et al. [76] have recommended

more aggressive surgery, akin to that for AIFS, for the non-granulomatous form of CIFS after their experience with 2 fatal cases. On the opposite end of the spectrum, Stringer and Ryan [14] reported a case with orbital invasion, visual loss, telecanthus, and diplopia treated only with biopsy and prolonged medical therapy with amphotericin B and itraconazole. After a period of months, the orbital and visual symptoms completely resolved, and CT showed resolution of intra-orbital disease. Sandison et al. [19] likewise reported cures of cases from the Sudan with only incomplete resection. It seems rational to surgically remove all grossly involved tissue, if possible, without violating protective tissue planes such as the periorbita and dura that may act as barriers to further spread of disease, and without sacrificing important structures. Widely opening the sinuses and removing polyps, other soft tissue inflammatory masses (granulomas) and fungal debris or mucin will permit post-operative endoscopic follow-up. Radical surgery should be reserved for the most aggressive cases. Given the chronic nature of this disease, the ability to follow it clinically and radiologically, and the availability of specific antifungal medical therapy, initial conservative surgical treatment is appropriate.

6.5.2 Medical Therapy

The extent and necessity of medical therapy after surgery for CIFS is uncertain. Antifungal therapy is intended to clear any residual invasive fungus and to decrease recurrences. However, it is unclear which patients will recur or whose disease will ultimately be fatal. Most patients with CIFS are treated with a combination of surgery and post-operative antifungal therapy [14, 17, 76]. Sandison et al. [19] reported a few cases that were apparently cured with surgery alone, though follow-up was short. deShazo et al. [76] quoting Milosev have stated that granulomatous CIFS responds well to surgery alone without antifungal treatment. In Milosev's series of 17 patients from the Sudan [20], conservative surgery to widely aerate the sinuses was performed without an attempt to completely remove the "*Aspergillus* granuloma". The authors state that "treatment is essentially surgical," though they did not report their length of follow-up or rates of recurrence [20]. Considering that some cases will recur after surgery, and that the clinical course of an individual patient is difficult to predict, most recent authors have stated that a post-operative course of antifungal therapy seems appropriate.

Diverse treatment recommendations have been made in the literature regarding antifungal therapy. Most cases of CIFS are due to the *Aspergillus* spp. and appropriate empiric or culture directed antifungal therapy should be employed. In general, antifungal medical therapy for CIFS should be continued until all objective signs of disease resolve. Washburn [15] and Zieske [77] both recommended treatment with amphotericin B up to a dose of 2 g. Following the amphotericin B course Washburn recommended up to a year with an oral antifungal agent such as ketoconazole. Zieske [77] made similar recommendations for treatment with itraconazole. In the best study of medical treatment for CIFS to date, Gumaa et al. [17] reported a treatment protocol for treatment of "paranasal *Aspergillus* granuloma" using post-operative itraconazole. Patients were treated with itraconazole 100 mg

twice daily for 6 weeks, and then re-evaluated clinically and serologically. The itraconazole dose was then modified up or down based on clinical and serological criteria (measuring precipitins to *A. flavus*). Itraconazole was stopped when patients were deemed to be in complete remission. After an average of 17 months follow-up, 63 % were in complete remission, 10% had suffered transient serologic relapses, and only one had no improvement, with increasing pain and proptosis despite itraconazole 300 mg daily [17]. This available evidence suggests that antifungal therapy for CIFS should be tailored to the patient's extent of disease, response to therapy, and tolerance of antifungal agents. Amphotericin B nasal lavage does not seem to offer any benefit [78].

6.6 Follow-Up

As previously emphasised, the prognosis of CIFS is uncertain. Regular follow-up therefore seems prudent so that further surgery or adjustments in medical therapy can be carried out. Ideally one would identify and treat recurrent disease before it involves contiguous structures like the brain and eye, or radical surgery is required. Repeated endoscopy at intervals may detect intranasal or intrasinus disease before symptoms develop or spread occurs. CT scanning is recommended 1 month after surgery for a baseline and at regular intervals to follow the regression/progression of disease [14, 72, 77]. Regular examination and imaging at intervals is an important component of management of CIFS that should not be overlooked.

7 Acute Invasive Fungal Sinusitis

7.1 Introduction

AIFS is a medical emergency. Once it is considered a possibility, the diagnosis should be thoroughly sought until it can be confidently ruled out. The challenge for the clinician is to catch the disease early before extension into the cranial cavity or other extra-sinus tissues. The clinical presentation of AIFS is sufficiently different from the other forms of fungal sinusitis that they aren't likely to be confused. The real danger is in confusing AIFS with bacterial or other non-life-threatening forms of rhinosinusitis. Patients who develop AIFS are usually, but not always, immunocompromised [79]. The diagnosis of AIFS must be considered in any immunocompromised patient with rhinosinusitis symptoms. The most important risk factor for developing acute invasive *Aspergillus* sinusitis is prolonged neutropenia. Other risk factors include prolonged treatment with systemic steroids or broad-spectrum antibiotics, transplant immunosuppression, and AIDS. Patients with AIFS develop acute symptoms that initially seem benign – such as clear rhinorrhoea or nasal congestion. The symptoms may initially be attributed to viral or bacterial rhinosinusitis and only later recognised as the manifestations of AIFS when symptoms

worsen in the face of "appropriate" medical therapy. As the disease progresses, fever, headache, facial pain and swelling are rapidly followed by decreased visual acuity, other cranial nerve deficits, and facial or palatal necrosis. Extension into the cranial vault may cause seizures, altered mental status and then death in a matter of hours to days. Unfortunately, AIFS may not be recognised until the diagnosis is all but confirmed.

Physical exam, especially nasal endoscopy, is extremely helpful for making the diagnosis in the early stages of disease. A complete head and neck exam including the cranial nerves and visual acuity should be completed. If nasal endoscopy is not performed, facial, ocular, and cranial nerve abnormalities may be the first indication that the patient suffers from more than "sinus". The middle turbinate is the most commonly involved intranasal site of disease [80], but the entire mucosa of the nasal cavity should be inspected. Nasal endoscopy will reveal areas of pallor and non-bleeding, insensate mucosa. As the disease progresses and mycotic thrombosis leads to ischemic necrosis, the nasal tissues will take on a grey to black appearance with areas of ulceration. The hard palate may become discoloured and ulcerated as well. In indeterminate cases, nasal endoscopy should be repeated at frequent intervals.

7.2 Diagnostic Criteria

The diagnosis of AIFS can be made in a patient with acute rhinosinusitis symptoms and histologic identification of fungal hyphae within tissue. As previously mentioned, the middle turbinate is the site most likely to appear abnormal on endoscopy and the most likely to yield a positive fungal biopsy. For this reason, the middle turbinate should be biopsied in all patients suspected of AIFS as tissue invasion and symptoms will precede the appearance of visible anomalies. Any other area of abnormal mucosa should be similarly biopsied. Acute invasive fungal sinusitis is characterised by invasion of fungal hyphae into the soft tissues with a variable amount of reactive inflammation depending upon the patient's underlying disease state. Perineural or vascular invasion may be seen and this leads to areas of anesthesia, mycotic thrombosis, and ischemic coagulative necrosis of tissue [40]. The histologic diagnosis must be sought emergently and may require multiple biopsies and special fungal stains.

Although galactomannan testing has been widely used as an indirect evidence of *Aspergillus* infection [81], experience with galactomannan testing in patients with sinusitis is very limited [82]. Galactomannan has also been tested in the nasal lavage fluid [83], although this approach has not yet been standardised.

7.3 Pathophysiology and Natural Course

The clinical risk factors for the development of AIFS are well defined. Neutropenia is the single most important permissive immune defect in most cases of acute

invasive fungal sinusitis [84]. This develops as a result of haematological malignancy, chemotherapy, HSCT and end-stage AIDS. Diabetic ketoacidosis has long been recognised as a risk factor for acute invasive fungal sinusitis caused by the mucoraceae. The hyperglycaemia and acidosis create a favourable environment for the proliferation of these fungi [10, 85] and cause qualitative neutrophil defects (impaired phagocytosis, reactive oxygen species generation). Together these put patients with diabetic ketoacidosis at significant risk for acute invasive fungal sinusitis (Interestingly, granulocyte colony stimulating factor [G-CSF] which is sometimes used in AIFS, increases neutrophil superoxide production). Other risk factors for AIFS include prolonged systemic steroid treatment, prolonged use of broad-spectrum antibacterial antibiotics, and iron chelation therapy [59]. There is some evidence from animal studies that prior viral infection within the nose enhances airway susceptibility to invasive *Aspergillus* sinusitis [86]. And an antecedent upper respiratory infection may cause perturbations of mucosal immunity that facilitate the development of invasive fungal disease [24].

Basic research into the defense mechanisms against invasive *Aspergillus* infection has shown that innate immunity is crucial to defend against invasive fungal disease. In *Aspergillus* infection, macrophages are the first line of defense, and are responsible for phagocytosis of inhaled conidia. Hyphae are targeted by granulocytes (e.g. neutrophils) which degranulate around the fungus, killing it by a myeloperoxidase-dependent mechanism (through generation of reactive oxygen species). This explains the clinical observation that systemic steroids, which impair macrophage function, and inhibit reactive oxygen species production, increase the risk of fungal invasion and why profound or prolonged neutropenia is a significant risk factor for the development of invasive fungal sinusitis [87]. The role of T cells and humoral immunity in resisting fungal tissue invasion is uncertain. Significant T cell depletion in AIDS is rarely associated with invasive fungal sinusitis, though patients with AIDS frequently develop other fungal infections. Cell-mediated immune defects typified by HIV infection may be important in some cases of AIFS, but these become more important with concomitant neutropenia [88]. Adaptive immune responses of any type appear less important than innate defenses.

7.4 Natural Course

Acute invasive fungal sinusitis is defined by its clinical course. A critical distinction between AIFS and the other forms of fungal sinusitis is that the patient with AIFS will die from their disease (in a matter of days) without appropriate treatment. This aggressive course necessitates a high index of suspicion for the disease, rapid pursuit of the diagnosis, and emergent administration of appropriate treatment. Unlike the other forms of fungal sinusitis, AIFS must be treated emergently. Even with appropriate treatment, mortality is significant and ranges from 50 to 90% [22, 89].

The most important factor in determining prognosis is the underlying disease state, though other prognostic factors have also been identified. Patients with diabetic ketoacidosis, (and its associated transient immunocompromise from neutrophil

dysfunction) have the best prognosis whilst patients with a failed HSCT or those who are undergoing chemotherapy for leukaemia relapse seem to have the worst prognosis [23, 89–91]. It is rational to suppose that early diagnosis and intervention improve prognosis, but limited disease at diagnosis may just be a marker of less aggressive disease. The most important prognostic factor is the recovery of normal immune function-without it, most patients will succumb to disease.

7.5 Radiologic Features

Radiologic imaging in AIFS is primarily useful to confirm the presence of rhinosinusitis. Computed tomography scanning is the most appropriate initial imaging study. The radiologic findings in AIFS may initially be non-specific or even normal. Benign appearing patches of mucosal thickening or air/fluid levels may be seen. Delgaudio et al. [92] found that severe unilateral nasal soft tissue swelling was the most common finding in a series of AIFS cases. Bone erosion or soft tissue infiltration in immunocompromised patients usually signal an invasive, aggressive process but are not noted until late in the course of the disease. Abnormalities also frequently involve the orbit or cranial cavities by the time images are obtained. If intracranial involvement is suspected (because of events such as stroke, seizure, and diplopia) an MRI may be helpful in ascertaining the extent of disease. Imaging findings may not correlate with endoscopic or pathologic findings in this disease [93] because soft tissue oedema and retained secretions do not reliably localize areas of fungal tissue invasion. For similar reasons, CT may not be particularly useful for following patients' disease progression (endoscopy is also probably better for this). Therefore CT is best used as an indicator of rhinosinusitis and a roadmap for surgery.

7.6 Treatment

The most effective treatment of AIFS is prevention. This is accomplished by limiting exposure to inhaled fungi. For patients undergoing immunosuppression a high efficiency particulate air (HEPA) filtered laminar flow room is the best way to reduce exposure to fungal spores. Plants and soil should be kept away from units housing immunocompromised patients, and dust from hospital remodelling must be sealed out of patient care areas. There have been reports of invasive fungal disease outbreaks in facilities undergoing remodelling. Efforts at elimination or filtration of fungal spores from inhaled air decrease the incidence of AIFS in patients with known immunocompromise.

The ideal treatment for AIFS is unclear. Newer surgical techniques like endoscopic surgery and newer medications like voriconazole, the lipid formulations of amphotericin B, and posaconazole have opened up new avenues of treatment that do not have extensive support in the literature. The relative rarity of the disease and the absence of comparative treatment trials also hamper decision-making. Nevertheless,

general recommendations can be made that currently reflect our understanding of the most helpful therapies.

7.6.1 Medical Therapy

Medical management of AIFS is directed toward reversing any immunocompromise and retarding the growth of invasive fungus. The most important medical intervention for AIFS is the rapid reversal (if possible) of the underlying responsible disease state. For patients undergoing cytotoxic chemotherapy, this may require adjustments in the treatment schedule, administration of G-CSF or even granulocyte transfusions [80, 91]. Some patients, such as those with failed HSCT or end-stage AIDS, may have irreversible disease. That changes their prognosis and should be considered in making decisions about surgical therapy.

The second most important aspect of medical management in AIFS is the use of specific antifungal therapy. Amphotericin B has been the mainstay for years and is usually used as IV monotherapy. The lipid formulations of amphotericin B, whilst costly, may allow higher dosing with decreased toxicity [94]. There are limited data supporting the use of echinocandins in *Aspergillus* sinusitis [95, 96], which is also true for voriconazole [97]. In the pivotal study by Herbrecht et al. *Aspergillus* sinusitis occurred for only 5.6 and 5.3% of patients in the voriconazole and amphotericin B arms, respectively [98]. Combination therapy with amphotericin and one of the newer azoles may be employed, and a benefit to combined treatment is suggested by isolated case reports [99]. Amphotericin may be used as a topical agent to soak packing materials post-operatively, or as a nasal spray [100, 101]. Topical amphotericin B seems to be well tolerated, although a decrease in ciliary beat frequency has been documented when this drug is diluted in distilled water [102]. Moreover, the benefit of this approach has been questioned, particularly nowadays that modern and less toxic antifungal compounds are available to be used systemically. Kavanagh [90] recommends that a neutropenic patient with facial pain or other rhinosinusitis symptoms and radiographic evidence of sinusitis should be immediately started on antifungal and antibacterial antibiotics, even in the absence of fever. The prophylactic use of antifungal agents is discussed elsewhere in this book. Patients with a history of invasive fungal sinusitis during a previous bout of immunosuppression have a high rate of recurrence that warrants prophylactic systemic administration of antifungal agents whenever they experience neutropenia or repeated immunosuppression [103]. Some may administer topical amphotericin B for treatment or prophylaxis in patients with immunosuppression, but the efficacy of this measure is uncertain.

An unresolved question about antifungal therapy is the ideal duration of treatment to control or eliminate the disease whilst avoiding or minimising toxicities. In most situations the desperate nature of the case dictates dosing and treatment duration, but once immunocompetence is restored the disease may take on behaviour similar to chronic invasive fungal sinusitis. A good general rule is to follow the patient's symptoms and endoscopic (or perhaps imaging) evidence of disease. Once objective and subjective disease is resolved, the medications may be stopped with

the expectation that they will be restarted should any evidence of disease reactivation present itself.

7.6.2 Surgical Treatment

The importance of surgery for AIFS has long been assumed [28]. The benefits of surgery include the acquisition of a specimen for biopsy and culture to secure the diagnosis (and direct appropriate medical treatment). Surgery may also decrease the local fungal burden in the tissues and thus augment the normal immune mechanisms for elimination of fungal organisms. A recent study in neutropenic patients showed that sinus surgery combined to antifungal therapy significantly improved the overall response to treatment ($p = 0.06$), in comparison to medical treatment only [104]. Early aggressive surgery may slow disease progression and allow time for bone marrow recovery. Widely opening the sinuses also facilitates the application of packings soaked in amphotericin B, or the placement of catheters for irrigation of antifungal solutions. A drawback to the surgical treatment of AIFS is the very real risk of haemorrhagic complications [84]. Neutropenic patients are usually anaemic and thrombocytopenic. Liberal use of blood products may be necessary to prevent exsanguination, and platelet transfusions may be required. Surgery also creates mucosal defects, which expose tissues to further fungal invasion. These issues must be considered when planning surgery for AIFS.

Available evidence suggests that patients treated with surgery have a better prognosis than those treated with medical therapy alone, though there are cases can be cured with medical therapy alone [89, 105]. The types of operations have changed over the years, but the general principle remains: surgery should remove all tissue grossly involved with invasive fungus. This may be accomplished by open or endoscopic techniques to debride back to healthy bleeding tissue. The endoscopic approach has been gaining credibility as an effective technique which minimizes the morbidity of surgery, yet frequently allows a complete operation. The external approach may be required, however, if there is extensive disease of the lateral nasal wall, or evidence of orbital, facial, or intracranial extension. These external procedures include medial maxillectomy, total maxillectomy with or without orbital exenteration, or craniofacial resection. Orbital contents should be saved, if at all possible, though the principle of complete resection should prevail. In patients with haematological malignancy or HSCT failures where recovery of neutrophil function is doubtful, even radical surgery may be useless. The extent of surgery undertaken should be modified by due consideration of the overall prognosis. Radical surgery in the face of unremitting immunocompromise may simply be “mutilating surgery”.

Surgery for AIFS should be undertaken on an emergency basis. Definitive surgery should be undertaken as soon as feasible once the diagnosis is confirmed. The patient, family, and consulting physicians should together agree with the operating surgeon on the extent of surgery to be undertaken once the diagnosis is established (recognising that intra-operative findings may reveal the extent of surgery necessary). The consulting physicians should provide input into the expectations for reversal of immunocompromise and prognosis for the underlying disease. The

patient must be presented with the options of aggressive or conservative surgical procedures given the available information about their overall prognosis. It bears repeating that aggressive surgery in the face of irreversible immunocompromise is probably futile.

7.7 Follow-Up

Follow-up evaluation of patients after successful treatment of AIFS should ensure that the disease process is arrested and monitor for any signs of recurrence. Once immunocompromise is reversed, the fungal sinusitis may resolve completely or take on attributes of CIFS with a chronic, indolent course. Regular, periodic examination should be performed and any suspicious areas biopsied, even in the absence of subjective symptoms. The patient should also be counselled to seek care for any symptoms such as facial pain, rhinorrhoea, or congestion, which may signal a recurrence. Return of any signs of disease should prompt consideration of re-starting antifungal therapy. Once a patient has experienced one episode of invasive fungal sinusitis, they are at risk of recurrence with any immunosuppression, and should be treated prophylactically and monitored accordingly.

8 Summary

Aspergillus sinusitis can be divided into distinct subtypes that are determined by the host's response to the fungus. Most cases of fungal sinusitis are due to *Aspergillus* species. The types of fungal sinusitis can be distinguished by their different clinical and radiologic presentations. Acute invasive fungal sinusitis is an aggressive, life-threatening condition that requires emergency treatment. The other forms of fungal sinusitis develop slowly, and patients present after failure of standard treatments for rhinosinusitis. Unlike acute invasive disease, these do not usually require emergency treatment. Confirmation of the diagnosis of fungal sinusitis requires histopathologic examination. In an aspergilloma (fungal ball), dense tangles of non-invasive hyphae are seen. Allergic fungal sinusitis is characterised by "allergic mucin": thick debris that microscopically consists of sheets and clumps of eosinophils with scant, non-invasive fungal hyphae.

In invasive fungal sinusitis, the fungal hyphae can be demonstrated within tissue. The degree of associated inflammation is determined by the immune competency of the host. Allergic fungal sinusitis tends to develop in patients with atopy and asthma. Acute invasive disease develops in patients immunocompromised by haematological malignancy, chemotherapy, HSCT or other medications like steroids.

Treatment for fungal sinusitis is both medical and surgical. Most surgery can now be performed endoscopically. Surgery for AFS is followed by topical and systemic steroids to suppress polypoid inflammation. Fungal ball (aspergilloma) can be cured with surgery and minimal post-operative therapy. Chronic invasive fun-

gal sinusitis is treated with conservative surgery and antifungal therapy. Surgery for acute invasive fungal sinusitis aims to remove all tissue grossly involved with fungus. Medical treatment consists of reversing immunocompromise if possible and aggressive antifungal therapy.

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PART VI
OVERLAP SYNDROMES

Aspergillus Overlap Syndromes

Ayman O. Soubani

Abstract *Aspergillus* causes a variety of clinical syndromes that are influenced by the patients' immune status and lung function and structure. Whilst these syndromes (such as chronic cavitary pulmonary aspergillosis, *Aspergillus* sinusitis, allergic bronchopulmonary aspergillosis – ABPA – and invasive aspergillosis) tend to have their unique clinical, serological, and radiological presentations, there are rare situations where these may overlap, co-exist or even progress from one entity to another. There are limited reports of patients with ABPA who developed cavities or fungal balls, or progressed to invasive aspergillosis. Also some patients may have ABPA and allergic *Aspergillus* sinusitis. *Aspergillus* species may also play a role in the pathogenesis of other conditions such as hypersensitivity pneumonitis, mucoid impaction in bronchi, and bronchocentric granulomatosis. These conditions have similar features to the classic *Aspergillus*-related syndromes. The *Aspergillus* overlap syndromes may be coincidental, as a result of specific host and fungal factors, or reflect the possibility that the *Aspergillus*-related syndromes represent a spectrum of the same disease rather than separate entities. Knowledge about the *Aspergillus* overlap syndromes may decrease the need for extensive investigations to exclude other conditions, which may have an impact on the management and follow up of patients with *Aspergillus*-related lung diseases. In this chapter the different *Aspergillus* related overlap scenarios reported in the literature are described, emphasising the clinical features and outcome of these patients.

Keywords ABPA · Aspergillosis · CCPA · Invasive aspergillosis · Sinusitis

A.O. Soubani (✉)
Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Wayne State University School of Medicine, Detroit, MI, USA
e-mail: asoubani@med.wayne.edu

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Contents

1 Introduction

Aspergillus species, mainly *A. fumigatus,* cause a variety of clinical syndromes that range from invasive pulmonary aspergillosis (IPA) – a severe disease and a major cause of mortality in severely immunocompromised patients – to chronic cavitary pulmonary aspergillosis (CCPA), typically a non- invasive disease that is seen mainly in patients with chronic lung diseases. CCPA (sometimes in association with fungal balls or aspergillomas) and allergic bronchopulmonary aspergillosis (ABPA) are two main non-invasive pulmonary diseases caused by *Aspergillus*. Similar conditions may occur in the paranasal sinuses (e.g., invasive, chronic non-invasive or allergic sinusitis). Whilst CCPA and fungal balls usually occur in patients with pre-existing lung cavities [1], ABPA is a hypersensitivity disease of the lungs that almost always affects patients with asthma or cystic fibrosis [2]. In addition to

Table 1 Respiratory disorders related to *Aspergillus* species

Respiratory disorders characteristic of Aspergillus *species*
– Allergic bronchopulmonary aspergillosis (ABPA)
– Chronic cavitary pulmonary aspergillosis (CCPA), with or without fungal balls (aspergillomas)
– Invasive aspergillosis
– Allergic *Aspergillus* sinusitis
Respiratory disorders where Aspergillus *may play a role*
– IgE-mediated asthma
– Hypersensitivity pneumonitis
– Eosinophilic pneumonia
– Mucoid impaction
– Bronchocentric granulomatosis
Aspergillus *overlap syndromes*
– Combinations from the above

these characteristic syndromes, *Aspergillus* may play a role in the pathogenesis of other pulmonary diseases such as hypersensitivity pneumonitis, mucoid impaction in bronchi (MIB) and bronchocentric granulomatosis (BG) (Table 1). Despite the fact that *Aspergillus*-related syndromes are frequently described as separate entities, there is considerable degree of overlap between them. These entities may coexist (e.g., fungal balls, cavities or fibrosis in patients with ABPA) or may progress from one to another (e.g., IPA in a patient with ABPA) (Fig. 1).

The available literature about the different *Aspergillus* overlap syndromes is largely based on case reports or small case series. The aetiologies for these overlap syndromes are not clear. The potential mechanisms for *Aspergillus* overlap syndromes are listed in Table 2. This chapter discusses the clinical scenarios in which *Aspergillus* overlap syndromes have been described.

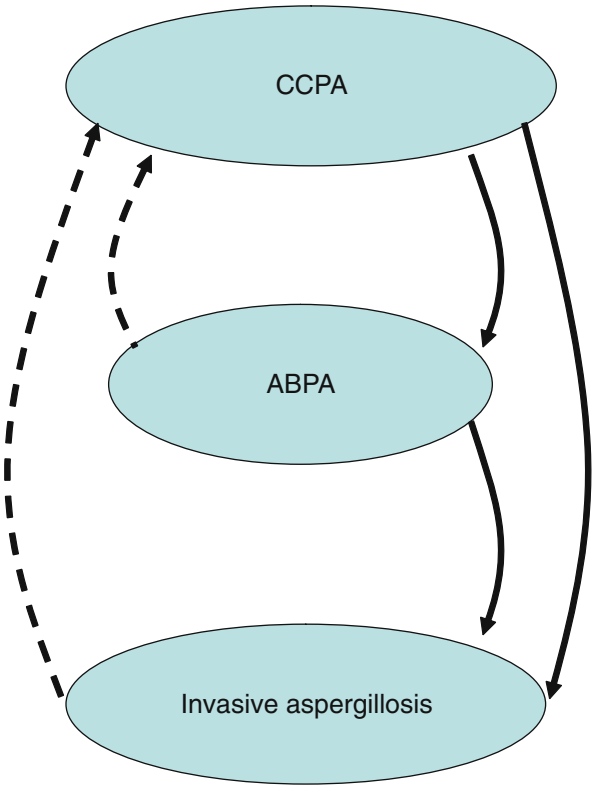

Fig. 1 Clinical scenarios of *Aspergillus* overlap syndromes in the lungs
Legend: CCPA, chronic cavitary pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis

Table 2 Proposed mechanisms for *Aspergillus* overlap syndromes

– Chronic underlying lung diseases (e.g., IPA in a patient with CCPA)
– Therapy with steroids (e.g., IPA or CNPA in a patient with CCPA or ABPA)
– Lung tissue destruction by *Aspergillus* (e.g., fungal balls in a patient with IPA)
– *Aspergillus* fungal load (e.g., IPA in a patient with ABPA)
– Host susceptible to develop hypersensitivity reaction to *Aspergillus* (e.g., ABPA in a patient with CCPA or AAS)
– Gene mutations such as CFTR gene (predisposing to ABPA and AAS) or mannose-binding lectin gene (predisposing to invasive diseases)
– Viral infections (e.g., IPA in a patient with ABPA)

Legend: AAS, allergic *Aspergillus* sinusitis; ABPA, allergic bronchopulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; CFTR, cystic fibrosis transmembrane conductance regulator; CNPA, chronic necrotising pulmonary aspergillosis (the same as subacute invasive aspergillosis); IPA, invasive pulmonary aspergillosis.

2 Fungal Balls and Allergic Bronchopulmonary Aspergillosis (ABPA)

The emergence of fungal balls in patients with ABPA has been reported infrequently in the literature. Safirstein et al. did not identify any patients with aspergillomas in a 5-year review of 50 patients with ABPA [3]. However, in another report by McCarthy et al. of 111 patients with ABPA followed over several years, fungal balls were present in 8 patients (7%) [4].

Fungal balls in ABPA patients may occur as an early event as well as a late phenomenon. In early cases, the bronchiectatetic areas affected by ABPA may enlarge to form cavities that colonize with *Aspergilli* forming fungal balls [5, 6]. In a report by Israel et al., a patient was diagnosed with ABPA and 7 months later developed haemoptysis and a left lower lobe cavitary mass. ABPA was in remission and serum IgE level was low. There was suspicion of malignancy, and the patient underwent thoracotomy. The pathological examination was consistent with a fungal ball [6]. Knowledge that aspergillomas may develop in patients with ABPA helps avoid unnecessary invasive procedures to rule out alternatives aetiologies for this condition.

Fungal balls may also be a late finding in patients with fibrosis and cavitation associated with long standing or poorly treated ABPA. At Wayne State University affiliated hospitals (Courtesy of Dr. Dana Kissner, USA) a 36 year-old female patient with a long-standing history of ABPA (Fig. 2a) developed a large cavitary lesion in the left upper lobe (Fig. 2b). Two years later she presented with haemoptysis and chest computed tomography (CT) revealed a left upper lobe aspergilloma (Fig. 2c). A lobectomy was eventually required for recurrent haemoptysis.

The fungal ball in some patients may not be directly related to ABPA rather due to concomitant fibrocavitary disease [4]. Few reports from India describe patients with history of tuberculosis and ABPA who later on developed CCPA and fungal

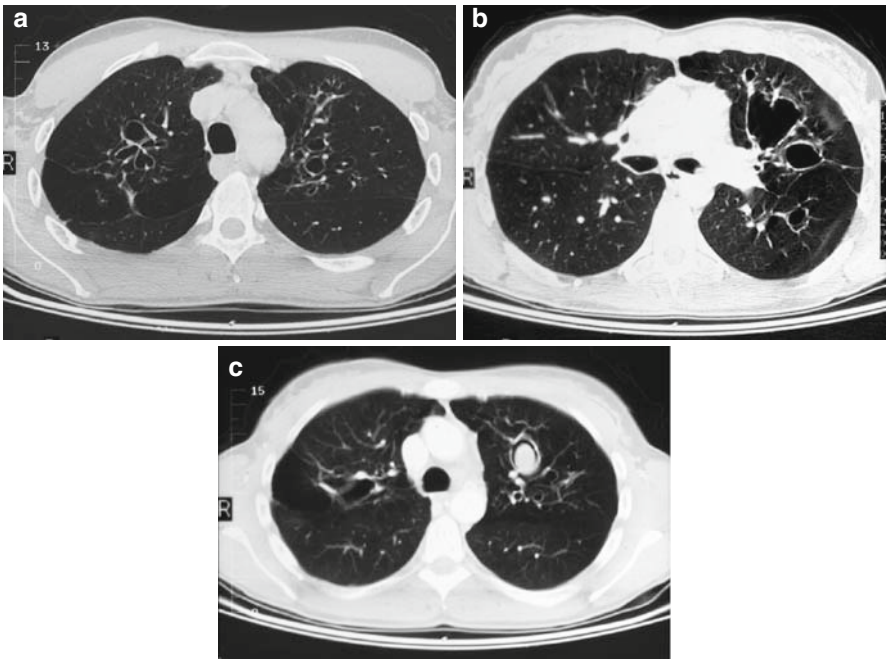

Fig. 2 (**a**) Chest computed tomography (CT) scan in a patient with allergic bronchopulmonary aspergillosis showing bilateral central bronchiectatic changes. Few years later, the chest CT showed cavitary areas in the *left upper* lobe (**b**). An aspergilloma developed at the site of the large cavity in the *left upper* lobe (**c**) (Courtesy of Dana Kissner, MD)

balls. Many of these patients were treated by anti-tuberculous medications for the suspicion of active tuberculosis. The authors suggested that the aspergilloma developed in the background of fibrocavitary lung disease associated with tuberculosis, and that therapy with steroid for ABPA may have accelerated the development of fungal balls [5, 7].

Fungal balls may also precede ABPA and may have a role in the pathogenesis of ABPA in the susceptible host. There are reports of patients developing CCPA secondary to pre-existing firbocavitary disease (such as tuberculosis or sarcoidosis) and later on develop ABPA. Ein et al. reported 2 patients with previous tuberculosis – but no history of asthma or allergic disease – who developed fungal balls which were followed by clinical, radiological, and biochemical features of ABPA. These patients received 0.5 mg/kg/day of prednisone and clinical and radiological improvement were observed, in addition to reduction in serum IgE level and eosinophilia [8]. It is possible that the overgrowth of *Aspergillus* species in these cavities triggers a hypersensitivity reaction in susceptible patients, leading to ABPA. This theory was suggested by reports that up to 75% of selected patients with aspergilloma had evidence of hypersensitivity reaction to *Aspergillus* [9]. Elevated IgE levels were observed for 78% of CCPA patients in the series by Denning et al. [10]., with some CCPA patients demonstrating a prominent eosinophilic infiltrate

surrounding lung cavities. Accordingly, IgE might also be used as an adjunct tool to monitor antifungal therapy in CCPA patients (Dr. Pasqualotto's unpublished data). Another study by Campbell and Clayton demonstrated that 5 out of 20 patients with fungal balls had positive immediate skin hypersensitivity to *Aspergillus* antigens and 9 of 19 patients had peripheral eosinophillia of > 500 cells/mm^3 [11]. Stevens reported 5 patients with aspergilloma who were challenged by *Aspergillus* antigens and demonstrated an immediate type I hypersensitivity reaction in one patient and late type III reaction in 4 patients [12]. It appears that the development of fungal balls in certain patients serves as a depot for the fungal antigens that could cross the cavity into the circulation or spill to communicating bronchi triggering a hypersensitivity reaction with subsequent features of ABPA.

The natural history of patients with co-existing ABPA and CCPA is not clear. Some reports suggest that the cavities and fungal balls remain stable in size. Rosenberg and Greenberger reported a patient with asthma who presented with haemoptysis and a large multiloculated cavity consistent with a fungal ball. The patient had peripheral eosinophillia and elevated serum IgE levels. *A. flavus* and *A. glaucus* were isolated from the sputum. The patient had sufficient features to diagnose ABPA and was treated with systemic steroids. A 2 year follow up documented significant improvement in ABPA, however the fungal ball remained stable in size [13]. Other reports document that the fungal ball actually decreases in size with treatment of ABPA with systemic steroids. Shah et al. described 2 patients with ABPA who presented with haemoptysis and fever. They were found to have cavitary lesions consistent with CCPA. The patients were treated with prednisone 0.5 mg/kg/day that was tapered gradually. In one patient the left upper lobe fungal ball decreased in size over 30 months follow up; in the second patient, the cavity remained stable in size after 18 months of follow up [14]. It should be noted in this context that there are very few reports –as will be outlined later – where IPA developed in patients with fungal balls or ABPA, and treatment with inhaled or systemic steroids may have contributed to the development of invasive disease [15]. In the presence of immunosuppressive therapies such as high dose steroids, CCPA patients might progress to a semi-invasive form of aspergillosis, named chronic necrotising pulmonary aspergillosis (CNPA) [10].

The limited available literature on the overlap between ABPA and CCPA suggests that these patients respond well to systemic steroids with improvement in the ABPA features and no detrimental effect on the fungal balls. If the patients require long term steroid therapy, or remain symptomatic, the addition of antifungal therapy or resection of the fungal ball may be necessary. Supportive measures for the management of aspergillomas are similar to other patients without ABPA – this is discussed in more detail elsewhere in this book.

3 ABPA and Invasive Aspergillosis

Invasive pulmonary aspergillosis (IPA) in patients with ABPA has been sporadically reported in the literature. The invasive disease appears to have two distinct syndromes. In some reports the invasion by *Aspergillus* species is restricted to the

tissues surrounding the bronchiectatic segments, with granulomatous reaction that contains *Aspergillus* hyphae. These patients do not have evidence of vascular invasion, necrosis or dissemination. In a report by Riley et al., a 46 year-old patient with typical feature of ABPA was on prednisone at 60 mg daily [16]. However the patient continued to have excessive sputum production with bronchiectasis so he underwent right upper lobe lobectomy. Histological examination of the surgical specimen revealed many necrotising granulomas with multinucleated giant cells, plasma cells and few eosinophils. The granulomas were present throughout the lobe. Around 10% of the granulomas contained *Aspergillus* hyphae, and tissue culture grew *A. fumigatus*. The patient did well after lobectomy and the steroid treatment was gradually discontinued. The patient did not require antifungal therapy. Similar cases were reported by others [16–18]. Due to their chronic course, it is likely these cases represent the semi-invasive form of CCPA (CNPA, as already explained) rather than the classic IPA. The former syndrome was not well-characterized when these cases were reported. Local invasion by *Aspergillus* probably develops as a result of chronic immunosuppression (steroids) and/or the presence of underlying chronic lung disease. The authors of one of the reports suggested that tissue growth by the fungus occur routinely during one phase of ABPA, however because of the hosts' robust immune system, invasion regresses or is limited to a localised granulomatous reaction [16].

On the other hand, disseminated IPA has been rarely described in patients with ABPA [19, 20]. Bodey and Glann reported that an ABPA patient on methylprednisolone (20–40 mg daily) developed seizures [21]. Brain CT scan revealed a large parietal brain abscess. The patient underwent craniotomy with resection of the abscess and tissue culture grew *A. fumigatus*. The patient was treated by amphotericin B with almost completed recovery. Anderson et al. reported another case of a patient with ABPA who has been on prednisone (10–20 mg daily) and inhaled steroids for 3 years [15]. The patient developed symptoms consistent with a viral upper respiratory tract infection that lead to exacerbation of asthma. The patient was admitted to the intensive care unit and was treated with high dose methyprednisolone. The chest radiograph showed diffuse infiltrates with multiple nodules in the left upper lobe. Few days later, lethal massive haemoptysis occurred. Autopsy revealed large bilateral apical fungal balls, central bronchiectasis and disseminated IPA involving the lungs, myocardium, thyroid and kidneys. The authors suggested that high dose of steroids was responsible for the disseminated disease. However there are few reports that describe IPA developing after a viral illness – including influenza – [15]. It is possible that these viral illnesses exacerbate the underlying ABPA and asthma leading to increased doses of systemic steroids, or may be associated with post-viral T cell suppression or direct damage to the affected respiratory epithelium that put these patients at risk for IPA [22–24].

Another group of patients that may be at risk of overgrowth of *Aspergillus* and IPA in the setting of ABPA are patients with cystic fibrosis. In vitro growth of *Aspergillus* species is inhibited by products of *Pseudomonas aeruginosa*. Aggressive treatment of *Pseudomonas* in patients with cystic fibrosis might promote *Aspergillus* airway colonisation, which in turn predisposes to ABPA and/or IPA in this patient population [25].

Given the paucity of literature about these cases, there are no validated predictors for the development of IPA in patients with ABPA. Vigilance is warranted in those patients with ABPA who develop sudden worsening of respiratory symptoms, have extrapulmonary findings, or have diffuse infiltrates or nodules on chest radiography. Chest CT scan with high resolution cuts has been shown to be more sensitive in detecting IPA, and should be considered early in patients who are suspected to have IPA [26]. The role of new non-invasive diagnostic methods (such a galactomannan, B-D-glucan or polymerase chain reaction testing) in early detection of IPA in patients with ABPA is not known and warrants further study. It is also not clear if adding an antifungal agent to the management of patients with ABPA who require prolonged or high dose steroid therapy prevents the development of an invasive disease.

In the discussion of the overlap syndromes with IPA, of note that there are few reports that describe a localised invasion of the tissue surrounding the cavity with a fungal ball. Chow et al. described a 29 year-old female patient with cystic fibrosis

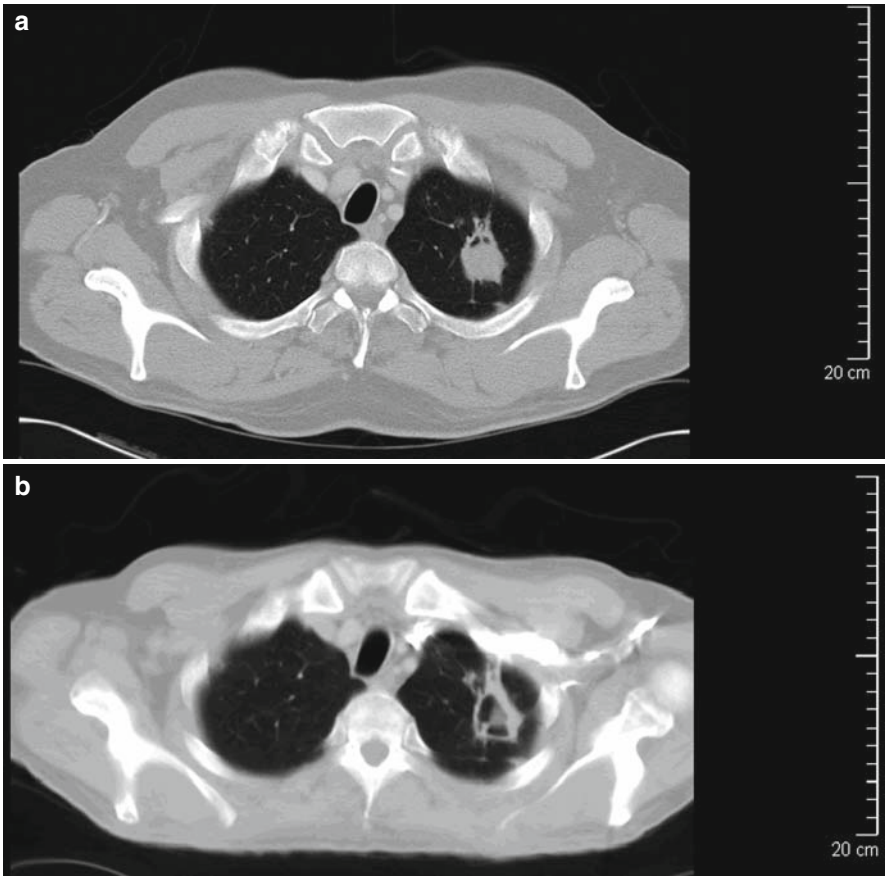

Fig. 3 (**a**) Chest computed tomography (CT) scan of a patient with acute myelogenous leukaemia showing a *left upper* lobe mass with air crescent cavitation consistent with invasive pulmonary aspergillosis. Few months after antifungal therapy, the chest CT showed a residual fungal ball (**b**)

and left lower lobe aspergilloma who presented several months later with massive haemoptysis and was diagnosed with IPA. The patients' condition worsened despite treatment with amphotericin B and itraconazole. She was subsequently successfully treated and cured with voriconazole [27]. On the other hand, fungal balls commonly develop in patients with IPA as a result of fungal invasion of blood vessels with the subsequent of thrombosis and necrosis. This leads to residual cavity and formation of fungal ball (Fig. 3).

4 Allergic *Aspergillus* Sinusitis Overlap Syndromes

Allergic *Aspergillus* sinusitis (AAS) is another allergic disease related to *Aspergillus* species which has been recently recognised as a separate entity. The diagnostic criteria for AAS are: (i) sinusitis of one or more paranasal sinus on x-ray film; (ii) necrotised amorphous tissue infiltrated with eosinophils on histological examination of material from the sinus; (iii) demonstration of fungal elements in nasal discharge or material obtained at the time of surgery either by stain or culture; (iv) absence of invasive fungal disease at the time of diagnosis or subsequently; (v) absence of diabetes mellitus, previous or subsequent immunodeficiency disease or treatment with immunosuppressive therapy; and (vi) presence of other features of allergy such as peripheral eosinophillia, types I and III hypersensitivity to *Aspergillus*, precipitating antibodies to *Aspergillus* antigens, and elevated total and *Aspergillus*-specific IgE levels [7].

As is evident from these diagnostic criteria, AAS has features similar to ABPA. Early reports described nasal symptoms in patients with ABPA. McCarthy and Pepys have reported that 10% of patients with ABPA produce nasal plugs that are similar to the airway casts that are present in the bronchi of patients with ABPA [9]. Safirstein also reported that nasal discharge containing airway casts improved when a patient with ABPA was treated with systemic steroids [28]. More recently Shah et al. reviewed the records of 95 patients with ABPA and found 22 patients with radiological evidence of sinusitis. The nasal symptoms preceded chest symptoms in 2 patients, visa-versa in 1 patient, and simultaneously in 4 patients. Nine of these patients underwent surgery and 7 fulfilled the diagnostic criteria for AAS. The probability of AAS could not be ruled out in the rest of patients who did not undergo surgery [29]. Awareness of the association between these two conditions is important since they are commonly treated by different specialists who may not be aware of the other condition. Furthermore, AAS usually does not respond to intranasal steroids, and commonly requires systemic steroids and surgical debridement.

The reports of the overlap between AAS and ABPA intrigued Vensarke and DeShazo who reviewed the records of 5 patients with both conditions [30]. The patients were 17–55 years of age. All had history of chronic sinusitis and asthma. Peripheral eosinophilia was present in all patients, and elevated serum IgE was documented in 4. Sinus surgery was performed on 4 patients prior to confirming the diagnosis. All 5 patients experienced clinical response of the pulmonary and sinus disease after treatment with systemic steroids (prednisone 0.5 mg/kg/day). The authors suggested

that these two conditions are manifestations of the same disease and named it Sinobronchial Allergic Mycosis (SAM). They also raised the possibility that mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene may contribute to the development of SAM syndrome [30].

In addition to the characteristic allergic mucin associated with AAS, some patients may have evidence of tissue invasion suggestive of invasive sinusitis. Bony expansion and erosion are frequent with AAS and are thought to be due to pressure remodelling rather than fungal invasion. However there are some reports of documented tissue invasion by *Aspergillus* hyphae in patients with AAS who are not immunocompromised. A retrospective analysis by Thakar et al. identified 28 cases of AAS based on pathological examination over a 32-month period. All histological specimens were re-evaluated for features of invasive pathology, and case records were correlated with clinical, radiological, or laboratory parameters associated with such invasion. Six cases (21%) had additional evidence of mucosal invasion as indicated by granulomatous inflammation and branching septate fungal hyphae in the submucosal tissues. Vascular invasion or vasculitis were not seen. The features of tissue invasion were associated with advanced disease as indicated by a higher incidence of orbital involvement on clinical evaluation ($p = 0.024$), and extrasinus spread (intraorbital or intracranial spread) on CT scan of the sinuses ($p = 0.003$). One patient with co-existing AAS and invasive disease died. The authors concluded that advanced AAS may be complicated by tissue invasion, and that the presence of clinical evidence of orbital involvement, or CT manifestations of extrasinus spread should alert the clinicians to the possibility of invasive disease. It is prudent to carefully review histological specimen obtained from such patients for evidence of invasion before starting steroids. Tissues should be appropriately stained for fungi for that purpose. The authors reported that many of these patients do well with surgical debridement alone, although the addition of an antifungal agent should be considered [31].

Another feature of overlap between AAS and other *Aspergillus* related syndromes is demonstrated by a case report of a patient with AAS, ABPA and aspergilloma [32]. A 26 year-old patient with history of asthma and rhinitis since childhood presented with haemoptysis. Chest CT scan revealed pulmonary infiltrates, central bronchiectasis and a fungal ball. Serological tests were consistent with the diagnosis of ABPA. CT of the sinuses confirmed sinus involvement. The patient underwent endoscopic sinus surgery and pathological material demonstrated allergic mucin that contained *A. fumigatus*. The patient responded to systemic therapy with steroids.

5 Overlap Between Aspergillosis and Other Respiratory Diseases

Aspergillus species are also implicated in other conditions that are not part of the characteristic diseases caused by this fungus. These conditions are thought to develop independently from the *Aspergillus* species, although there is evidence

that *Aspergillus* species may play a role in some cases or have features similar to one of the typical aspergillosis syndromes. The most important diseases in this entity are IgE-mediated asthma, hypersensitivity pneumonitis, mucoid impaction in bronchus, and bronchocentric granulomatosis (Table 1). The overlap between severe asthma with fungal sensitisation (SAFS) and ABPA is discussed elsewhere in this book.

5.1 Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis is an interstitial and/or alveolar infiltrative disorder occurring after exposure to a wide range of organic and non-organic antigens including thermophilic actinomycetes, fungi, animal proteins, and a variety of chemicals. The clinical presentation may be acute, subacute, or chronic depending on the intensity and duration of exposure. Classically, the patient with acute intermittent exposure to one of these antigens develops symptoms within 4–6 hours after inhalation. Pathological examination characteristically reveals a mononuclear infiltrate with or without granuloma formation or bronchiolitis obliterans. Hypersensitivity pneumonitis has been rarely reported due to *Aspergillus* species following exposure to damp hay (sometimes called Farmer's lung). Hypersensitivity pneumonitis due to *Aspergillus* species is distinct from IPA, although some reports suggested an overlap between the two conditions [33, 34]. A report by Meeker et al. describes 2 brothers who were exposed to mouldy damp hay. One patient presented 4 days later with features of hypersensitivity pneumonitis that resolved spontaneously. The second patient presented 2 weeks later with acute illness associated with fever, hypoxemia, and diffuse interstitial and alveolar infiltrates. The patient also had increased serum IgE level, peripheral eosinophilia, and increased eosinophils on bronchoalveolar lavage examination. The patient underwent open lung biopsy that demonstrated suppurative granulomata that contained *A. fumigatus* with destruction of the lung parenchyma and prominent tissue eosinophilia. The patient was diagnosed to have an overlap between hypersensitivity pneumonitis and IPA and was treated with systemic steroids and amphotericin B. However he died 12 days later of multiorgan system failure [35].

There were other cases reported in the literature of hypersensitivity pneumonitis associated with features of tissue invasion by *Aspergillus* species [33, 34, 36]. Most of these cases differ from classic hypersensitivity pneumonitis by virtue of the prolonged interval between exposure and onset of symptoms (2–3 weeks rather than hours). The reason for tissue invasion by *Aspergillus* in some patients with hypersensitivity pneumonitis is not clear. It is possible that the host hypersensitivity reaction may predispose to tissue invasion or that hypersensitivity pneumonitis simply represents a low grade invasive disease by *Aspergillus* species. Treatment of hypersensitivity pneumonitis with steroids may also play a role in tissue invasion by *Aspergillus* species in those patients. Hence, caution may be warranted during treatment of hypersensitivity pneumonitis due to *Aspergillus* species by steroids alone.

5.2 Mucoid Impaction of the Bronchus (MIB)

This condition results in obstruction of the bronchi by plugs of inspissated mucus and exudate. There are several features of overlap between this syndrome and ABPA. Almost all patients with MIB have asthma and have peripheral eosinophilia. The patients produce large mucus plugs or casts, however the mucus impaction usually involves larger bronchi, and more in the upper lobes with distal atelectasis forming V or Y shaped densities with the apex toward the hilum. Some of the patients have hypersensitivity against *Aspergillus* species, but do not have enough criteria to diagnose ABPA [37]. The exact aetiology of MIB is not known but theories about its pathogenesis include a hypersensitivity reaction to a fungus [38]. The management of mucoid impaction is supportive with mucolytic agents and pulmonary toileting. Contrary to ABPA, steroids are rarely of benefit [37].

5.3 Bronchocentric Granulomatosis (BG)

BG is a unique clinicopathological entity characterized by focal granulomatous and destructive lesions of the bronchi and bronchioles. BG has features that overlap with ABPA although it is believed to be a separate entity. Approximately one third of patients with BG have asthma. Radiologically, the patients present with segmental or subsegemental atelectasis. They may also have masses or nodules – that represent the granulomatous reaction of the involved bronchi – mimicking neoplasms [39]. Pathological specimens show thickening of the bronchial walls that are dilated and saccular. The bronchi are filled with inspissated yellowish viscous material that contains eosinophils. *Aspergillus* hyphae are seen in the lumen of resected bronchi of 75% of patients with BG and asthma and 29% of those with BG without asthma [38]. The aetiology of BG is not known, however some reports suggested a hypersensitivity reaction to *Aspergillus* species or other fungi [37]. The patients with BG may have spontaneous remission [38]. Systemic steroids are effective resulting in improvement of the clinical symptoms and clearance of the radiological findings.

A representative case is in a 24 year-old male patient with history of asthma presented to our pulmonary clinic with haemoptysis. Chest CT scan revealed collapse of the left upper and right lower lobes with bronchiectatic segments (Fig. 4a) The patient had peripheral eosinophilia (1,400 cells/mm^3) and elevated serum IgE level (6,429 IU/ml). *Aspergillus* specific IgE and IgG were positive. The patient underwent bronchoscopy that revealed severely inflamed airways in the radiologically abnormal areas with thick, inspissated mucus. Bronchial biopsies revealed mucosal ulcerations with epithelioid granulomas and eosinophilic infiltrates. Bronchial washings cultures grew *A. fumigatus*. The patient was diagnosed to have an overlap syndrome of ABPA and BG. He was treated with steroids and itraconazole that lead to remarkable clinical and radiological improvement (Fig. 3b).

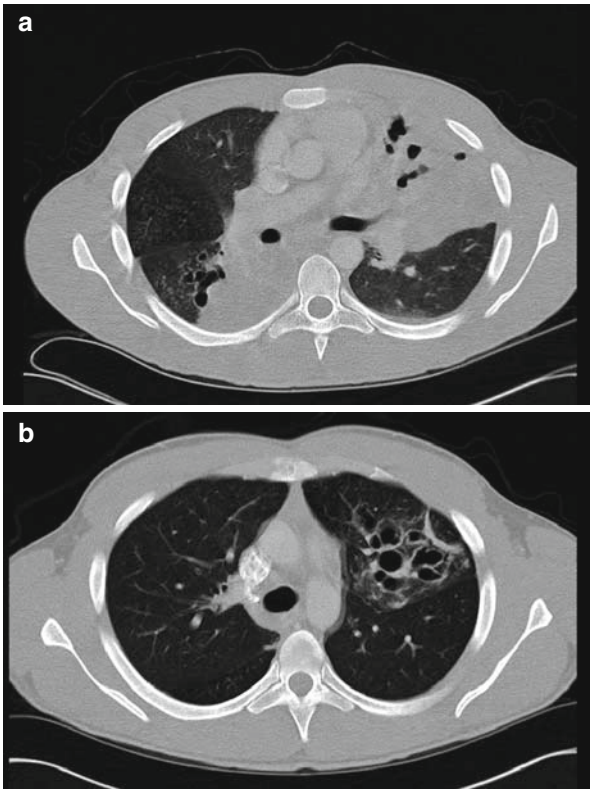

Fig. 4 (**a**) Chest computed tomography (CT) scan showing segmental atelectasis of the *left upper* lobe and *right lower* lobe with bronchiectatic changes. After treatment with systemic steroids and an antifungal agent, the chest CT scan showed resolution of the atelectasis with residual bronchiectasis in the *left upper* lobe (**b**)

6 Conclusions

Aspergillus-related lung diseases are diverse depending on the patients' immune status and the underlying lung function and structure. Whilst these syndromes have characteristic clinical, radiological and serological features, there are sporadic reports that document significant overlap between these syndromes. The *Aspergillus*-related illnesses may co-exist or progress from one entity to the other under certain circumstances. These reports suggest that *Aspergillus*-related lung disease may represent a spectrum rather than separate entities. A better knowledge about the *Aspergillus* overlap syndromes may decrease the need for extensive investigations to exclude other conditions, and may impact the management and follow up of patients with *Aspergillus*-related diseases.

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PART VII
OTHER PRESENTATIONS OF ASPERGILLOSIS

Cerebral *Aspergillus* Infections and Meningitis

Stefan Schwartz

Abstract Cerebral aspergillosis is notoriously difficult to treat and increasingly recognised in immunocompromised patients. These infections frequently result in brain abscess formation, but can also cause cerebral embolism or vasculitis, fungal aneurysms, cerebral granuloma or meningitis. Cerebral aspergillosis carries a particular poor prognosis with an almost 100% mortality in patients treated with either amphotericin B or itraconazole. Penetration of most available antifungal agents into the central nervous system (CNS) is largely limited by their molecular size, physicochemical properties or P-glycoprotein-mediated efflux at the blood-brain barrier. Voriconazole has a small molecular size (349 Da) favouring penetration into the CNS. Data from animal models and humans confirm that fungicidal concentrations of voriconazole may be attained not only in cerebrospinal fluid and brain tissue, but also in brain abscess material. In a retrospective study evaluating 81 patients with cerebral aspergillosis, voriconazole therapy was associated with an improved 35% response- and a 31% survival-rate. Neurosurgical interventions also had a positive impact on survival. Thus, voriconazole therapy and neurosurgical management, if considered feasible in individual patients, is currently the best approach to treat patients with cerebral aspergillosis. Animal model data indicate that higher drug doses or selected combination therapies could further enhance treatment efficacy, which should be explored in future studies to further improve the still unsatisfactory prognosis of cerebral aspergillosis.

Keywords Antifungal agents · Blood-brain barrier · Neuroaspergillosis · Neuropharmacology · Voriconazole

S. Schwartz (✉)
Medizinische Klinik III, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, 12200 Berlin, Germany
e-mail: stefan.schwartz@charite.de

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Contents

1 Introduction

The first description of cerebral aspergillosis was published in the late nineteenth century, but this particular type of *Aspergillus* infection was observed infrequently during subsequent decades with less than 100 cases published before 1978 [1, 2]. In a large autopsy survey from Japan, covering the era from 1969 to 1994 and evaluating patients with visceral mycoses, only 65 (3.3%) patients were identified to have meningeal or brain involvement amongst 1,967 patients with aspergillosis [3]. A higher frequency (20%) of cerebral infections in patients with aspergillosis was reported from a multinational autopsy study analysing fungal infections in cancer patients [4]. In addition, cerebral involvement has been reported in 14–42% of patients in two more recent series of patients with invasive aspergillosis (IA) and acute leukaemia or allogeneic haematopoietic stem cell transplantation (HSCT), indicating that fungal dissemination to the central nervous system (CNS) is not a rare condition in severely immunocompromised patients [5, 6]. Likewise, in patients who developed a brain abscess after solid organ transplantation or HSCT, the most common infecting agents were *Aspergillus* species [7, 8].

Cerebral aspergillosis was almost a deadly infection in the past and data on successfully treated patients with this devastating fungal infection were limited to anecdotal case reports [9–12]. Repeatedly, a 100% mortality was seen in retrospective studies evaluating patients with cerebral aspergillosis [5, 6, 13, 14]. Moreover, only a single survivor was found amongst 141 patients with cerebral aspergillosis in an extensive, literature-based study of 1,223 cases with IA extracted from published case series or studies [15].

A major factor, which likely contributes to the limited efficacy of most antifungal agents in cerebral aspergillosis, is their limited ability to penetrate across the blood-brain barrier. The available data indicate that concentrations of antifungal agents in cerebrospinal fluid (CSF) and brain tissue rarely exceed minimal inhibitory concentrations required for most *Aspergillus* isolates, except for voriconazole. Animal model data and studies in humans demonstrate that fungicidal concentrations of voriconazole may be attained in CSF, brain tissue as well as in brain abscess material [16–18]. In a recent retrospective study of 81 patients treated with voriconazole for cerebral aspergillosis, responses were seen in 35% of patients and 31% of patients survived this life-threatening fungal infection [19]. Neurosurgery has been repeatedly applied to remove infected tissue or areas of low drug penetration as well as for

the management of complications (e.g., cerebral haemorrhage, increased intracranial pressure). In a review of 26 patients successfully treated for cerebral aspergillosis, the majority of patients (73%) underwent neurosurgical interventions, which suggests that adjunct neurosurgical management might contribute to an improved outcome [20]. Noteworthy, neurosurgical management was significantly associated with an improved survival in the recent retrospective study evaluating voriconazole therapy in cerebral aspergillosis [19].

2 Clinical Characteristics and Diagnostic Approach

Cerebral aspergillosis typically affects patients with marked immunosuppression (e.g., patients with profound and sustained neutropenia, after HSCT or solid organ transplantation, or patients with chronic granulomatous disease), but this type of CNS fungal disease has also been observed occasionally in patients with a less severe degree of immunosuppression (e.g., short-term therapy with steroids) and non-immunocompromised patients [5, 8, 21–25].

Symptoms in patients with cerebral aspergillosis are protean and may include fever refractory to antibacterial therapy, confusion, lethargy, headache, meningism, nausea, vomiting, focal neurological deficits, tremor, ataxia or seizures [6, 13]. However, none of these findings – neither alone nor in combination – is sufficiently specific for the diagnosis of a fungal CNS infection [7]. In the majority of patients with cerebral aspergillosis, the disease tends to deteriorate rapidly with a reported median survival of only 5–7 days [5, 6]. Thus, a rapid diagnostic approach with evaluation of neurosurgical options and early initiation of antifungal treatment should be attempted always in risk patients with symptoms compatible with cerebral aspergillosis.

Neuroradiological studies in patients with suspected cerebral aspergillosis should always include magnetic resonance imaging, which facilitates the detection of small size lesions [26]. In addition, diffusion-weighted imaging has been found to be useful for early detection of ischemic areas and differentiating cerebral aspergillosis from other CNS infections in immunocompromised patients [27]. Results from routine CSF analyses are rarely helpful in establishing the diagnosis of a cerebral aspergillosis and might show mild to moderate elevations of white blood cell counts and protein concentrations with normal or decreased glucose content. Noteworthy, elevated red blood cell counts in CSF samples have been repeatedly observed in patients with cerebral aspergillosis [13, 28]. In order to increase the diagnostic yield of microscopy and culture in CSF samples, the study of large quantities of CSF has been advocated [29]. Nevertheless, hyphae are very rarely detectable in CSF specimens and CSF cultures infrequently yield growth of *Aspergillus* species in patients with cerebral aspergillosis [9, 30–32]. Conversely, positive CSF cultures have been reported in up to 20% of cases of CNS aspergillosis following neurosurgical procedures [33].

Indirect assays, which detect *Aspergillus* galactomannan or *Aspergillus*-specific DNA in CSF samples, have been used successfully to affirm the diagnosis of

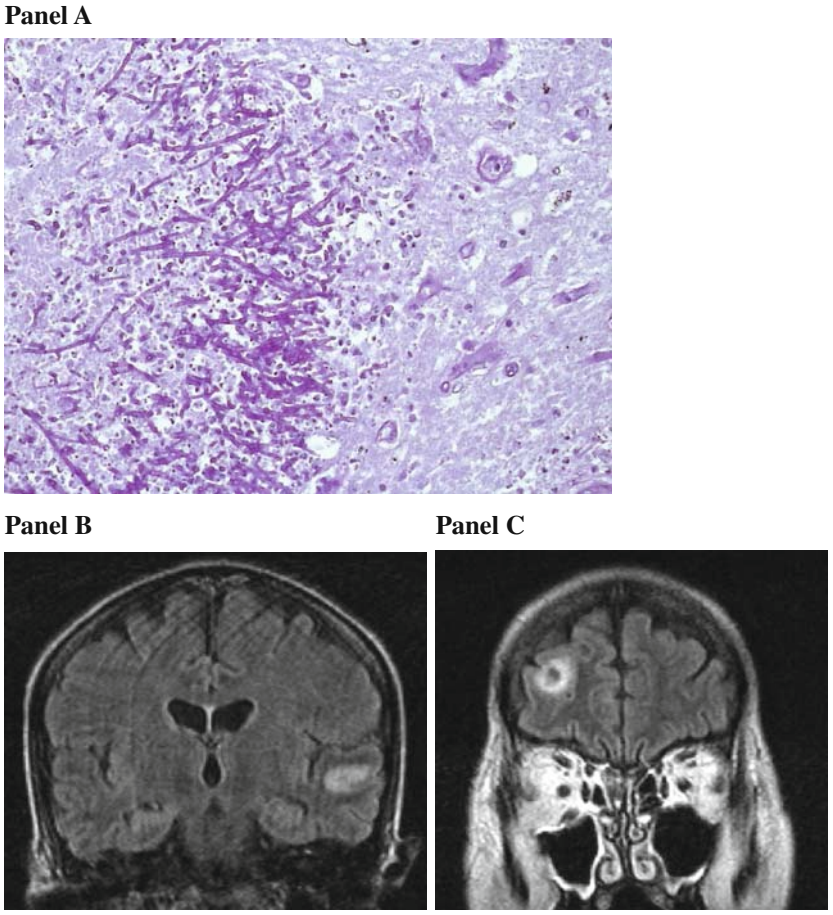

Fig. 1 Cerebral aspergillosis in a haematopoietic stem cell transplant recipient. **Panel A**: Histological section of a brain tissue specimen showing hyphal invasion and tissue necrosis (PAS stain; kindly provided by Dr. A. Harder, Berlin, Germany). **Panels B** and **C**: Brain abscesses visualized in both hemispheres by magnetic resonance scan (fluid-attenuated inversion recovery-imaging)

cerebral aspergillosis [31, 34]. However, there is consensus that proven IA requires demonstration of damaging tissue invasion by the fungus plus fungal growth in specimens from a normally sterile site, which is involved into the infectious disease process (Fig. 1a) [35].

Cerebral aspergillosis is mostly caused by haematogenous seeding from the primary sites of the infection such as the lungs and may result in bihemispheric lesions (Fig. 1b and c) [5, 6]. In addition, IA of the paranasal sinuses or *Aspergillus* otitis can also cause cerebral aspergillosis by destructive invasion into neighbouring structures [21, 36, 37]. Rarely, fungal embolism arising from *Aspergillus* endocarditis or arteritis has been reported as a source of cerebral aspergillosis [38, 39]. Direct inoculation of fungi acquired through invasive

procedures, such as spinal anesthesia, or neurosurgery has been infrequently observed as a condition, which could cause cerebral aspergillosis [21, 25, 33, 40].

In patients with cerebral aspergillosis, brain abscess formation is the predominant type of manifestation of the disease (Fig. 1b and c) [5, 7]. The angioinvasive nature of *Aspergillus* species could cause vasculitis and formation of fungal aneurysms, which could promote cerebral infarcts by septic embolism or vascular thrombosis, as well as cerebral haemorrhage due to a destructive invasion of vascular walls [30, 41–44]. Chronic granulomatous *Aspergillus* infections of the paranasal sinsuses are discussed in the chapters about *Aspergillus* sinusitis – these were first recognised in Sudanese patients, but have also been reported from India, Saudi Arabia and rarely from other climatic regions [37, 45–47]. This particular type of *Aspergillus* infection mostly affects immunocompetent individuals, tends to invade neighbouring structures and could result in a destructive CNS invasion with formation of tumour-like granulomatous masses [37, 48, 49]. Pure meningitis is an uncommon presentation of cerebral aspergillosis [9, 25, 31, 32]. However, meningeal involvement or meningism as the leading symptom has been repeatedly reported in patients with other forms of cerebral aspergillosis [11, 50, 51]. In addition, various other manifestations of cerebral aspergillosis may occur simultaneously or evolve at varying time points during the course in individual patients, indicating an overlapping spectrum of the disease in at least a subset of patients [41, 42, 44, 52]. Due to the non-specific nature of the clinical manifestations of CNS aspergillosis, a high index of suspicion is therefore required amongst patients at higher risk for IA.

3 Aspects of Central Nervous System Pharmacokinetics

The CNS is protected by various barriers, which prevent accumulation of neurotoxic substances and limit penetration of several drugs, including most antifungal agents. The human blood-brain barrier has an amazing vessel length of around 600 km and is situated at the interface between blood and brain parenchyma. Despite its estimated surface area of approximately 20 m^2, penetration of substances across the blood-brain barrier is limited, as the endothelial cells of brain capillaries are largely connected with tight junctions. In contrast, the epithelium of the blood-CSF barrier is fenestrated, which facilitates penetration of substances into the CSF. However, further uptake into brain parenchyma is limited by a layer of epithelial cells (ependyma), which covers the ventricles [53]. At the blood-brain barrier, substances mainly enter the CNS through transcytosis using active transporter systems. In addition, hydrophilic substances may cross the blood-brain barrier to some extent by paracellular diffusion, whereas small lipophilic compounds (< 400–600 Da) may freely enter the CNS by transcellular diffusion. Various active transport systems mediate influx of substances at the blood-brain barrier. In addition, active efflux transporter systems also maintain brain homeostasis. Of these, P-glycoprotein, which is an ATP-dependent transmembrane protein, displays high affinity to various cationic and lipophilic compounds. P-glycoprotein is predominantly expressed at the apical membrane of brain capillary endothelial cells and

antagonizes delivery of various drugs into the CNS [54]. Amongst various factors, which determine CNS drug concentrations, disruption of the blood-brain barrier by an underlying infectious disease process likely has a major impact, but the available data on this, at least in humans, are limited.

Amphotericin B is a large molecule (924 Da), which consists of a hydrophilic polyhydroxyl chain and a lipophilic polyene chain and displays moderate lipophilicity with an octanol to water partition coefficient (LogP) of 0.95 [55]. Despite its rather low lipophilicity, amphotericin B is virtually unsoluble in water and intravenous delivery requires solubilization with deoxycholate or by lipid encapsulation. The CNS penetration of all available amphotericin B preparations have been investigated in a rabbit model with *Candida* meningoencephalitis. In this study, mean drug concentrations in the CSF and brain tissue did not exceed 0.033 μg/ml and 1.99 μg/g, respectively (Table 1). The mean brain tissue drug concentrations were significantly higher in animals treated with liposomal amphotericin B compared to those found in animals treated with any other amphotericin B preparation ($\geq$ 1.84 μg/g vs. $\leq$ 0.51 μg/g). Interestingly, the residual fungal burden in brain tissue was significantly reduced only in animals treated with liposomal amphotericin B or amphotericin B deoxycholate, despite the rather limited CNS penetration of amphotericin B deoxycholate [56]. In humans, amphotericin B CSF concentrations rarely exceed 4% of corresponding blood concentrations [57]. In a single patient with cryptococcal meningoencephalitis, CSF concentrations of amphotericin B increased from 0.068 to 0.11 μg/ml during therapy with amphotericin B deoxycholate and subsequent switch to liposomal amphotericin B, confirming an improved CNS penetration of the liposomal drug preparation [58]. In a pharmacokinetic study with infants, CSF concentrations of amphotericin B were 40–90% of corresponding serum concentrations, but sampling time points and absolute drug concentrations were not given (Table 1) [59]. Data on the deposition of amphotericin B in human tissues are available from two autopsy studies. In these studies, total brain tissue concentrations of amphotericin B ranged from 0.2 to 5.8 μg/g (median: 0.5 μg/g) and from undetectable to 1.6 μg/g (median: 0.7 μg/g) after therapy with amphotericin B deoxycholate and liposomal amphotericin B, respectively (Table 1). In both studies, concentrations of amphotericin B determined by a bioassay in tissue homogenates were only 15–41% of those determined by high-performance liquid chromatography after methanolic extraction, demonstrating that large amounts of amphotericin B in tissues are inactive [60, 61].

Itraconazole was the first available triazole with activity against *Aspergillus* species. The molecule of itraconazole is rather large (705 Da) and exhibits a pronounced lipophilicity (LogP 6.99) with high protein binding [55]. In several animal studies exploring CNS fungal diseases or the effects of experimental CNS or eye infections on cerebral penetration of azoles, low ($\leq$ 0.156 μg/ml) to undetectable itraconazole CSF concentrations were repeatedly found [62–65]. Brain tissue concentrations of itraconazole are also remarkably low and were $<$ 0.3 μg/g in an early study with rats treated with a single dose of 10 mg/kg itraconazole (Table 1) [66]. A more rapid clearance of itraconazole from brain tissue compared to plasma and liver tissue (half-life of 0.4 vs. 5 hours) was observed in rats after intravenous

Table 1 Published data on concentrations of various antifungal agents in cerebrospinal fluid and brain tissue

Agent	Cerebrospinal fluid	Source	Brain tissue	Source	References
Amphotericin B	≤ 0.033 μg/ml[a]	Animal	≤ 1.99 μg/g[c]	Animal	[56]
	≤ 0.11 μg/ml	Human	≤ 0.5 μg/g[d]	Human	[58, 60]
	0.4–0.9[b]	Neonates	≤ 0.7 μg/g[d]	Human	[59, 61]
Itraconazole	≤ 0.156 μg/ml[a]	Animal	< 0.3 μg/g	Animal	[62, 66]
	≤ 0.07 μg/ml	Human	0.262 μg/g[a] (hydroxy-itraconazole)	Animal	[31, 75]
Posaconazole	Undetectable	Animal	Unknown		[76]
	< 0.01–0.221 μg/ml	Human			[77]
Voriconazole	~ 0.5, 0.68[b]	Animal	~ 2 tissue/plasma ratio, ≤ 6.8 μg/g	Animal	[16, 78]
	0.04–3.93 μg/ml	Human	11.8 μg/g, 58.5 μg/g	Human	[31, 50]
	0.22–1[b]	Human	1.2–1.4 μg/g, 5.1 μg/ml[e]	Human	[16–18]
Anidulafungin	Unknown		≤ 3.9 μg/g[a]	Animal	[81]
Caspofungin	Unknown		≤ 0.08 μg/g[a], ≤ 0.164 μg/g[a]	Animal	[82, 83]
			≤ 0.2 tissue/plasma ratio	Animal	[82]
Micafungin	< 1 μg/ml	Animal	< 1 – ~4 μg/g[f]	Animal	[80, 84]
	0.007–0.017 μg/ml	Human			[85]

Legend: [a]Mean values; [b]percent of plasma level; [c]higher concentrations with liposomal amphotericin B; [d]median values; [e]brain abscess; [f]mean values, dose dependency (0.25–16 mg/kg).

administration of 5 mg/kg itraconazole, indicating active redistribution of itraconazole from the CNS into circulation [67]. In this study, co-administration of verapamil, which is a known P-glycoprotein inhibitor, had no relevant effects on itraconazole plasma concentrations, but significantly increased the ratio of brain tissue to plasma concentrations of itraconazole. Similarly, brain tissue concentrations of itraconazole were significantly higher in P-glycoprotein deficient knockout mice (mdr1–/–) compared to control animals with normal P-glycoprotein expression [67]. These findings were corroborated in a subsequent study evaluating CNS efflux inhibition and brain concentrations of itraconazole in mice intracerebrally infected with *Cryptococcus* neoformans. In this study, pre-treatment with a P-glycoprotein inhibitor (GF120918) resulted in significant increases of itraconazole brain concentrations and there was a trend towards an improved survival in animals with experimentally P-glycoprotein inhibition compared to controls [68]. In humans with or without CNS fungal diseases, only low CSF concentrations of itraconazole have been detected ($\leq$ 0.07 μg/ml, Table 1) and published data on itraconazole concentrations in human brain tissue are lacking [31, 66, 69].

Despite its rather limited CNS penetration, itraconazole displays clinical activity in various CNS fungal diseases (e.g., coccidioidal or cryptococcal meningoencephalitis, cerebral blastomycosis) [70–72]. In addition, responses to itraconazole have been occasionally observed in patients with cerebral aspergillosis [12, 73]. The reasons for this discrepancy are not well understood. The major metabolite of itraconazole, hydroxy-itraconazole, exhibits in vitro antifungal activity, which is comparable to the parent compound [74]. However, published CNS pharmacokinetic data on hydroxy-itraconazole are scarce. In an animal model with experimentally CNS histoplasmosis, mean brain tissue concentrations of itraconazole were below the lower limit of detection, whereas mean concentrations of hydroxy-itraconazole in brain tissue were 0.262 μg/g (Table 1) [75]. Thus, CNS penetration of hydroxy-itraconazole could at least in part explain the activity of itraconazole therapy in CNS fungal diseases.

Posaconazole shares some physicochemical characteristics with itraconazole. Posaconazole is the largest triazole (708 Da) and, like itraconazole, displays pronounced lipophilicity (LogP 6.1) and high protein binding [55]. In an animal study comparing the effects of posaconazole with fluconazole in experimental cryptococcal meningoencephalitis, posaconazole CSF concentrations were undetectable (< 0.05 μg/ml). Interestingly, the efficacy of posaconazole was equivalent to fluconazole in this study [76]. Recently, posaconzole CSF levels have been reported from 3 patients. Concentrations of posaconazole in CSF specimens varied largely and ranged from undetectable (< 0.01 μg/ml) to 0.221 μg/ml with a ratio between CSF and serum concentration ranging from 0.41 to 2.3 (Table 1) [77]. Interestingly, posaconazole was only detectable in 2 patients with CNS infection (1 bacterial brain abscess, 1 brain abscess with ventriculitis caused by *A. fumigatus*), whereas the remaining patient with undetectable posaconazole CSF levels suffered from invasive fungal sinusitis without CNS infection. Until now, data on posaconazole brain tissue concentrations have not been published.

Voriconazole, which is structurally related to fluconazole, is the smallest (349 Da) available compound with activity against *Aspergillus* species. Voriconazole is moderately lipophilic (LogP 2.56) and displays intermediate protein binding [55]. Like fluconazole, voriconazole penetrates well into the CNS. Concentrations of voriconazole were found to be around half and double in CSF and brain tissue of corresponding plasma concentrations under steady state conditions in an early study with guinea pigs using radiolabeled voriconazole [78]. In a subsequent study with healthy guinea pigs, the ratio of the mean voriconazole concentrations in CSF samples to the mean total voriconazole concentrations in plasma was 0.68 (Table 1) [16]. In this animal model, voriconazole peak concentrations in brain tissue were rapidly attained with 6.8 μg/g at 15 minutes and 2.7 μg/g at 1 hour after dosing (10 mg/kg). In humans, reported CSF concentrations of voriconazole ranged from 0.04 to 3.93 μg/ml with a CSF to plasma ratio of 0.22–1 [16, 31, 50, 79]. High brain tissue concentrations (11.8 and 58.5 μg/g) were found in two patients treated with voriconazole for pulmonary aspergillosis [16]. In addition, concentrations of voriconazole in solid and liquid brain abscess material were 1.2, 1.4, and 5.1 μg/ml in two patients, who were treated successfully with voriconazole for cerebral aspergillosis and *Candida* meningoencephalitis (Table 1) [17, 18]. Thus, fungicidal concentrations of voriconazole may rapidly be achieved in CSF, as well as in infected and non-infected brain tissue. It is likely, that the low molecular size and moderate lipophilicity of voriconazole are key factors contributing to the observed, excellent CNS penetration of this compound.

The available echinocandins (anidulafungin, caspofungin, micafungin) are very large molecules (1,140–1,292 Da) and are highly protein bound, which makes a relevant CNS penetration unlikely. Published data on CSF levels are only available for micafungin. Concentrations of micafungin in CSF were not reliably detectable (< 1 μg/ml) in a rabbit model with *Candida* meningoencephalitis, although daily doses of micafungin ranged from 0.25 to 16 mg/kg (Table 1) [80]. Brain tissue concentrations of echinocandins have been investigated in animal studies. In one study with neutropenic rabbits, intravenously challenged with *C. albicans*, mean brain tissue concentrations of anidulafungin were undetectable at daily doses ≤ 0.25 mg/kg, but increased in a dose-dependent fashion from 0.247 to 3.907 μg/g at daily doses ranging from 0.5 to 10 mg/kg [81]. Mean brain tissue concentrations were only ≤ 0.164 μg/g in two studies with healthy rodents treated with 1 and 2 mg/kg caspofungin [82, 83]. Similar low mean brain tissue concentrations (≤ 0.18 μg/g) were found in another study with healthy rabbits treated with 0.5–2 mg/kg micafungin per day for 8 days [84]. However, escalated doses of micafungin resulted in increased brain tissue concentrations in a rabbit model with *Candida* meningoencephalitis. Mean brain tissue concentrations of micafungin increased in a dose dependent fashion and were slightly above 4 μg/g at daily doses of 16 mg/kg [80]. Data on echinocandin concentrations in specimens from human CNS are restricted to a single case report. Concentrations of micafungin in CSF samples from a patient treated with 300 mg micafungin per day for cerebral aspergillosis ranged from 0.007 to 0.017 μg/ml (Table 1) [85].

4 Treatment Strategies

Sole medical treatment of cerebral aspergillosis has produced very disappointing results in the past. In a retrospective survey, only 3 of 34 patients with cerebral aspergillosis responded to amphotericin B- or itraconazole-based therapies [86]. Likewise, an extensive literature-based analysis confirmed the devastating prognosis of cerebral aspergillosis with only a single survivor amongst 141 patients with cerebral aspergillosis [15]. In retrospective series, rapid progression of cerebral aspergillosis was repeatedly observed with median survival times of only 5–10 days [5, 6, 14].

Successful outcomes in single patients treated with high doses of liposomal amphotericin B ($\geq$ 10 mg/kg per day) for cerebral aspergillosis have been repeatedly reported [20, 87]. Enhanced CNS penetration with high doses of liposomal amphotericin B could be anticipated, which possibly contributed to the therapeutic success in these patients, but meaningful data to support this approach are lacking. A recent, randomised trial comparing initial treatment with 10 mg/kg per day liposomal amphotericin B with the standard dose (3 mg/kg per day) of liposomal amphotericin B in patients with invasive filamentous fungal infection showed no significant differences in response rates and survival, but enhanced treatment-related toxicity [88]. However, the number of patients with cerebral aspergillosis included in this trial was low. Successful treatment of cerebral aspergillosis with itraconazole has been repeatedly observed in single patients [11, 12, 69]. Higher daily doses of itraconazole (800 mg) have been proposed based on a retrospective analysis of 12 patients with 3 survivors amongst 4 patients with high dose itraconazole and only one survivor amongst 8 patients treated with daily doses of 400 mg itraconazole [73]. However, a subsequent analysis of these patients disclosed a clear association between survival and presence of granulocytopenia [89]. In view of lacking meaningful data on the use of itraconazole in cerebral aspergillosis from case series, itraconazole should not be considered as the first-choice drug for treating this disease. Treatment with posaconazole has shown a promising 49% response rate in an open-label clinical trial evaluating 39 patients with various CNS fungal diseases. Of these, 29 patients had cryptococcal meningoencephalitis and 4 had cerebral aspergillosis. Only a single patient with cerebral aspergillosis partially responded to posaconazole with deterioration after treatment discontinuation [90]. Like itraconazole, posaconazole is both a substrate and inhibitor of P-glycoprotein [91]. However, it is unknown whether this affects CNS penetration of posaconazole. Data from patients treated with echinocandins for cerebral aspergillosis are scarce. In the first study evaluating caspofungin in patients with refractory IA or intolerance to previous therapies, six patients had cerebral involvement. Of these, two (33%) patients responded to standard doses of caspofungin [92]. In addition, a single patient was treated successfully with 300 mg micafungin per day [85].

Successful outcomes in patients treated with voriconazole for cerebral aspergillosis have been repeatedly reported prior to the publication of the well-acknowledged, pivotal trial, which demonstrated that voriconazole is superior to amphotericin B deoxycholate in treating IA [31, 50, 93]. These promising reports

prompted a retrospective analysis of patients who were treated with voriconazole for cerebral aspergillosis. In this largest series of patients with cerebral aspergillosis published to date, 28 (35%) of 81 patients responded (complete and partial responses) to voriconazole treatment [19]. The majority of patients (96%, 78/81) had received prior antifungal therapy with agents other than voriconazole. In 62 patients, voriconazole was given because prior antifungal therapy was considered ineffective. Twenty-five (31%) of 81 patients were alive at last follow-up and the reported median survival time of these 25 surviving patients was 390 days. Interestingly, 15 patients had a reported median survival of 237 days after termination of voriconazole therapy, which suggests that some of these patients were potentially cured.

Neurosurgical management of patients with cerebral aspergillosis could reduce the mass effects of a fungal brain abscess and allows removal of tissue that might contain viable fungi – which is also of diagnostic importance. In an early review of 26 patients, who were treated successfully for cerebral aspergillosis, antifungal therapy mainly consisted of amphotericin B and itraconazole [20]. It is noteworthy, that 19 (73%) of 26 patients in this review received neurosurgical interventions (abscess resections, craniotomies, stereotactic drainages, intracavitary catheters). In the more recent series of patients with cerebral aspergillosis and voriconazole therapy, 31 patients received neurosurgical management (abscess resection/craniotomy 14, abscess drainage 12, ventricular shunt 4, Ommaya reservoir 1) and this was significantly associated with an improved survival [19]. Stereotactic or neuronavigated abscess aspiration or resection may be considered as less invasive procedures. However, any invasive neurosurgical intervention carries the unwanted risk of haemorrhage, particularly in patients with low platelet counts, and the optimal neurosurgical approach in patients with cerebral aspergillosis needs to be defined. Currently, decisions on neurosurgery in these patients should be made interdisciplinary and weigh the risks of neurosurgery against the potential benefits in individual patients.

5 Future Developments

Although voriconazole treatment improved the prognosis of patients with cerebral aspergillosis, overall treatment results in this CNS fungal disease are still far from satisfactory. The available data indicate that patients with cerebral aspergillosis could benefit from adjunct neurosurgery, but it is unclear which patients benefit from what type of neurosurgical intervention. Thus, further studies are needed to explore a refined neurosurgical approach in different patterns of this CNS fungal disease.

Results from a growing number of studies suggest that combination therapies may be superior over single agent treatments in IA, but the clinical effects of combination therapies in cerebral aspergillosis are largely unknown [94]. Animal models of cerebral aspergillosis have been developed to study the pathophysiology of this CNS fungal disease and to evaluate treatment strategies [95, 96]. A prolonged survival compared to controls was demonstrated with single agent therapies,

including antifungal agents with a low to negligible CNS penetration (e.g., amphotericin B deoxycholate, itraconazole, echinocandins), in an immunocompromised mouse model with cerebral aspergillosis [97–101]. Interestingly, dose escalation of echinocandins (caspofungin, micafungin) did not result in an improved outcome, whereas lipid preparations of amphotericin B showed a dose responsiveness, except for very high doses (> 15 mg/kg) of liposomal amphotericin B [100]. Of various combination therapies tested in this immunocompromised mouse model, only a combination of voriconazole with liposomal amphotericin B significantly prolonged survival compared to single agent therapies. Interestingly, this effect was only seen with suboptimal doses of both combination partners [100]. Future clinical studies should evaluate the effects of escalated doses of selected antifungal agents and antifungal combination therapies to further improve treatment results in patients with cerebral aspergillosis.

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Osteoarticular and Epidural Infections

Emmanuel Roilides and John Dotis

Abstract Osteoarticular infections are uncommon manifestations of invasive aspergillosis. Bone and joint involvement can occur in the setting of local extension or as part of disseminated infection and rarely as primary infection in immunocompromised patients. *Aspergillus fumigatus* constitutes the most common pathogen, and vertebral osteomyelitis is the most frequent bone infection. Delay in the onset of symptoms frequently complicates diagnosis. Isolation of *Aspergillus* spp. from the infected area with the help of imaging studies, mainly magnetic resonance imaging, can establish the diagnosis. Use of sensitive and rapid non-culture-based diagnostic assays, such as detection of galactomannan and β-D-glucan or detection of genomic DNA sequences by polymerase chain reaction (PCR), are helpful. Aggressive antifungal therapy constitutes the cornerstone for the management of osteoarticular aspergillosis. Treatment with amphotericin B, azoles or echinocandins may result in a favourable clinical outcome. Surgery is appropriate for the management of complications or therapeutic failure. Epidural abscesses caused by *Aspergillus* spp. are rare and occur mostly in immunocompromised patients. Spinal epidural abscesses often are developed by contiguous spread from an adjacent tissue involved. Symptoms are nonspecific and biopsy is required for the confirmation of diagnosis. Treatment is multi-modal with one arm being aggressive therapy using antifungal drugs and the other being decompressive surgery.

Keywords Arthritis · Epidural abscess · Invasive aspergillosis · Osteomyelitis · Vertebral osteomyelitis

E. Roilides (✉)
Third Department of Pediatrics, Hippokration Hospital, Aristotle University, Thessaloniki, Greece
e-mail: roilides@med.auth.gr

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Contents

1 Osteoarticular Aspergillosis

Osteoarticular infection constitutes the 4th most common site of invasive aspergillosis (IA) following pulmonary, sinus and cerebral infections [1, 2]. Vertebral osteomyelitis and spondylodiscitis are the most frequent bone infections caused by *Aspergillus* spp [1, 3]. By comparison, joint infections and osteomyelitis of long bones and cranium due to *Aspergillus* spp. have been relatively uncommon [3]. In a series of cases, the lumbar vertebrae were infected in 53%, the thoracic vertebrae in 46%, whereas the cervical vertebrae in only 2% of cases [1]. In one third of the patients no underlying disease was found, although in some of these patients laminectomy and discectomy had been previously performed [1].

Host defense against *Aspergillus* spp. is primarily a function of neutrophils with help from local macrophages and monocytes. Thus, patients at risk to develop osteoarticular and epidural aspergillosis are immunocompromised by previous steroid therapy, transplant immunosuppression and cytotoxic chemotherapy or have neutropenia or chronic granulomatous disease (CGD). *Aspergillus* bone infections are considered to occur through direct extension, traumatic injury, inoculation by a surgical intervention or haematogenous spread, especially in patients with predisposing risk factors or intravenous drug users [1, 4]. In children contiguous spread from an adjacent pulmonary infection is most common, whereas in adults haematogenous spread is the rule. In addition, there is a bimodal age distribution among children with CGD and older adults with other underlying disorders [5].

Aspergillus fumigatus is the most frequent filamentous fungus causing 80–90% of IA cases in immunocompromised patients [6]. Similarly, *A. fumigatus* constitutes the most commonly isolated pathogen in osteoarticular aspergillosis followed by *A. flavus*, *A. nidulans*, *A. terreus* and *A. niger*. However, while *A. fumigatus* is the most frequent cause of IA in CGD patients, *A. nidulans* is more frequent cause of osteomyelitis than *A. fumigatus* in these patients [3]. Dr Segal discusses IA occurring in CGD patients in a separate chapter in this book. Furthemore, *A. flavus* seems to be the main aetiological agent of *Aspergillus* osteomyelitis following trauma, a

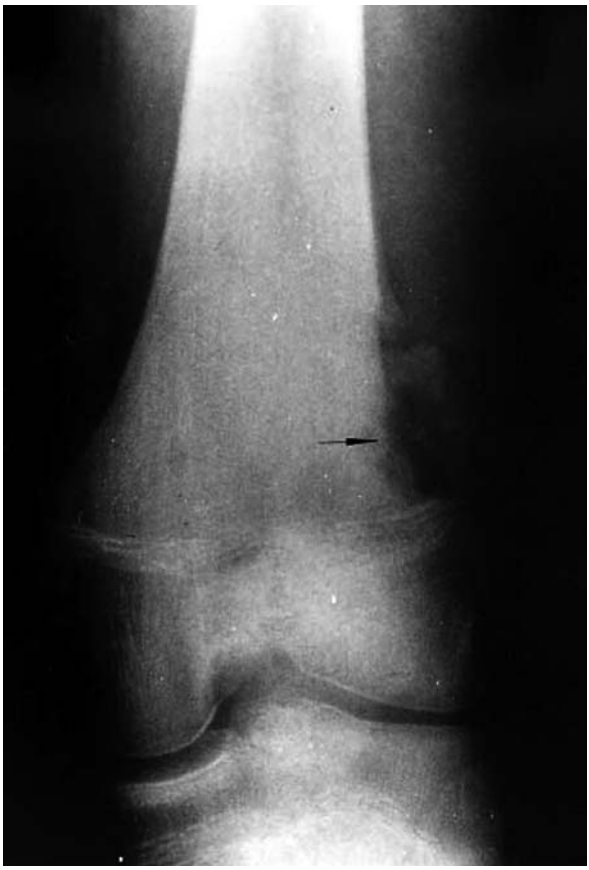

Fig. 1 Roentgenogram of the *lower* part of *left* femur and the knee showing an osteolytic of a patient with CGD and osteomyelitis due to A. nidulans lesion of the distal metaphysis of the femur. *Arrow* indicates the site of the lesion [7]

situation which resembles the elevated frequency at which *A. flavus* causes primary cutaneous aspergillosis and wound infections (Fig. 1) [8].

1.1 Clinical Presentation

Aspergillus osteoarticular infection is more frequently seen as part of disseminated infection than as primary infection. In both cases there is a delay of the onset of symptoms complicating diagnosis. This may be partly associated with the fact that more than 50% of patients do not have fever. Back pain is an almost universal initial symptom of vertebral osteomyelitis, while neurological deficits are present in less than one third of the cases. Indeed, some patients can present with paraparesis, in which neurological prognosis is extremely poor. Lameness with/without pain can be an early sign of an osteoarticular infection especially in patients with predisposing risk factors for IA [9].

In a study of *Aspergillus* spinal osteomyelitis the overall mortality rate was 26.8% [1]. In another study of osteomyelitis in CGD patients due to *A. fumigatus* and *A. nidulans* the overall mortality was 29.2%. However, mortality was 50% amongst patients with osteomyelitis due to *A. nidulans* as compared to no mortality in cases due to *A. fumigatus* [3].

1.2 Diagnosis

The diagnosis of *Aspergillus* osteomyelitis should be based on isolation of *Aspergillus* spp. from the infected area. However, the diagnosis is often delayed due to the late onset of symptoms. An adequate sample is very important for the correct diagnosis. Even though percutaneous needle aspiration may be helpful, many cases remain undiagnosed until a good sample is obtained by open surgery. In addition, histopathological findings of hyphal invasion in tissue specimens constitute an important clue for the diagnosis. Tissue specimens can be either infected bone segments or smear from the infected area [10, 11].

The diagnosis of *Aspergillus* arthritis is primarily based on isolation of *Aspergillus* spp. from the synovial fluid. Histopathologic findings include thickened and hyperemic synovial linings, nonspecific acute and chronic inflammatory infiltrates and erosion of cartilage, but it is not required for the confirmation of the diagnosis. Synovial fluid studies show the classic changes of septic arthritis including elevated protein concentration, high white blood counts, and polymorphonuclear predominance.

In a series of *Aspergillus* osteomyelitis cases, only few of the patients had elevated peripheral white blood cell count (28% of those reported had a white blood cell count greater than 11,000mm^3). However, the majority had an elevated erythrocyte sedimentation rate (although occasional patients were noted with an erythrocyte sedimentation rate < 40 mmh) [1]. These findings suggest that peripheral white blood cell count and erythrocyte sedimentation rate are not specific laboratory surrogate markers to help diagnosis of *Aspergillus* osteoarticular infections.

Imaging studies such as magnetic resonance imaging (MRI) and computed tomography (CT) can be helpful in the diagnosis of osteoarticular infections. In osteomyelitis there are specific findings in contrast to arthritis in which findings are nonspecific and include soft tissue swelling and effusion (Fig. 2). Small areas of radiolucency in the cortex of the metaphyseal zone surrounded by a slight sclerotic reaction are thought to suggest osteomyelitis in this setting. In *Aspergillus* arthritis, an adjacent focus of osteomyelitis is often present. However, although MRI is the most sensitive, specific and accurate imaging tool, it does not allow differentiation of changes due to *Aspergillus* spp. from those due to other infectious agents. The use of routine bone scintigraphy is less important with limited specificity, because some conditions such as trauma and surgery can give false-positive results. Positron emission tomography (PET) with fluorine-18-fluoro-D-deoxyglucose (FDG) seems to be a very promising technique. Uptake of the agent occurs in inflammatory cells such as macrophages and leucocytes. FDG PET combined with CT scan appears

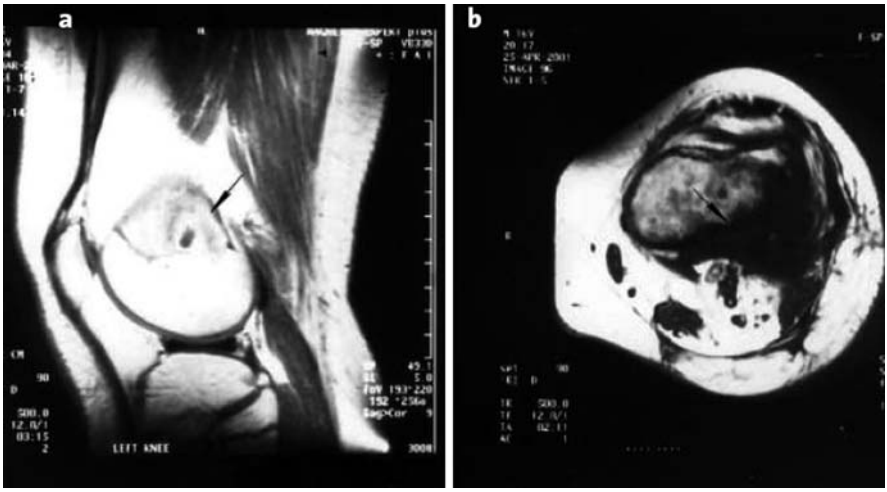

Fig. 2 Magnetic resonance imaging (MRI) of the distal part of *left* femur and the knee in a patient with CGD and osteomyelitis due to A. niduland. *Arrows* indicate the sites of the lesions. (**a**) At the beginning of the infection (day 3 of hospitalization), MRI shows an osteomyelitis of the distal metaphysis of the femur without participation of the surrounding soft tissues. (**b**) On day 60 of hospitalization, MRI shows increased bone destruction compared to the previous examination [7]

particularly promising for delineation of lesions and their concomitant inflammatory or infectious activity [12].

The use of sensitive and rapid non-culture-based diagnostic assays, such as detection of *Aspergillus* cell wall components (galactomannan, β-D-glucan) or detection of genomic DNA sequences may allow a shift in emphasis from empirical to preemptive therapy, especially when substantiated by suggestive radiological findings. These newer tools may be used to confirm a presumed diagnosis of IA including an osteoarticular infection or may identify an infection at the early stage of disease [13].

In particular, the commercially available immunoassays for the detection of galactomannan and β-D-glucan appear promising as these *Aspergillus* antigens can be detected in the blood, urine, joint fluid, cerebrospinal fluid (CSF) and bronchoalveolar lavage of infected patients before conventional methods, even before the onset of clinical symptoms. Serial screening for these antigens in conjunction with radiological studies may be used to guide the initiation of antifungal therapy in patients with risk factors such as neutropenic and haematopoietic stem cell transplant recipients [14].

In addition, polymerase chain reaction (PCR)-based methods have been reported to detect fragments within the 18S rRNA and the 135-bp fragment in the mDNA of *Aspergillus* spp., and a 401-bp fragment in the rDNA complex of *A. fumigatus*. Although PCR assays may prove more sensitive than antigen detection, few of these assays have been tested with body fluids in prospective trials of IA, and reproducibility has not been verified yet. There is also interest in using metabolites, such as mannitol, for diagnostic identification of invasive disease [4].

1.3 Treatment

For the treatment of osteoarticular aspergillosis combined medical and surgical intervention, where feasible, is recommended. Traditionally, the management of osteoarticular infections due to *Aspergillus* spp. has based on conventional amphotericin B and more recently on its lipid formulations. However, conventional amphotericin B has not received reliable evidence for significant concentrations in bone and joint tissues. In relation to this, an apparently poor response to amphotericin B alone has been noted. By comparison, lipid formulations of amphotericin B may achieve better concentrations. In a neonate with *Candida* arthritis receiving daily doses of 3.5 mg/kg of liposomal amphotericin B, random drug concentration was measured in joint fluid and found to be 0.79 μg/ml [15]. New azoles, such as voriconazole and posaconazole have the potential to be successfully used in this role [4]. Although limited experience currently exists with regard to voriconazole for the treatment of *Aspergillus* osteomyelitis, it appears to be effective for this indication [4]. In addition, high concentrations of voriconazole are achieved in the synovial fluid and bone tissues, particularly in the medullar bone [16].

In the past, medical treatment alone using amphotericin B plus 5-flucytosine, or itraconazole alone, or amphotericin B followed by oral itraconazole, has been partially successful in the management of *Aspergillus* spp. osteomyelitis [17]. However, more recently, voriconazole has been successfully used as salvage and primary therapy, either alone or in combination with surgical debridement [18, 19].

Experience on treatment of *Aspergillus* osteomyelitis with posaconazole is very limited. However, this azole can be useful in some cases with progression in which surgery fails or is contraindicated and prolonged treatment with amphotericin B is not efficacious [20]. In addition, echinocandins, mainly caspofungin, have good activity against *Aspergillus* spp., but penetration to bone and joint tissues is likely to be poor. It should not be used as monotherapy ahead of alternatives, but may have a role as part of a combination therapy with an agent with good bone penetration.

In the case of *Aspergillus* arthritis most of the successfully treated cases have responded to combined medical therapy and drainage of the joint. In the majority of reported cases, amphotericin B has been used as primary therapy, with azoles being less commonly used in this role [21].

Haematopoietic growth factors and T helper-1 cytokines may play an important adjunctive role in the treatment of IA especially in patients with CGD. In parallel with the use of antifungal agents, improving immune response by either exogenous modulation of enhancing/regulatory cytokines or by transfusion of cytokine-elicited allogeneic phagocytes appears to be a promising adjunct to antifungal chemotherapy [3, 2].

Careful assessment before and after discontinuation of treatment is mandatory in the management of osteoarticular aspergillosis. While optimal duration of therapy is a difficult issue, treatment for a minimum of 6–8 weeks is warranted in immunocompetent patients. In immunocompromised patients, consideration of long-term suppressive therapy or treatment throughout the duration of immunosuppression is appropriate. A prolonged treatment of months is recommended especially due to the

availability of well-tolerated azoles. It is worth noting that after apparent cure and discontinuation of the treatment the infection often relapses [4].

1.4 Surgical Management

Surgical intervention is limited to the management of complications or for therapy failure. However, surgical intervention can rapidly reduce fungal load, remove necrotic material and abscesses increasing drug penetration. It can also prevent mass effects of the infections, such as paralysis, which may occur as a result of the mass effect of spinal abscesses on the spinal cord. For that reason, it is undertaken mainly to relieve compression of the spinal cord or to drain epidural or paravertebral abscesses and to improve spinal stability. In addition, some infections are refractory to antifungal treatment, and require combined medical-surgical intervention to be cured [12, 17].

2 Epidural Infections due to *Aspergillus*

While cerebral aspergillosis occurs in about 20% of all cases of IA, usually associated with disseminated disease, epidural infections are rare events in both adults and children. Epidural abscesses are even more uncommon as the initial manifestation of IA and in children there may be pathophysiologic processes distinct from adults [23].

The species involved include *A. fumigatus*, *A. flavus*, *A. niger* and *A. nidulans*. Patients at risk for developing an epidural abscess are immunocompromised patients with malignancies undergoing chemotherapy, acquired immunodeficiency syndrome (AIDS) or CGD, those on steroids for other pre-existing diseases such as asthma and those with fungal balls elsewhere in the body [23–25]. However, there are rare cases in which epidural involvement has been noted in immunocompetent patients.

The three possible origins of an epidural abscess are: (i) contiguous spread from an adjacent bone or involved organ most commonly the lungs; (ii) haematogenous spread from a distant focus; and (iii) iatrogenic inoculation. Most spinal epidural lesions are granulomatous and arise from contiguity, whereas haematogenous dissemination results in abscess formation. Cord compression in spinal aspergillosis may be a result of a combination of vertebral osteomyelitis, granuloma, and/or abscess formation in either the epidural or disc spaces. In addition, D4–D5 level of spinal cord appears to be frequently affected by contiguous spread from the lungs.

2.1 Clinical Presentation

The clinical presentation of epidural abscesses is generally nonspecific characterized by focal neurological signs. Spinal epidural abscesses are manifested with

complications varying from rapidly progressive paraparesis to gradually progressive myelopathy without sensory loss or dysautonomia [26]. In some cases sensory loss has also been noted. In contrast, epidural brain abscesses commonly present with minor neurological symptoms often following a trauma or a brain surgery. However, the time from the onset of symptoms to the definite diagnosis can range between 1 and 7 months due to the fact that the signs and symptoms in most cases are not specific [25].

2.2 Diagnosis

Diagnosis depends on a high index of suspicion with biopsy being the only definitive approach. A good biopsy specimen can confirm the diagnosis after histological examination and culture. Histopathologically, marked necrosis and haemorrhage in the spinal cord can be observed with extensive granulomatous and purulent lesions in the epidural and subdural spaces. *Aspergillus* hyphae probably penetrate the myelin sheath and result in myelomalacia. However, cultures of blood, sputum or bone marrow are rarely positive [25]. Although analysis of CSF is usually nondiagnostic, detection of *Aspergillus* cell wall components such as galactomannan and β-D-glucan or detection of genomic DNA sequences with PCR methodology in CSF seems to be very promising for the early diagnosis.

2.3 Treatment

Therapy is multi-modal with one arm being decompressive surgery with or without spinal stabilization, which usually provides the histopathology in cases where the diagnosis is not established; the other arm being aggressive drug therapy. However, most of the experience in managing epidural aspergillosis is based on single case reports and brief case series.

Systemic antifungal agents, such as mainly amphotericin B, itraconazole and flucytosine or combinations of these drugs have been found to be effective in few cases but even with increased doses patients have dismal prognosis [27, 28]. This is probably due to the fact that the nature of the disease is widespread, and surgery is unlikely to clear involved tissues from the disease pathology. No data exist so far with use of newer triazoles and echinocandins.

Surgical intervention should be limited to cases of failure of medical management [29]. However, excision of the lung lesion and a decompressive laminectomy along with anti-*Aspergillus* therapy in pulmonary aspergillosis and cord compression due to its extension has been reported [28, 30]. Furthermore, the role of simultaneous spinal stabilization is unclear as certain reports favor the use of simple decompression of purulent, necrotic and exuberant granulation tissue. This perception results from the observation that more extensive procedures, especially in immunocompromised patients, would result in more complications. Mortality is about 95% [24]. Yet, there may be an age-related difference in surgical

outcome with younger children faring better than adults irrespective of their immune status.

Despite the development of new antifungal drugs, diagnostic delays or difficulties adversely affect the prognosis of *Aspergillus* epidural abscesses. Dismal outcome is the usual consequence of these infections.

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Aspergillosis of the Digestive Tract

Philippe Eggimann, David Bracco, and Didier Pittet

Abstract Most cases of invasive aspergillosis are reported as a primary disease of the lung from which disseminated aspergillosis is thought to occur as a result of vascular invasion with subsequent bloodstream dissemination. Currently, portals of entry other than sinuses and/or the respiratory tract remain speculative. However, spores may not only be inhaled, but also ingested and cases of primary invasive aspergillosis of the digestive tract have been reported. We review 2 amongst 43 and 24 reported cases of primary aspergillosis of the upper and lower digestive tract, respectively, for which detailed data are available. This analysis provides convincing evidence to support that the digestive tract may represent a portal of entry for *Aspergillus* species in immunocompromised patients.

Keywords Aspergillosis · Invasive aspergillosis · Digestive aspergillosis · Immunosuppression · Fungal infection · Oesophagitis · Typhilitis

Contents

1 Introduction

The spectrum of infections related to *Aspergillus* species in humans is wide. It includes benign and usually self-limited forms such as superficial skin infections (ecthyma infectiosum), allergic bronchopulmonary aspergillosis with a variety of

P. Eggimann (✉)
Department of Intensive Care Medicine and Burn Center, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland
e-mail: philippe.eggimann@chuv.ch

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clinical expressions, fungal balls complicating pre-existing lung cavities, and life-threatening invasive diseases with secondary dissemination in immunocompromised patients [1–3]. *Aspergillus* spores are ubiquitous. Once aerosolised, they may colonise the airways and related structures such as the nose and facial sinuses [1, 2]. Further immunosuppression, particularly severe and prolonged neutropenia, markedly increases the risk for invasive disease characterised by tissue invasion and eventual secondary bloodstream dissemination [1–3]. *Aspergillus fumigatus, A. flavus* and *A. niger* are responsible for most invasive diseases [4–6]. Aspergillosis is reported to occur in fewer than 1% of patients infected by human immunodeficiency virus (HIV), 1–5% of liver transplant recipients, 3–7% of allogenic haematopoietic stem cell transplantation (HSCT), but up to 10% in patients with haematological malignancies or lung transplants, and as high as 14% of heart transplant recipients [6–10] .

In immunocompromised patients, invasive pulmonary aspergillosis (IPA) accounts for 90–98% of infections. Extra-pulmonary aspergillosis may be present in 25–60% of cases and is almost universally described in the context of disseminated diseases [11–18]. Although rarely documented before autopsy, these conditions usually result from bloodstream dissemination secondary to vascular invasion at the primary site of infection [19, 20]. Isolated extra-pulmonary aspergillosis located in the central nervous system, the skin, the liver, and the digestive tract has only been mentioned in case reports or small cases series, and potential portals of entry other than the respiratory tract are speculative [3, 6, 21, 22].

In this chapter, we identify digestive tract involvement reported in large series of more than 25 cases of invasive aspergillosis (IA) and published cases with a possible diagnosis of aspergillosis at this site. We searched the English language literature from January 1970 to December 2007 for reports of aspergillosis with digestive tract involvement using the MEDLINE (National Library of Medicine, Bethesda, MD) database with key words “aspergillosis”, “invasive aspergillosis”, “disseminated disease”, “extra-pulmonary aspergillosis” and “digestive, tract aspergillosis” “bowel, aspergillosis” “esophagus, aspergillosis” and the reference lists of retrieved articles, including reviews.

2 Aspergillosis of the Digestive Tract

Extra-pulmonary IA is almost exclusively considered as a pulmonary disease with secondary haematogenous dissemination [3, 10]. Fungal infections of the upper or of the lower digestive tract are rare [21]. However, *Aspergillus* spores are not only inhaled but also ingested, and isolated aspergillosis of the digestive tract has been described [23]. In two reviews covering more than 3,000 cases collected from several hundreds of articles over three decades, Denning suggested in the early 1990s that the gastrointestinal tract may be considered as a potential portal of entry for *Aspergillus* spp., although never demonstrated [6, 8].

Aspergillus peritonitis is described in patients with peritoneal dialysis [24, 25]. This is also the case in immunocompromised patients after liver transplant. In these

cases of localised aspergillosis, colonisation may have occurred during surgery and they could be considered as surgical infections [26, 27]. We will not review this particular topic in this chapter.

Of 140 clinical studies retrieved from our review of the literature, 53 included more than 25 cases of IA with some details on the distribution of organs involved ($n = 6{,}086$). An involvement of the digestive tract is reported in 183 cases, representing 5.7% of the 3,184 cases included in 32 series out of these 53.

There were 13 autopsy series [11–13, 16, 28–36] (Table 1), 17 retrospective series [10, 17, 20, 22, 37–48] ulcreation (Table 2) and 21 prospective series [49–67] (Table 3).

Mortality averaged 71% (27–88%) in retrospective, and 48% (32–64%) in prospective series. This may indicate that patients included in prospective studies were less critically ill, or may have benefited from early presumptive antifungal therapy. The case-fatality rate was recently confirmed to be highest in HSCT (87%) and amongst patients with central nervous system involvement (88%) [68]. A large number of patients with solid organ transplant and combined antifungal treatments may have contributed to the higher survival rate reported in the most recent prospective series.

IPA accounted for a majority of cases. Bowel involvement during disseminated disease was reported in only 1 of 671 cases in prospective series and in only 1.3% of cases ($n = 18$) in retrospective series (0.3–28%). However, it was reported in 13% (2–53%) of cases included in the autopsy series (164 of 1,237 cases). Apart from nine cases for which no details were available, invasion of the digestive tract was always reported in the context of disseminated disease [11, 12, 16, 17, 30, 37, 66, 67, 69–72]. The upper digestive tract and, in particular, the oesophagus, is the most common involved site of the digestive tract. Most histological lesions consist of ulcerative lesions which may result in haemorrhage and perforation. In these series, although responsible for signs and symptoms such as abdominal pain and haemorrhage, involvement of the digestive tract was only confirmed at autopsy. However, several authors suggested that aspergillosis should be included in the differential diagnosis of digestive haemorrhage in severely immunocompromised patients [12, 13, 16, 73].

A mixed infection with *Candida* species was reported in series where more details were available [12, 21]. A digestive haemorrhage was reported in most cases, but was not considered specific.

2.1 Primary Digestive Aspergillosis

In the above-mentioned literature review, we identified cases where detailed descriptions allowed a more specific analysis. Cases were included if the diagnosis of primary invasive aspergillosis of the digestive tract was defined as histological evidence of *Aspergillus* hyphae in a segment of the bowel biopsy with mucosal alteration and tissue destruction and/or tissue invasion with microvascular involvement.

Table 1 Invasive aspergillosis: organ distribution amongst *autopsy series* including more than 25 cases, 1970–2007

References	Period studied	*n*	Mortality y (%)	Organ distribution						
				Lung	Disseminated	CNS	Upper digestive	Lower digestive	Primary digestive	Total digestive
[12][a]	1953–1968	98	100	92 (94%)	34 (35%)	13 (13%)	NS	NS	7 (7%)	21 (21%)
[16]	1964–1971	93	100	90 (98%)	23 (35%)	9 (10%)	3 (3%)	5 (5%)	1 (1%)	9 (10%)
[11]	1980–1989	32	100	32 (100%)	20 (63%)	16 (50%)	10 (31%)	5 (16%)	0	15 (53%)
[28]	1980–1988	137	100	107 (78%)	NS	20 (15%)	13 (10%)	6 (4%)	0	19 (14%)
[29]	1978–1992	119	100	112 (94%)	63 (53%)	28 (24%)	NS	NS	0	11 (17%)
[30]	1994–1995	27	100	23 (85%)	16 (59%)	12 (44%)	5(19%)	2 (7%)	1 (4%)	7 (26%)
[31]	1993–1996	48	100	41 (85%)	28 (58%)	21 (44%)	0	1 (2%)	0	1 (2%)
[33]	1997–1998	27	100	26 (96%)	8 (30%)	2 (7%)	1 (4%)	0	0	1 (4%)
[32][b]	1981–2000	71	100	68 (96%)	47 (66%)	42 (59%)	8 (12%)	5 (8%)	0	10 (14%)
[13][c]	1980–1998	107	100	104 (98%)	55 (51%)	22 (21%)	21 (20%)	12 (11%)	0	25 (23%)
[34]	1989, 1993, 1997	412	100	310 (75%)	NS	16 (4%)	19 (5%)	22 (5%)	0	41 (10%)
[35]	1991–2002	39	100	39 (100%)	8 (21%)	5 (13%)	2 (5%)	2 (5%)	0	4 (10%)
[36]	1994–2003	27	100	25 (93%)	NS	2 (7%)	0	0	0	0
Total		1,237	100	870 (86%)	302/661 (46%)	208 (17%)	82/1,118 (7%)	60/1,145 (5%)	9 (0.7%)	164 (13%)

Legend: CNS, central nervous system; NS, not specified.

[a]Of the 21 patients with involvement of the digestive tract, 11 had disease in multiple sites. Oesophagus 10, stomach 5, large intestine 9, small intestine 7.

[b]Concomitant upper and lower digestive tract reported in three cases.

[c]Information extracted from several publications on the same cohort of patients [73, 120]. Not included 2 cases with isolated tracheal invasive aspergillosis and 2 cases with disseminated aspergillosis with involvement of the pharynx ($n = 2$) and the tongue ($n = 1$). Concomitant upper and lower digestive tract reported in several cases.

Table 2 Invasive aspergillosis: organ distribution amongst *retrospective studies* including more than 25 patients, 1970–2007

References	Period studied	*n*	Mortality	Organ distribution						
				Lung	Disseminated	CNS	Upper digestive	Lower digestive	Primary digestive	Total digestive
[17]	1971–1976	91	88%	83 (91%)	20 (22%)	13 (14%)	NS	NS	1 (1%)	6 (6%)
[10]	1974–1989	93	62%	87 (95%)	43 (47%)	21 (23%)	NS	NS	1 (1%)	1 (1%)
[37]	1988–1994	25	92%	25 (100%)	8 (32%)	3 (12%)	2 (14%)	4 (28%)	4 (28%)	8 (32%)
[38]	1988–1996	37	27%	37 (100%)	8 (22%)	0	0	0	0	0
[22][a]	1989–1993	35	97%	33 (94%)	8 (23%)	1 (3%)	0	0	0	0
[39][b]	1962–1996	66	85%	46 (70%)	23 (35%)	9 (14%)	0	0	0	0
[40][c]	1990–1998	144	76%	114 (79%)	26 (18%)	0	0	0	0	0
[50][d]	1994–1995	595	NS	444 (74%)	114 (19%)	34 (6%)	NS	NS	NS	NS
[41][e]	1984–1996	31	45%	28 (90%)	7 (23%)	4 (13%)	0	0	0	0
[44][f]	1985–1997	34	29%	30 (88%)	13 (25%)	2 (6%)	0	0	0	0
[42]	1989–1999	61	48%	56 (92%)	20 (33%)	5 (8%)	NS	NS	NS	NS
[43][g]	1988–1997	391	74%	332 (85%)	NS	37 (9%)	1 (0.3%)	5 (1%)	5 (1%)	11 (2%)
[46][h]	1985–1999	342	82%	297 (87%)	NS	NS	0	1 (0.3%)	1 (0.3%)	0
[45][i]	1990–1999	33	61%	30 (91%)	NS	1 (3%)	0	0	0	0

Table 2 (continued)

References	Period studied	*n*	Mortality	Organ distribution						
				Lung	Disseminated	CNS	Upper digestive	Lower digestive	Primary digestive	Total digestive
[47]	1998–1999	102	48%	87 (85%)	7 (7%)	1 (1%)	0	0	0	0
[48]	2002	51	62%	42 (82%)	7 (14%)	NS	NS	NS	NS	NS
[20][j]	1999–2003	91	70%	68 (75%)	14 (15%)	3 (3%)	NS	NS	NS	NS
Total		2,222	1,158/1,627 (71%)	1,839 (83%)	318/1,456 (22%)	134/1,829 (7%)	3/1,240 (0.2%)	10/1,485 (0.8%)	12/1,485 (0.8%)	26/1,424 (1.8%)

Legend: CNS, central nervous system; NS, not specified.

[a]Two patients had both upper and lower digestive tract involvement.

[b]Out of 9,500 children and adolescent receiving therapy for malignancies or haematologic disorders.

[c]144 patients out of 228 cases of aspergillosis for whom details were available among 35,252 HIV patients included in the Adult and Adolescent Spetrum of HIV Disease Project.

[d]Large retrospective international survey from the Aspergillus Study Group on current treatment practice and outcome for invasive aspergillosis. A treatment failure was scored for 340 of 595 patients (57%). Crude death rate was 51% for the subgroup of 338 patients treated with ampho B, ampho B to itraconazole and itraconazole, respectively.

[e]Cases collected from 15 centres among patients with multiple myeloma.

[f]Amongst 1963 thoracic organ transplant.

[g]From 14 centres from the GIMEMA Infection Program. Filamentous fungi including 296 *Aspergillus* spp, 45 *Mucorales* spp, 6 Fusarium spp, 4 other filamentous fungi, 40 non-identifiable filamentous fungi.

[h]Data extracted from the 218 and 124 patients with proven and probable invasive aspergillosis, respectively.

[i]Among 2046 solid organ transplant recipients (695 kidney, attack rate: 0.4%; 686 heart, attack rate: 0.4%; 439 liver, attack rate: 0.7%; 183 lung, attack rate: 12.8%; 38 kidney-pancreas; 5 heart-lung).

[j]Including 86 invasive aspergillosis and 5 other moulds infections (3 *Fusarium* spp., 1 *Zygomycetes* spp. and 1 *Scedosporidium* spp.).

Table 3 Invasive aspergillosis: organ distribution amongst *prospective studies* including more than 25 patients, 1970–2007/

References	Period studied	*n*	Mortality	Organ distribution						
				Lung	Disseminated	CNS	Upper digestive	Lower digestive	Primary digestive	Total digestive
[66]	NS	32	34%	29 (91%)	NS	0	0	0	0	0
[67]	NS	76	32%	51 (67%)	25 (33%)	8 (11%)	NS	NS	NS	NS
[110][a]	NS	125	34%	90 (72%)	24 (19%)	8 (6%)	0	0	0	0
[49]	1993–1994	123	64%	106 (87%)	33 (27%)	10 (8%)	0	0	1 (0.8%)	1 (0.8%)
[54]	1993–1996	87	63%	67 (77%)	20 (23%)	8 (9%)	NS	NS	NS	NS
[50]	1994–1995	595	51%	330 (56%)	148 (25%)	34 (6%)	NS	NS	NS	NS
[51]	1994–1996	116	58%	81 (70%)	6 (5%)	19 (16%)	0	0	0	0
[53]	1993–1997	174	53%	107 (62%)	NS	12 (7%)	NS	NS	NS	NS
[52]	1997–2000	277	35%	240 (87%)	15 (5%)	10 (4%)	NS	NS	NS	NS
[55]	NS	42	40%	32 (76%)	7 (17%)	6 (14%)	0	0	0	0
[56]	NS	83	48%	64 (77%)	13 (16%)	5 (6%)	NS	NS	NS	NS
[57]	2001–2004	32	47%	32 (100%)	5 (15%)	1 (3%)	0	0	0	0
[58]	1999–2002	47	49%	37 (93%)	4 (10%)	1 (3%)	NS	NS	NS	NS
[58]	2003–2005	40	33%	41 (87%)	6 (13%)	4 (4%)	NS	NS	NS	NS
[59]	1998–2002	225	56%	183 (81%)	18 (8%)	1 (0.4%)	NS	NS	NS	NS
[60]	1993–1995	36	NS	25 (69%)	NS	3 (8%)	NS	NS	NS	NS
[61]	2003–NS	40	33%	37 (93%)	4 (10%)	1 (3%)	NS	NS	NS	NS
[62]	2003–2004	53	45%	43 (81%)	7 (13%)	2 (4%)	NS	NS	NS	NS
[63]	1999–2001	193	74%	146 (76%)	17 (9%)	6 (3%)	NS	NS	NS	NS
[64]	2003–2004	201	34%	180 (90%)	7 (4%)	NS	0	0	0	0
[65]	2004–2005	30	10%	30 (100%)	NS	NS	NS	NS	NS	NS
Total		2,627	1,256/2,591 (48%)	1,951 (74%)	359/2,355 (15%)	139/2,396 (6%)	0/671	0/671	1/671 (0.1%)	1/671 (0.1%)

Legend: CNS, central nervous system; NS, not specified.

[a]Including 1 case with *Aspergillus* peritonitis, not reported as digestive tract involvement in the table.

2.1.1 Oral Cavity

Few cases of primary invasive oral aspergillosis or stomatitis are reported in the literature [74]. However, in any case, it should be investigated if it does not correspond or result from a local invasion from a paranasal sinus aspergillosis which are more commonly reported [75, 76]. Myoken et al. reported a unique series of 12 cases of true primary *Aspergillus* stomatitis collected in a single centre over 8 years [23]. The infection presented in neutropenic patients during induction chemotherapy for haematological malignancies as solitary painful oral cavity ulceration covered with a gray necrotic pseudomembrane. All patients had a history of previous periodontal infection, with a lesion originating from the marginal gingival. It spread to the adjacent tissues and invaded the alveolar bone in half of all cases. Overall, combined antifungals and surgery cured the infection in 10 of the 12 patients. Surgery was not possible in two patients who died due to the rapid progression of the disease with extensive noma-like (cancrum oris) infections. In contrast to other types of IA, *A. fumigatus* is usually the most frequent pathogen which was responsible for most cases, *A. flavus* was responsible for 9 of these 12 cases. The authors hypothesise that this may be possibly due to food contamination, already described elsewhere by another group [77]. The larger size of *A. flavus* conidia (3–6 μm in diameter in comparison to 2–3.5 μm for *A. fumigatus*) might explain why *A. flavus* is usually involved in cases of aspergillosis involving the sinuses, skin and the oral mucosa, while *A. fumigatus* conidia may reach the alveoli more easily to cause IPA [78].

2.1.2 Upper Digestive Tract

Involvement of the upper digestive tract is usually reported with clinical signs and symptoms related to oesophagitis and gastritis. The investigations showed locally IA, mainly in the context of a disseminated disease for which this represents the first clinical manifestation [79, 80]. However, in contrast to *Candida* spp., *Aspergillus* spores might be less likely to survive or meet favourable conditions to develop on the mucosal surfaces of the digestive tract. In this context, ulcerations of the mucosa may be further colonised by *Aspergillus* spores [23].

Of 15 cases of possible primary IA of the upper digestive tract, defined as structures common to the upper respiratory tract, the oesophagus and the stomach, details were available for 12 patients [81–91] (Table 4). These cases of primary aspergillosis of the upper digestive tract are relatively homogenous. Eleven of 12 patients (92%) were severely immunocompromised. Eight received aplastic chemotherapy, two were on T-cell suppressors and one was at a terminal stage of an HIV infection. Almost all had received broad spectrum antibiotics and antifungal prophylaxis, potentially active against *Aspergillus,* is reported in only two cases. Identification of the strain was reported in only one of 12 cases, and it is not possible to comment on the fact that it was an *A. terreus* which is only rarely reported as compared to *A. fumigatus* [92]. A secondary dissemination was documented in only three patients (25%). Antifungal therapy was started in only 6 of 12 patients (50%) and combined in two, but the impact of therapy cannot be assessed in these cases. Despite uniform

Table 4 Selected characteristics of 12 patients with documented primary invasive aspergillosis of the upper digestive tract

	Obrecht [81]	Cappell [82][a]	Nakamura [83]	Prescott [84]	Yoo [85]	Choi [86]	Komanduri [87][b]	Sanders [88][c]	Bergman [89][d]	Bergman [89][d]	Chionh [90]	Alioglu [91][e]
Age (years)	22	50	16	62	50	35	18	41	79	29	71	15
Underlying disease	AML	AIDS	ALL	CLL	AML (M2)	AML (M4)	AML	multiple trauma, splenectomy	AML (M2) (relapse)	AML (M3)	AML (M1)	AML (M7) (relapse)
Documentation	Histology at autopsy	Histology at autopsy	Histology at autopsy	Histology at autopsy	Histology of biopsies	Histology of biopsies	Histology of biopsies	Histology + cultures	Cytology	Cytology	Histology of biopsies	Histology of biopsies
Aspergillus species identified	–	–	–	–	–	–	–	*A. terreus*	–	–	–	–
Previous infection	Oesophagitis	None documented before autopsy	Fever of unknown origin	Cholecystitis	None	Pulmonary tuberculosis (surgically excised)	Fever of unknown origin	Bacteraemia and multiple organ failure	Not specified	Not specified	Fever of unknown origin	Fever of unknown origin
Microorganisms	*Candida albicans*, Herpes virus	none		none	none			not specified	not specified	not specified	none	none
Chemotherapy	cytarabine, daunorubicin, 6-thioguanin	None	cytarabine, daunorubicin, vincristine, prednisone	azathioprine, prednisone	cytarabine, idarubicin, 6-thioguanin	cyclophosphamide, methotrexate, cyclosporine	cytarabine, mitoxantrone, amifostine	High dose steroids	Not specified	Not specified	cytarabine, fludarabine	cytarabine, idarubicin, fludarabine
Days after chemotherapy	34	–	Not specified	2 months	30	25	Not specified	Several weeks	Not specified	Not specified	24	Not specified
Neutropenia at diagnosis	No	No	No	No	Yes	No	Yes	No	Yes	Yes	No	Yes
Antifungal prophylaxis	Oral-mycostatin	No	No	No	Not specified	Not specified	fluconazole	Yes	Not specified	Not specified	fluconazole	L-ampho b

Table 4 (continued)

	Obrecht [81]	Cappell [82][a]	Nakamura [83]	Prescott [84]	Yoo [85]	Choi [86]	Komanduri [87][b]	Sanders [88][c]	Bergman [89][d]	Bergman [89][d]	Chionh [90]	Alioglu [91][e]
Number of previous antibiotics	1	Several	4	Several	None	4	2	Several	Not specified	Not specified	3	2
Antimicrobial agents	acyclovir	Not specified	mezlocillin gent ceftazidime van-comycin	Not specified	–	isoniazid rifampicin ethambutol pyrazi-namide	cefepime vancomycin	Not specified	Not specified	Not specified	cefepime vancomycin meropenem	efepime amik-acin
Clinical diagnosis	Oesophago-tracheal fistula	Fever of unknown origin	Fever of unknown origin	Fever of unknown origin	Oesophagitis	Oesophagitis	Oesophagitis	Gastritis	Oesophagitis	Oesophagitis	Gasttitis	Oesopha-gitis
Clinical pre-sentation	Sore throat Cough Odynopha-gia	Weight loss vomiting persistent fever	Epigastric discomfort dysphagia	Malabsorption Diarrhoea Abdominal pain	Odynophagia	Odynophagia nausea vomiting	Dysphagia	Upper gastroin-testinal bleeding	Oesophagitis	Oesophagitis	Epigastric pain haematheme-sis	Dysphagia low back pain
Final diagnosis	Oesotracheal fistula	Oesophagitis (ulcer) + large and small bowel	Oesophagitis Oesoaortic fistula	Oesogastric Inflitration	Oesophagitis (ulcer)	Oesophagitis (ulcer)	Oesophagitis (exophytic lesion oesoatrial fistula)	Pseudomem-branous gastric aspergillosis	Oesophagitis (ulcer)	Oesophagitis (ulcer)	Oesophagitis (exophytic lesion)	Oesopha-gitis (ulcer)
Serology	–	–	–	Negative	–	–	–	–	–	–	–	–
Antige-naemia	–	–	–	–	–	–	–	–	–	–	–	–
Secondary dissemina-tion	No	No	No	Lung	No	No	Heart, CNS	No	No	Spleen	No	No

Table 4 (continued)

	Obrecht [81]	Cappell [82][a]	Nakamura [83]	Prescott [84]	Yoo [85]	Choi [86]	Komanduri [87][b]	Sanders [88][c]	Bergman [89][d]	Bergman [89][d]	Chionh [90]	Alioglu [91][e]
No. of surgical interventions	0	0	0	0	0	0	0	1	0	0	0	1
Bowel excision	–	–	–	–	–	–		Gastrectomy	–	–	–	Gastrectomy
Antifungal treatment	No	No	No	amphotericin B	amphotericin B	amphotericin B	No	Combined	No	No	voriconazole	Combined
Outcome	Death	Death	Death	Death	Survival	Survival	Death	Death	Death	Death	Survival	Death

Legend: ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; CNS, central nervous system; LLC, chronic lymphocytic leukaemia.

[a]Autopsy revealed *Mycobacterium avium* infection of the lungs, liver, kidney, spleen and bone marrow. The brain was infected with cytomegalovirus and *Toxoplasma gondii* and contained a primary lymphoma. Diffuse ulceration of the oesophagus, stomach, small intestine and colon with histological demonstration of dichotomous branched hyphae.

[b]The patient was not treated. He died from cardiac arrest 24 h after the diagnosis by endoscopy, before the results of the biopsies.

[c]Exposure to empirical antifungal treatment. Combined antifungal treatment: amphotericin B + itraconazole for pseudomembranous gastric aspergillosis. Patient died in multiple organ failure after withdrawal of care.

[d]Both patients did not respond to chemotherapy.

[e]Combined antifungal treatment: L-amphotericin B + caspofungin. Clinical cure, death from non responding AML with multiple organ leukaemic infiltration.

and clear clinical manifestations suggestive of upper digestive tract involvement that should lead, in theory, to earlier investigations and diagnosis, the overall mortality is high (75%).

2.1.3 Lower Digestive Tract

Amongst 890 consecutive autopsies performed amongst patients with malignancies, Prescott et al. reported 14 cases of fungal infections involving the lower intestinal tract. A mixed infection with *Candida* species was reported in the 10 cases with *Aspergillus* infection. Coexistent lung involvement was present in seven patients [21].

Details were available for 24 [72, 93–108] of 43 cases of possible primary IA of the lower digestive tract identified in the literature (Table 5) [10, 12, 16, 17, 30, 43, 49, 67, 70, 71, 82, 109–112].

Sixteen of 24 patients with bowel involvement presented with acute haematologic malignancy, and 15 received high-dose cytarabine or other combination therapies known to be associated with diffuse mucositis. Fifteen were neutropenic and six presented a prior episode of bacteraemia during neutropenia. Twenty (83%) received a combination of antibiotics with broad spectrum coverage within a few weeks before the onset of the abdominal complication, and all but one presented with abdominal symptoms suggestive of typhlitis or peritonitis. A digestive haemorrhage was present in only four patients. Laparotomy was performed for acute peritonitis and showed transmural necrosis of the small bowel requiring segmental resection in all cases except one AIDS patient in whom the diagnosis of extended *Aspergillus* ulcers of both the upper and lower digestive tract was only made at the autopsy. Histology consisted in multiple lesions from superficial ulceration to transmural necrosis and vascular thrombosis with tissue invasion by branched hyphae of *Aspergillus* spp. was present in all cases (Fig. 1). All cases were characterised by the absence of pulmonary disease at the time of histologically-confirmed digestive aspergillosis. Serology and antigenemia was either not available or not performed in the majority of cases. A strain could be identified in only 10 of these 24 cases (42%), A. fumigatus in 8 and *A. flavus* in two cases. This distribution did not differ from that of IPA [92, 4–6]. Antifungals were administered to 15 patients. Overall, death occurred in 15 of 24 patients (63%), for a fatality rate lower than reported for IA of the upper digestive tract. Of note, 9 of 16 patients (56%) for whom information about antifungal treatment is available survived. This survival rate, which is comparable to that of those reported in prospective series, suggests that aggressive management combining antifungals with surgery should be proposed in aspergillosis of the bowel.

As mentioned, *Aspergillus* spores may not survive or meet favourable conditions to develop on the mucosal surfaces of the digestive tract (Fig. 2). However, large ulcers, such as those described as occurring after high-dose cytarabine-related mucositis, may become colonised with *Aspergillus* spores [23]. This may also be the case for patients developing a neutropenic or necrotising enterocolitis, also referred to as typhlitis. This defines an abdominal complication occurring during neutropenia and induced by chemotherapy [113]. It complicates 5–30% of treatments, including

Table 5 Selected characteristics of 24 patients with documented primary invasive aspergillosis of the bowel

	Scully [93]	Weingard [94]	Rogers [109]	Cappell [82]	Cohen [95]	Marterre [96]	Catalano [97]	Ibrahim [101]	Shah [98]	Sousa [99]	Safieddine [112]	Tay [102]
Age (years)	4 weeks	38	21	50	33	8	58	21 months	68	21	62	57
Underlying disease	AML	ALL	AML	AIDS	AML	AML	AML	None	AML	Aplastic anemia	Lymphoma[a]	Breast tumour
Documentation	Histology	Histology + culture	Histology + culture	Histology at autopsy	Histology + culture	Histology	Histology + culture	Histology	Histology	Histology	Histology	Histology
Aspergillus species identified	–	*A. flavus*	*A. flavus*	–	*A. flavus*	–	*A. flavus*	–	–	–	–	
Pevious infection	Bacteraemia	Bacteraemia	Fever of unknown origin	None documented before autopsy	Fever of unknown origin	Bacteraemia	Fever of unknown origin	None	Suspected line infection	Fever of unknown origin	CMV pneumonia	Bacteraemia
Microorganisms	*S. aureus*	*K. oxytoca*; *E. faecalis*	None	None	–	*P. aeruginosa*	–	–	–	–	CMV	*E.coli*; *C. krusei*
Chemotherapy	cytarabine, daunorubicin	cytarabine, adriamicin	cytarabine, daunorubicin	None	cytarabine, daunorubicin	vincristine	cytarabine, daunorubicin	None	cytarabine, idarubicin	cyclosporine a	cyclosporine and radiotherapy	Doxorubicin, docetaxel, capecitabine
Days after chemotherapy	8 (second cycle)	19 (second cycle)	30	–	24	23	15	–	11	28	3 months	34
Neutropenia at diagnosis	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes	Yes	No	Yes
Antifungal prophylaxis	No	Not specified	Not specified	No	Not specified	Not specified	Yes	No	Not specified	Not specified	No	Not specified
Number of previous antibiotics	3	2	4	Several	5	3	Several	None	4	Several	Several	3

Table 5 (continued)

	Scully [93]	Weingard [94]	Rogers [109]	Cappell [82]	Cohen [95]	Marterre [96]	Catalano [97]	Ibrahim [101]	Shah [98]	Sousa [99]	Safieddine [112]	Tay [102]
Antimicrobial agents	carbenicillin gentamicin oxacillin	ceftazidime amikacin	vancomycin ceftazidime gentamycin azlocilin	Not specified	ceftazidime amikacin	ceftazidime tobramycin Ticarcillin	Not specified	–	cefepime/ genta Metronida-zole Van-comycin	Not specified	Not specified	meropenem amikacin van-comycin
Prior exposure to amphotericin B	No	No	No		Yes	No	No	No	No	No	No	No
Clinical presentation	Relapsing fever peritonitis	Relapsing fever, bloody diarrhoea, ileus, peritonitis	Paralytic ileus, abdominal pain	Weight loss, vomiting, persistent fever	Relapsing fever, watery diarrhoea, peritonitis	Relapsing fever, ileus, peritonitis	Relapsing fever haemor-rhage, peritonitis	Constipation Abdominal pain Weight loss	Persisting fever, peritonitis	Persisting fever, peritonitis	Acute abdomen, abdominal pain, weight loss	Not specified
Serology Antige-naemia	NS NS	NS NS	Negative NS	NS NS	NS NS	NS NS	NS NS	NS NS	NS NS	NS NS	NS NS	NS NS
Secondary dis-semination	Lung, liver	Lung	Small bowel	Oesophagus	Lung, liver	No	NS	No	NS	NS	NS	No
No. of surgical interventions	1	1	3	0	2	5	1	3	1	1	2	1
Bowel excision	Yes	Yes	Yes	–	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Antifungal treatment	No	amphotericin B	amphotericin B	No	amphotericin B	amphotericin B	Not specified	amphotericin B	Not specified	Not specified	No	amphotericin B
Outcome	Death	Survival	Death	Death	Death	Survival	Death	Death	Death	Death	Death	Survival

Legend: AML, acute myeloid leukaemia; ALL, acute lymphoid leukaemia; CMV, cytomegalovirus; NS, not specified.
[a]Cervical lymphoproliferative disorder post cardiac transplantation under cyclosporine A and prednisone.

Table 5 (continued)

	Chambon-Pautas [121]	Ouaussi [103]	Trésallet [100]	Eggimann [122]	Eggimann [122]	Lehrnbecher [72]	Finn [104]	Mohite [105]	Gonzalez-Vincent [106]	Kayiran [107]	Kayiran [107]	Andres [108]
Age (years)	54	52	57	63	52	10	75	46	19	6 months	4 months	42
Underlying disease	AML	AML	Lymphoma	AML	AML	HSCT[b]	Palendromic rheumatism	Granulitic sarcoma[e]	HSCT for relapse of Willms tumour	Immuno-deficiency[f]	Immuno-deficiency[g]	Extensive burn
Documentation	Histology + PCR	Histology	Histology + culture	Histology + culture	Histology + culture	Histology + culture	Histology	Histology	Histology + culture	Histology	Histology	Histology + culture
Aspergillus species identified	*A. flavus*	–	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*	–	–	*A. fumigatus*	–	–	*Aspergillus* spp.
Previous infection	Fever of unknown origin	Fever of unknown origin	Fever of unknown origin	Bacteraemia	Bacteraemia	Fever of unknown origin	No	Fever of unknown origin	Fever of unknown origin	No	No	Super-infection of burned skin
Microorganisms	–	–	–	*P. vulgaris*	*P. aeruginosa*	–	–	–	–	No	No	*P. aeruginosa*
Chemotherapy	cytarabine, adriamycin	cytarabine, daunorubicin	cytarabine, etoposide	cytarabine, idarubicin	cytarabine, idarubicin	thietepa, carboplatin, etoposide	Steroids, methotrexate, TNF-alpha	arabinoside, daunorubicin, etoposide	topotecan/ thietepa, carboplatin, etoposide	No	No	No
Days after chemotherapy	13	14	Induction phase	13	14	30	3 months	16	24	–	–	65
Neutropenia at diagnosis	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No	No
Antifungal prophylaxis	NS	NS	NS	No	No	Fluconazole	No	No	No	No	No	No
Number of previous antibiotics	NS	Several	3	3	3	4	Several	2	3	No	No	1
Antimicrobial agents	Yes (NS)	NS	imipenem, gentamicin, metronidazole	ciprofloxacin, gentamicin, metronidazole	meropenem, amikacin, teicoplanin	pip-tazo/ amikacyn, meropenem vancomycin	NS	pip-tazo/ amikacyn	ceftazidime/ amikacyn, teicoplanin	–	–	pip-tazo

Table 5 (continued)

	Chambon-Pautas [121]	Ouaussi [103]	Trésallet [100]	Eggimann [122]	Eggimann [122]	Lehrnbecher [72]	Finn [104]	Mohite [105]	Gonzalez-Vincent [106]	Kayiran [107]	Kayiran [107]	Andres [108]
Prior exposure to amphotericin B	Currently treated	Currently treated	No	No	Yes	Yes	No	Yes, liposomal	Yes, liposomal	No	No	No
Clinical presentation	Abdominal pain, peritonitis	Persisting fever, peritonitis	Persisting fever, peritonitis	Relapsing fever, ileus, peritonitis	Relapsing fever, bloody diarrhoea, ileus, peritonitis	Persisting fever, abdominal pain, diarrhoea	Right hypochondria firm tender swelling	Persisting fever, abdominal pain and distension	Abdominal pain, bleeding, peritonitis	Weight loss, diarrhoea	Weight loss, distended abdomen	Bleeding, enterocolitis, peritonitis
Serology Antigenaemia	NS Index >5	NS Negative	NS 6.2 ng/ml	NS NS	NS NS	NS 11.8 ng/ml	NS NS	NS NS	NS NS	NS NS	NS NS	NS NS
Secondary dissemination	No	NS	No	Lung	No	No	No	No	No	No	No	No
No. of surgical interventions	1	1	1	1	2	1	1	1	1	0	0	1
Bowel excision	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes
Antifungal treatment	amphotericin B	amphotericin B	voriconazole	amphotericin B	Amphotericin B	Combined[c]	Combined[d]	caspofungin	amphotericin B	Combined	Combined	No
Outcome	Death	Death	Survival	Death	Survival	Survival	Death	Survival	Death	Survival	Survival	Death

Legend: AML, acute myeloid leukaemia; ALL, acute lymphoid leukaemia; CMV, cytomegalovirus; HSCT, haematopoietic stem cell transplantation; NS, not specified; PCR, polymerase chain reaction testing; Pip-tazo, piperacillin-tazobactam; TNF, tumour necrosis factor.

[b]Autologous HSCT rescue after high dose chemotherapy for a primitive neuroectodermal tumour of the central nervous system.

[c]Liposomal amphotericin B + voriconazole. Voriconazole replaced by caspofungin for elevated liver enzymes.

[d]Amphotericin B + voriconazole.

[e]Disease located at the left testis and a retroperitoneal involvement. Considered as a localised form of acute myeloid leukaemia.

[f]Bare lymphocyte syndrome (autosomal recessive lack of expression of MHC class II genes located on the short arm of human chromosome 6). Combined treatment included intravenous immunoglobulin and liposomal amphotericin B.

[g]Transient hypogammaglobulinemia of infancy (primary immunodeficiency disorder which usually resolve by age of 2–6). Combined treatment included intravenous immunoglobulin and liposomal amphotericin B.

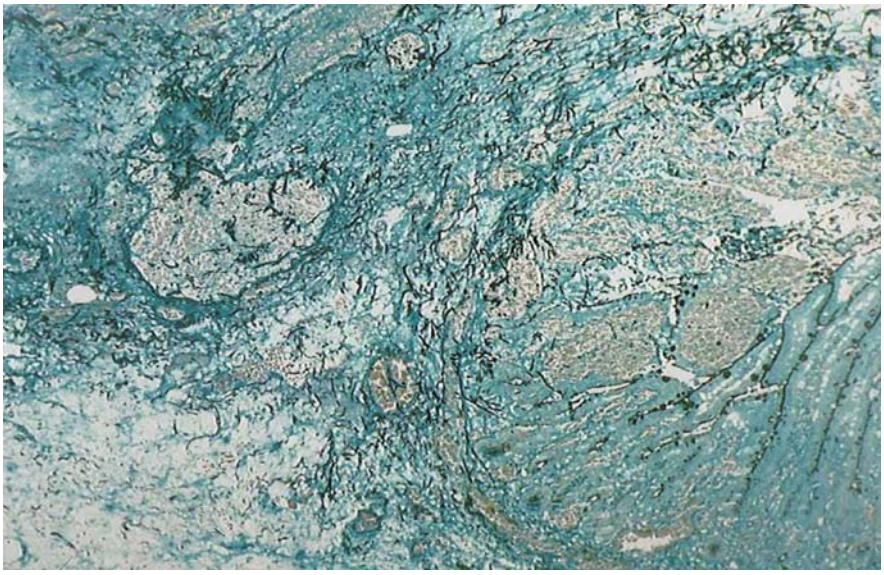

Fig. 1 Giemsa-Silver coloration (original magnification, ×200). Diffuse haemorrhagic necrosis surrounding an ulceration of the wall of a surgically-resected segment of jejuna with loss of the architectural organization of the layers. Vascular occlusion by branched *A. fumigatus* hyphae (black structures)

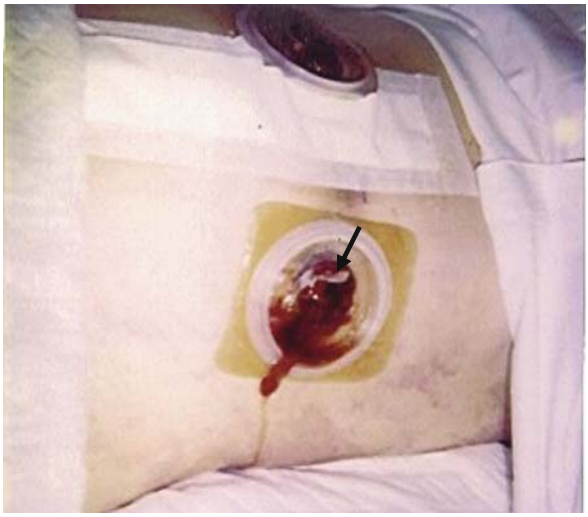

Fig. 2 Ileostomy of a patient after surgery for primary invasive aspergillosis of the lower digestive tract. Colony of *Aspergillus fumigatus* (*arrow*)

those with high-dose cytarabine, with variable clinical manifestations ranging from mild gastrointestinal symptoms to life-threatening intestinal necrosis with perforation and secondary peritonitis [114, 115]. The latter condition mandates a surgical approach and has been associated with mortality rates greater than 50% [116, 117].

Although not specific, abdominal computed tomography (CT) scan may be suggestive. The possible value of *Aspergillus* serology and antigenaemia has not been studied in this context, and only limited data are available from the cases summarised in Table 4. Diffuse dilatation with oedema of the bowel walls, predominantly located in the caecum, with some degree of haemorrhage and necrosis are common pathological findings [118].

As described in the 24 above-mentioned cases, bowel necrosis is associated with the invasion of a poorly vascularized wall by gram-negative bacilli and yeast after breakdown of the normal microbiota [116, 119].

These cases add further evidence that the digestive tract may be a portal of entry for aspergillosis.

3 Conclusion

In conclusion, in this literature review, we identified detailed information on 36 cases of primary aspergillosis of the digestive tract. A detailed analysis of the 12 upper digestive tract and 24 cases of lower digestive tract for which details are available strongly suggests that the digestive tract may represent a portal of entry for *Aspergillus* species in immunocompromised patients.

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Cardiac Aspergillosis

Philippe Lagacé-Wiens and Ethan Rubinstein

Abstract Cardiac aspergillosis has been increasingly recognized as a complication of immunocompromise in recent times. The use of progressively more potent immunosuppressive agents and the longer survival times of transplant recipients is likely contributing to an increasing prevalence of the disease. Although still uncommon, the disease has an extremely high mortality rate and management remains difficult. Cardiac aspergillosis can present as combinations of endocarditis, myocardial invasion or pericarditis and commonly occurs in the context of disseminated aspergillosis. Myocardial involvement is most common, followed by endocarditis although the various forms frequently co-exist. Diagnosis is challenging and is frequently only done post-mortem. Culture diagnosis is best achieved with tissue (valve or biopsy), since blood culture is frequently negative. Any cardiac findings on imaging or physical examination in the context of disseminated aspergillosis suspected by culture, histology or antigen assay should be assumed to be cardiac aspergillosis and investigations for endocarditis should be undertaken. Treatment of cardiac aspergillosis is usually not successful and has not been systematically studied. For endocarditis, surgical and medical therapy are indicated, and medical therapy with either combination amphotericin B and flucytocine or the newer triazoles is suggested. Other forms of cardiac involvement are best managed as disseminated aspergillosis, with a low threshold for diagnosis of endocarditis.

Keywords *Aspergillus* · Endocarditis · Immunocompromise · Aspergillosis · Treatment · Myocarditis · Disseminated

P. Lagacé-Wiens (✉)
Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada
e-mail: rubinste@cc.umanitoba.ca

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Contents

1 Introduction

Cardiac aspergillosis is a disease that has arisen in the latter half of the twentieth century. The recognition of this disease in the past 50 years has largely been the result of advances in medical practice. It now occurs in a variety of clinical situations and either as an isolated occurrence or as a manifestation of disseminated disease. Characteristically, cardiac aspergillosis occurs mainly in immunocompromised hosts, either due to disease (e.g. HIV, malignancy) or iatrogenic immunosuppression (e.g. chemotherapy, corticosteroid therapy, bone marrow transplant). Exceptionally, it occurs in so-called immunocompetent hosts in the context of intensive therapy and prolonged hospitalisation, surgeries or intravenous drug use. Although uncommon, cardiac aspergillosis occurs with regularity in certain clinical settings and should always be considered in the context of disseminated aspergillosis or when a patient with known risk factors presents compatible clinical findings. Furthermore, the high mortality of this condition makes early diagnosis and treatment imperative.

2 Aetiology and Epidemiology

The species of *Aspergillus* involved in cardiac infection varies and has not been systematically studied. Nevertheless, when identification is available, these reflect

the species that are most commonly found in the environment: *A. fumigatus* and *A. flavus* are most commonly isolated while *A. terreus* and *A. niger* are less common [1–3]. There are only few case reports of cardiac aspergillosis implicating the less common species, *A. ustus*, *A. granulosus*, *A. restrictus* and *A. clavatus*. Antimicrobial susceptibility is known to vary by species. Partial or full resistance to amphotericin B in achievable serum concentrations are very common in *A. terreus* and variable susceptibility to voriconazole has been documented in *A. fumigatus* [4].

Accurate incidence data for cardiac aspergillosis are not available, but there is agreement that the rate of invasive fungal diseases of the heart is at an increase [1, 5, 6]. The cause of this increasing prevalence is likely to be multifactorial, including more potent immunosuppressive agents, aggressive intensive care, better treatments for bacterial infections and improved diagnostics. In addition, cardiac aspergillosis as a proportion of fungal carditis appears to be increasing, an observation attributed to the increasing use of yeast-active agents (fluconazole) for prophylaxis of immunosuppressed and particularly in neutropenic patients [5, 7, 8]. However, cardiac aspergillosis still accounts for a minority of fungal cardiac infections, ranging from 12 to 25% [5, 7, 9].

Cardiac aspergillosis occurs most commonly in the context of disseminated aspergillosis. In autopsy series, 85–90% of patients with cardiac mycosis of any type also had disseminated disease [5, 10]. Furthermore, a study of 107 patients with invasive aspergillosis (IA) revealed that the heart was the most common site of extra-pulmonary aspergillosis, occurring in 60% of patients with disseminated disease and in 28% of patients with IA [11].

3 Risk Factors

Risk factors for cardiac aspergillosis are similar to those that predispose to IA (Table 1). These include solid and haematological organ transplantation, anti-neoplastic therapy, myeloablative and suppressive therapies, steroid therapy, immunosuppressive therapy, myeloid disorders, HIV and malignancy [3, 5, 7, 9, 12]. To a lesser extent, diabetes mellitus, end-stage renal impairment, alcoholism, intra-venous drug use, corrective cardiac surgery or extremes of age may be the only risk factor identified [3, 5, 8]. Risk factors specific to cardiac aspergillosis are pre-existing structural and valvular heart disease, rheumatic heart disease, prosthetic valves, pace-maker wires, left ventricular assist device and other cardiac foreign bodies, central venous catheters, intravenous drug use and parenteral nutrition [5, 7–9]. However, most cases of cardiac aspergillosis do not report significant pre-existing cardiac or valvular disease, suggesting that the degree of pre-existing valvular disease required to initiate infection is less that which is required for many other pathogens. Rarely, cardiac aspergillosis occurs in individuals in the absence of immunosuppression [3]. In particular, it occurs with surprising frequency in immunocompetent patients following cardiac surgery [8]. The topic of post-surgical aspergillosis, including prosthetic valve endocarditis, is covered

Table 1 Risk factors for cardiac aspergillosis

Common	Rare	Specific to cardiac aspergillosis
Solid organ transplant	Diabetes mellitus	Structural and valvular heart disease
Anti-neoplastic therapy	End stage renal impairment	Rheumatic heart disease
Myeloablative/suppressive drugs	Alcoholism	Prosthetic valves and other intracardiac foreign bodies
Immunosuppressive therapy	Extremes of age	Pace-maker wires
Myeloid disorders		Central venous catheters
HIV/AIDS		Intravenous drug use
Malignancy		Parenteral nutrition

elsewhere by Dr. Pasqualotto in this book. Lastly, cardiac aspergillosis is also known to occur in otherwise immunocompetent intravenous drug users regardless of HIV status [13].

4 Outcomes

Accurate mortality rates for cardiac aspergillosis are not known, due to the difficulty in establishing accurate pre-mortem diagnosis and relatively low incidence. However, cardiac aspergillosis and endocarditis have a very high mortality and mortality rates from most case series approach 100% [3, 6, 12]. These rates are likely inflated since much of the published literature is bases on autopsy series or post-mortem diagnoses. There have indeed been a number of cases published where patients have survived, which suggests that cardiac aspergillosis can be successfully treated [3]. It is also likely that many cases of successfully treated disseminated aspergillosis involved undiagnosed cardiac disease. Although other prognostic data specific to cardiac aspergillosis are not available, outcomes have been better studied in IA. In a large study of thoracic transplant recipients, mortality for IA was 29.4% [1]. Higher mortality rates are reported for by others for solid organ transplant patients with IA (66.7% for heart and kidney, 20% for lung) and haematological stem cell transplant recipients (53.8% for matched, 84.6% for unmatched) [2]. These studies confirm that mortality rates for IA are high, and involvement of cardiac structures is likely to increase mortality even further.

5 Pathogenesis

The pathophysiology of cardiac aspergillosis is not well understood. Exposure to environmental moulds spores appears to be the critical factor in disease [14]. The inhalational route is likely the primary means by which infection occurs. This is particularly true in cases of disseminated aspergillosis. In disseminated aspergillosis with a primary pulmonary focus, inhaled spores are taken to distal alveoli where

infection occurs in immunosuppressed patients. Fungal proteases are produced and allow hyphal invasion of the blood vessels (angioinvasion) as the infection progresses [15]. Dissemination occurs when hyphal segments break off and seed other tissues, which may include cardiac structures [16]. Contiguous invasion of cardiac and mediastinal structures from a pulmonary focus is another route by which pericardial and myocardial disease occurs [16]. The pathogenesis of disease in intravenous drug users appears to be direct infection of cardiac structures following intravenous injection of spores [16]. On the other hand, post-operative cases may follow contamination with *Aspergillus* conidia in the theatre during the surgical procedures [8].

6 Clinical Presentation

The clinical presentation of cardiac aspergillosis varies depending on which structures of the heart are affected and whether or not disseminated aspergillosis coexists. There are no specific clinical findings and diagnosis is dependant on a high degree of suspicion and appropriate investigations.

6.1 Native Valve Endocarditis

Native valve endocarditis accounts for approximately 17% of cases or cardiac aspergillosis [11]. Native valve endocarditis typically occurs in individuals with profound immune dysfunction without previous cardiac surgery [3]. However, the disease also occurs in intravenous drug users without significant immunocompromise [13] and one study revealed no significant immunosuppression in 31% of 61 patients with *Aspergillus* endocarditis, although 85% had immunosuppression, malignancy, intravenous drug use, extensive surgery or chronic disease as an underlying condition [3]. Vegetations are typically large and friable and embolic events are commonplace [3]. The mitral valve is most commonly involved, followed by mural and aortic involvement [3]. Embolisation to large arteries (aortic and femoral) is especially characteristic of native valve aspergillosis [17]. Large vegetations may occur due to difficulties to establish an early diagnosis. These may obstruct coronary ostia, outflow tracts and venous return leading to coronary artery insufficiency and venous occlusion syndromes of major vessels which may lead to haemodynamic collapse [16]. Metastatic foci occur with high frequency and may involve any organ. In particular, cerebral aspergillosis in the form of cerebrovascular aspergillosis, abscesses, infarcts or mycotic aneurysms often occur with pre-existing native valve aspergillosis [16].

6.2 Prosthetic Valve Endocarditis

This topic is discussed in the chapter about aspergillosis in surgical patients.

6.3 Myocarditis and Mural Endocarditis

Myocardial aspergillosis, whether isolated or not, is the most common form of cardiac aspergillosis in autopsy series and occurs in 83% of cases of cardiac aspergillosis [11]. This is likely due to the highly vascular nature of the myocardium and the organism's propensity for invading vascular tissues (Fig. 1). Myocardial aspergillosis may occur with pericardial or endocardial involvement or as isolated finding [11]. Myocardial disease is common with disseminated disease and occurs in up to 25% of cases of disseminated aspergillosis [11]. However, the disease is often clinically silent and ante-mortem diagnosis of myocarditis is difficult for practical reasons. Although there are no specific clinical findings of myocardial aspergillosis, there are some characteristic findings that should lead the clinician to suspect myocardial aspergillosis in the proper clinical setting (i.e. immune suppression). Conduction abnormalities may occur with involvement of the sinoatrial or atrioventricular nodes and other structures of the conduction apparatus [18]. These manifest as various degrees of heart-block and bundle branch block. Destruction of coronary vessels (Fig. 1) may present as myocardial infarction, although infarction may also occur with isolated endocarditis [18]. In cases of advanced disease, myocardial aspergillosis may present with catastrophic mural rupture and tamponade which is invariably fatal [19].

Mural endocarditis occurs when the infection involves the endovascular surface of non-valvular endocardium. It may be isolated but typically occurs with contiguous myocardial involvement with or without valvular disease [20]. Although findings are typically non-specific, vegetations are often smaller than those that involve the valves and embolic aspergillosis (usually in the form of micro-emboli) without significant vavular disease or vegetations should raise the suspicious of mural endocarditis [3, 20]. Although uncommonly diagnosed ante-mortem, mural endocarditis is highly characteristic of cardiac aspergillosis [3, 20].

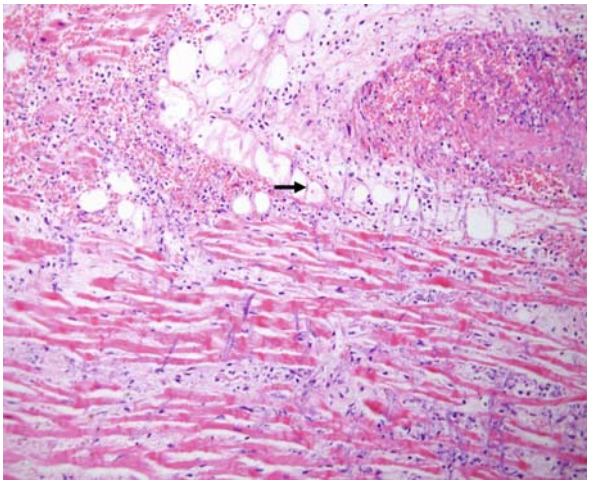

Fig. 1 Mycardial aspergillosis (H&E, ×200) showing extensive invasion of myocardial tissue with hyphea and invasion of a coronary vessel (*arrow*). Image courtesy of Dr. T. Balachandra

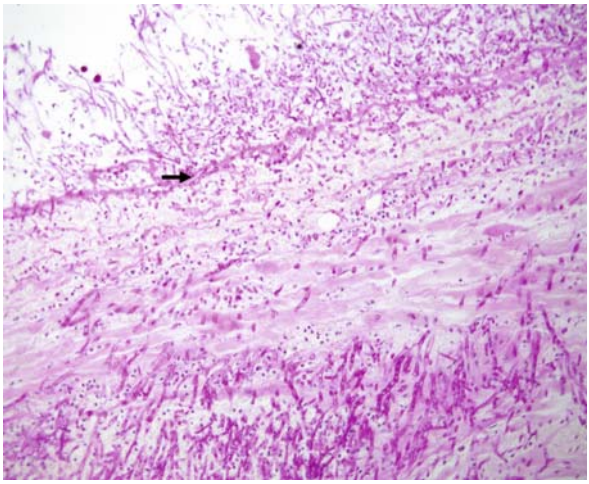

Fig. 2 Histopathological section (PAS, ×200) of pericardium showing extensive hypheal invasion of the pericardium (*arrow*). The patient also had a fibrinous serous pericardial effusion. Image courtesy of Dr. T. Balachandra

6.4 Pericarditis

Pericardial aspergillosis is an uncommonly isolated finding and occurs in 17% of cases of cardiac aspergillosis [11]. It occurs most commonly with invasion of the pericaridium by the hyphae of a contiguous mediastinal or pulmonary focus (Fig. 2) [19]. It may also occur when a myocardial focus of infection extends into the pericardium. Primary pericarditis has not been reported. Clinical findings are non-specific. Effusions may or may not occur, may be serous, sanguinous or purulent [19, 21]. Large effusions present with haemodynamic aberrations, pulsus paradoxus and mufflued heart sounds have been reported, but these are not specific [19]. Pericardiocentesis may show a variety of cellular and biochemical characteristics and are not diagnostic [19, 21]. Microscopic examination and culture may reveal the presence of *Aspergillus* species in the fluid [19, 21], although reactive pericarditis may occur, in which case the fluid will be sterile.

7 Diagnosis

The diagnosis of cardiac aspergillosis requires a high degree of suspicion in a host at risk of aspergillosis. In practice, clinicians will most often encounter cardiac aspergillosis in transplant recipients and other highly immunocompromised patients. Although the Duke's criteria [22] provide a framework for the diagnosis of endocarditis, they may not apply to unusual agents of endocarditis and should not be used to exclude *Aspergillus* endocarditis. Vegetations are often absent in mural endocarditis, blood cultures are almost always negative, pre-existing valvular disease is less common than for other pathogens of endocarditis and many of the other manifestations of endocarditis may be masked by the immunocompromised state [3]. A study of 61 cases of *Aspergillus* endocarditis demonstrated that the most

common clinical findings are fever (74%), embolic phenomena (69%), and a new murmur (41%) none of which are specific [3].

7.1 Imaging

Imaging is useful for the diagnosis of cardiac aspergillosis. In valvular endocarditis, vegetations caused by *Aspergillus* are typically large and are diagnostic in the setting of disseminated aspergillosis [3]. Approximately 78% of patients with *Aspergillus* endocarditis have visible vegetations on echocardiography [3]. Sequelae to endocarditis, such as septic emboli, mycotic aneurysms and distal organ involvement may be diagnosed by computed tomography (CT) or magnetic resonance imaging (MRI), although these are not diagnostic unless other clinical features are present. Imaging is less useful in myocardial forms of the disease. Changes in the myocardium may not be visible on echocardiography or CT [23]. Nuclear scans and electrocardiograms may show dysfunction of the myocardium, but these are not specific [24, 25]. Mural endocarditis, is characterized by multiple small vegetations and observation of these in the appropriate setting with appropriate imaging is highly suggestive of *Aspergillus* myoendocarditis [26]. Anatomical findings of pericardial aspergillosis are highly variable and traditional imaging methods such as echocardiography, CT and MRI may show the presence of fibrinous pericardium, restrictive pericarditis, effusion, tamponade or no anatomical abnormalities [19, 21]. CT and MRI are especially useful in identifying pericardial aspergillosis originating from contiguous pulmonary or mediastinal aspergillosis as these will often present as a detectable lesion in proximity to the pericardium.

7.2 Microbiology

Microbiology often provides the definitive diagnosis of aspergillosis. In the presence of radiographic or clinical evidence of disseminated aspergillosis with cardiac involvement, any suspect specimen can be submitted. Pulmonary tissues and aspirates are most common, but skin lesions, thrombi and emboli, bronchial secretions and biopsies of diseased solid organs are suitable for diagnosis [27]. These are often more practical to obtain than cardiac tissues and will usually suffice for a presumptive diagnosis of cardiac aspergillosis if pericarditis, myocarditis or endocarditis is present. In endovascular infection, blood cultures are typically negative even with prolonged incubation times and positivity rates are <5% [6]. Definitive diagnosis can only be achieved with demonstration of the organism in cardiac tissues. Diseased valves removed at autopsy or surgery, valvular vegetations and myocardium provide the best tissues for culture. Since pericarditis may be reactive, isolation of the organism from this tissue is not as reliable [19, 21].

Specimens should be submitted to a laboratory with expertise in mycology and species identification. Specimens should specifically be submitted fresh for fungal culture (moulds and yeasts) as well as routine, fastidious and anaerobic bacteriology and mycobacteriology and viral studies (if indicated) as these may be the

primary cause of disease or co-infecting agents. Some specimen should be preserved in formalin for histopathological analysis if possible. Fresh specimens should be examined with a 10% KOH preparation and the fluorescent dye calcofluor for the selective visulization of fungi [28]. The presence of hyphe should be reported, but accurate genus identification is not possible with direct visualisation only. Tissues for mould culture should be thoroughly macerated and incubated on media for moulds (e.g., brain heart infusion, Sabhi or similar) and inhibitory media if bacterial contamination is expected (Inhibitory Mould Agar) [28]. Identification of *Aspergillus*, should be at the species level if possible as this may determine choice and response to antifungal therapy [29]. Macroscopic characteristics, microscopic appearance of hyphae and conidial head usually provide species identification in experienced hands. Molecular methods have also been described for the speciation of most *Aspergillus* spp. but these are not practical for most laboratories at the present time [30]. Microbiological identification of *Aspergillus* spp. has been discussed in the initial sections of this book. Some experiments have demonstrated that *Aspergillus* spp. can be identified directly from blood, lung and cardiac tissues by polymerase chain reaction (PCR) amplification of specific DNA targets of *Aspergillus* [31, 32]. Although this method is attractive because of its sensitivity, specificity and speed, both the cost and the risk of specimen contamination with the spores of environmental moulds must be considered and are therefore unlikely to fully replace the more laborious techniques. Early diagnosis of IA by the means of novel technologies such as galactomannan, β-glucan and PCR testing might eventually result in a reduced incidence of disseminated forms of aspergillosis, including heart involvement.

7.3 Histopathology

In cases where microbiology expertise or facilities are not available, histopathology of diseased cardiac tissues may provide a definitive diagnosis. Tissue specimens of valves, vegetations, myocardium, pericardium prepared using simple fungal stains are sensitive and show characteristic narrow septate hyphae with acute angle branches invading tissues and blood vessels (Figs. 1, 2 and 3) [28]. These may be seen in any affected tissue during disseminated aspergillosis and serve to provide a tentative diagnosis of cardiac aspergillosis if additional clinical findings suggest this. Unfortunately, histopathology alone cannot provide a definitive species or genus in most cases, as several other moulds display similar hyphal arrangements in tissue (*Fusarium*, *Paecilomyces* and *Scedosporium* species) [28]. Immunohistochemistry, fluorescent in-situ hybridization and PCR have all been used directly on fixed tissues to confirm and speciate *Aspergillus* spp. but these techniques are laborious, costly and access is limited to referral centres.

7.4 Antigen Detection

Recent developments in the diagnosis of invasive mould and fungal infections have arisen with assays that reliably detect and quantitate the presence of fungal structural

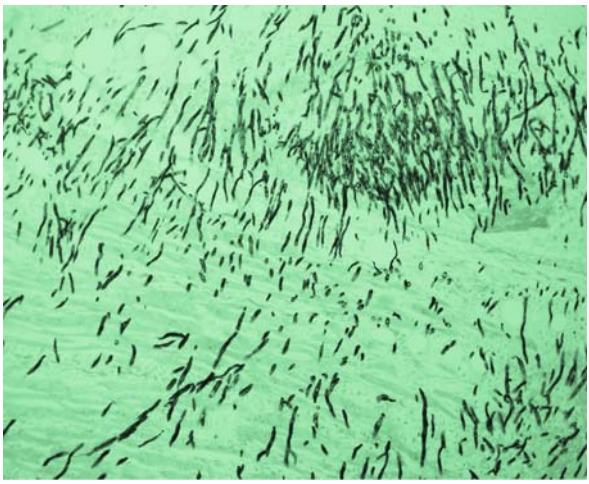

Fig. 3 Histopathological section (GMS, ×200) showing myocardial invasion of *Aspergillus* hyphae and characteristic acute (45°) angle hyphaeal branching. Image courtesy of Dr. T. Balachandra

components in patient's blood and secretions during the course of invasive disease. The galactomannan assay (see specific chapter) is one such assay. Although not specifically studied in cardiac aspergillosis, sensitivity and specificity of the galactomannan assay in patients with IA in a large meta-analysis was 71 and 89%, respectively [33]. Since most patients with cardiac aspergillosis have disseminated disease, galactomannan testing should perform well in this context. However, cross-reactions with other moulds and false-negative results, especially in patients on treatment, do occur [34, 35], and there has been a case report of galactomannan assays giving negative results in a patient with *Aspergillus* endocarditis despite positive blood cultures [36]. The reader is encouraged to review the chapter pertaining to these assays for more detail.

8 Treatment

Treatment of cardiac aspergillosis has not been systematically studied in randomised controlled trials. In vitro data, case reports, literature reviews and inference from IA trials form the bulk of the evidence for treatment of cardiac aspergillosis.

8.1 Endocarditis

Treatment approaches for *Aspergillus* endocarditis are best represented in the published literature. A number of regimens have been reported, but most of the available evidence is for Amphotericin B (AmB) and 5-flucytosine (5-FC) preparations, since other mould-active antifungals agents (triazoles and echinocandins) are relatively recent additions to the treatment armamentarium. No trials for treatment of *Aspergillus* spp. endocarditis exist, but experimental models [37, 38] suggest that the combination of amphotericin B with 5-FC results in better outcomes than AmB

Table 2 Treatment outcomes of *Aspergillus* endocarditis in experimental animals

Drug (dose) (references)	Survival rate	Vegetations sterilized (%)
Amphotericin B (3 mg/kg IP once daily) [38]	0% (14 days)	0
AmB + 5FU (3 mg/kg + 35 mg/kg IP once daily) [38]	10% (14 days)	30
Intraconazole (5 mg/kg IP once daily) [38]	100% (14 days)	100
Intraconazole (10 mg/kg IP once daily) [43]	0% (10 days)	0
Voriconazole (5 mg/kg PO twice daily) [43]	100% (10 days)	0
Voriconazole (7.5 mg/kg PO twice daily) [43]	100% (10 days)	70
Voriconazole (10 mg/kg PO twice daily) [43]	100% (10 days)	100

Legend: 5-FC, 5-flucytosine; AmB, amphotericin B; IP, intraperitoneally; PO, oral route.

alone (Table 2). Evidence from case series [3, 9, 39–41] has not substantiated these observations, but the numbers have been too small. Despite lack of human evidence, many experts continue to recommend combination therapy on the basis of animal data (Table 2), correlates with IA and pharmacokinetic data [40]. It is noteworthy to mention that resistance to AmB, particularly in *A. terreus* is likely to lead to AmB treatment failures [4, 42]. Early experimental use of itraconazole for the treatment of *Aspergillus* endocarditis in animals showed promise [38] but more recent studies have suggested itraconazole is not the drug of choice for experimental *Aspergillus* endocarditis [43] (Table 2).

Newer agents, including the triazoles voriconazole and posaconazole offer potential alternatives and advantages in treating *Aspergillus* endocarditis. However, only anecdotal evidence for their use in human endocarditis exists [44, 45]. Nevertheless, the potent in vitro activity of voriconazole [4], clearly superior outcomes in IA [46, 47] and acceptable side-effect profile [46] has led many experts to recommend its use over AmB combinations for the treatment of endocarditis. Furthermore, voriconazole has been successfully used in animal models of endocarditis [43]. The concomitant use of triazoles with other antifungals has only once been reported for the treatment of endocarditis [45]· but better outcomes with triazole combination therapy (using AmB or echinocandins) have been reported for IA [48–50]. Although caspofungin has a place in the treatment of IA [51], no studies or reports exist to support its use in *Aspergillus* endocarditis. Duration of treatment has not been well studied in *Aspergillus* endocarditis, although many experts recommend a protracted 6–12 week course of initial therapy with long term or even life-long antifungal suppression [9].

Regardless of the choice of agent for medical treatment of endocarditis, concomitant surgical therapy is essential for successful outcome [9, 40]. There are very few reports of successful medical therapy alone and only in immunocompetent hosts [52].

8.2 Myocardial and Pericardial Aspergillosis

The treatment of other forms of cardiac aspergillosis is largely based on the treatment of IA. As for IA, aggressive antifungal therapy and where indicated, surgical

therapy, are the mainstay of therapy. The newer agents, triazoles and echinocandins may offer survival benefit in IA over AmB, but no data are available for cardiac involvement [46, 47, 49]. In severe IA, published evidence seems to support combination therapy with AmB and 5-FC and more recently with combinations of AmB, voriconazole and/or caspofungin [48–50, 53]. *Aspergillus* pericarditis is nearly always fatal, but a few successful outcomes have been reported with a combination of AmB therapy, surgery and long-term azole (itraconazole) suppression [21].

9 Conclusion

Cardiac aspergillosis is a disease that primarily affects immunocompromised persons. Early diagnosis with appropriate investigations and initiation of appropriate therapy are associated with better survival, but mortality remains very high. New antifungal agents, in particular the triazoles, may offer a treatment advantage, but studies are urgently needed to confirm these findings in cardiac aspergillosis.

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Aspergillus Thyroiditis

Luciano Z. Goldani and Ana Luiza Maia

Abstract *Aspergillus* species are by far the most common agents of fungal thyroiditis. Immunocompromised patients, such as those with leukaemia, lymphoma, autoimmune diseases, organ-transplant recipients and pharmacologically immunosuppressed patients are particularly at risk. Fungal thyroiditis is diagnosed at autopsy in the context of disseminated infection in a substantial number of patients without clinical manifestations or laboratory evidence of thyroid dysfunction. Local signs and symptoms of infection are indistinguishable from other infectious thyroiditis and included fever, anterior cervical pain, and thyroid enlargement, which may be associated with dysphagia and dysphonia. Clinical and laboratory features of transient hyperthyroidism may be seen due to the release of thyroid hormones following damage to the follicular cells, which may result in hypothyroidism. Antemortem diagnosis of fungal thyroiditis is usually made by direct microscopy and culture of a fine-needle aspirates, or/and gland biopsies. Since most patients with *Aspergillus* thyroiditis have disseminated aspergillosis the diagnosis is frequently delay and the associated mortality very high.

Keywords *Aspergillus* · Fungi · Thyroid · Thyroiditis

Contents

L.Z. Goldani (✉)
Section of Infectious Diseases, Hospital das Clinicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
e-mail: lgoldani@ufrgs.br

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1 Introduction

Fungal infections of the thyroid are uncommon. Immunosuppressed patients, such as those with human immunodeficiency virus infection, leukaemia, patients with auto-immune diseases, and transplant patients are particularly at risk for fungal thyroiditis. In contrast to most other organs in the body, the thyroid gland is remarkably resistant to infection [1, 2]. For instance, direct inoculation with either *Staphylococcus aureus* or *Streptococcus pyogenes* into the carotid arteries of dogs rarely result in thyroid infection [3]. Protective mechanisms contributing to the relative resistance of the thyroid to infections include the rich blood supply to and lymphatic drainage from the thyroid, the high glandular content of bactericidal agents such as iodine, and separation of the thyroid from other structures of the neck by fascia planes with complete, protective fibrous capsules surrounding the gland.

Over 40 cases of fungal thyroiditis have been reported in the world's literature [4]. Case reports of fungal infections of the thyroid have included *Aspergillus* species, *Candida* spp., *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Pseudallescheria boydii*, and *Pneumocystis jirovecii* [4–8].

2 *Aspergillosis* and the Thyroid

Invasive aspergillosis caused by the hyaline moulds *Aspergillus* is a major cause of morbidity and mortality in immunosuppressed patients [9, 10]. Clinical syndromes associated with aspergillosis are diverse, ranging from allergic responses to the organism, asymptomatic colonisation, and superficial infection to acute or subacute invasive diseases. Generally, the clinical presentation reflects the underlying immune defects and risk factors associated with each patient group, with greater immunosuppression correlating with greater risk of invasive disease [9, 10].

Aspergillus species are the most frequent aetiologies of fungal thyroiditis. Reviewing the world literature [4], we were able to find 21 reports of *Aspergillus* thyroiditis (Table 1) [6, 11–30]. Patients' ages ranged from newborn to 95 years, with a slight predominance of males (13 out of 21 patients). Cases of thyroiditis were often described in the context of widespread *Aspergillus* infection in immunocompromised individuals. The most frequent underlying conditions were haematological malignancies, organ transplantation, and autoimmune diseases treated with steroids and cytotoxic drugs. Thyroid involvement by *Aspergillus* spp. was usually found at autopsy as part of disseminated aspergillosis in patients with no clinical or laboratorial manifestation of thyroid dysfunction (62% of cases) (Table 1). Except for one patient with hypothyroidism, a cervical mass due to diffuse goitre with thyrotoxicosis was the initial presentation most frequently seen in patients with clinical manifestations of thyroiditis. In those cases, thyroid involvement by *Aspergillus* spp. was often diagnosed by direct microscopy and culture through fine-needle aspiration (FNA) and biopsy of the thyroid. The overall mortality for patients with *Aspergillus* thyroiditis was 90%. The elevated mortality associated with these conditions results

Table 1 Baseline characteristics of patients with *Aspergillus* thyroiditis

Agent	Age, sex	Underlying diseases	Clinical features	Diagnosis	Antifungal treatment	Outcome	References
Aspergillus fumigatus	53 y, M	Renal TX, steroids, AZA	Disseminated aspergillosis	Autopsy	None	Death	[11]
Aspergillus spp.	24 y, F	SLE, HD, steroids	Hyperthyroidism, fever, cough, dyspnoea	FNA	d-AmB, Flu	Death	[12]
Aspergillus spp.	26 y, M	Lymphoma, QT	Hyperthyroidism, airway obstruction by a tender cervical mass, DA	Autopsy	d-AmB	Death	[13]
Aspergillus fumigatus	46 y, M	Myelodysplasia	Hyperthyroidism, oesophageal obstruction by a tender cervical mass	FNA	L-AmB	Death	[14]
Aspergillus fumigatus	65 y, M	Heart TX, steroids, tacrolimus	Hyperthyroidism	Biopsy	d-AmB, Flu, Itra	Death	[15]
Aspergillus fumigatus	11 y, M	Granulomatous disease	Hyperthyroidism, cervical mass, fever	Drainage	d-AmB	Survival	[16]
Aspergillus spp.	29 y, M	Kidney TX, steroids, AZA	Thyromegaly, neck pain, DA	Autopsy	d-AmB	Death	[17]
Aspergillus fumigatus	31 y, F	AIDS	Hyperthyroidism, DA	FNA	?	Death	[18]
Aspergillus spp.	55 y, M	Necrotizing fasciitis	DA	Autopsy	?	Death	[19]
Aspergillus flavus	64 y, F	Neutropenia, dermatitis	Hypothyroidism, DA	Autopsy	None	Death	[20]
Aspergillus ustus	46 y, F	Myelodysplasia, steroids, QT, Allo-HSCT	DA	Autopsy	L-AmB	Death	[21]
Aspergillus terreus	65 y, F	Leukaemia	Endophthalmitis, DA	Autopsy	?	Death	[22]
Aspergillus flavus	41 y, M	Leukaemia	DA	Autopsy	?	Death	[23]

Table 1 (continued)

Agent	Age, sex	Underlying diseases	Clinical features	Diagnosis	Antifungal treatment	Outcome	References
Aspergillus flavus	57 y, F	Leukaemia, neutropenia	Thyroid mass, DA	Biopsy	d-AmB, Itra	Survival	[24]
Aspergillus fumigatus	74 y, M	Corticosteroids	Tender neck mass, thyroid abscess, DA	FNA	d-AmB, Fluco	Death	[25]
Aspergillus fumigatus	41 y, M	Sarcoma, steroids, QT, RT	DA	Autopsy	None	Death	[26]
Aspergillus fumigatus	NB, M	None	DA	Autopsy	None	Death	[27]
Aspergillus spp.	NB, F	Erythroblastosis faetalis	DA	Autopsy	None	Death	[28]
Aspergillus spp.	14 y, M	SLE, nephritis, steroids	Tender thyroid, thyroid abscess, DA (cerebral aspergillosis)	Biopsy	d-AmB, surgical drainage	?	[6]
Aspergillus flavus	2 m, F	Steroids	DA	Autopsy	None	Death	[29]
Aspergillus fumigatus	43 y, M	Thyroid cancer	DA	Autopsy	?	Death	[30]

Legend: AZA, Azathioprine; DA, Disseminated aspergillosis; d-AMB, deoxycholate amphotericin B; F, Female; Flu, Fluconazole; FNA, Fine needle aspiration; HD, Haemodialysis; HSCT, Haematopoietic stem cell transplantation; Itra, Itraconazole; L-AmB, liposomal amphotericin B; M, Male; NB, Newborn; QT, Chemotherapy; RT, Radiotherapy; SLE, Systemic erythematous lupus; TX, Transplantation.

from a combination of marked immunosuppression, disseminated fungal infection and a delayed diagnosis and treatment.

In addition to the case reports, thyroid involvement by *Aspergillus* has been described in a few other studies in which immunocompromised patients underwent autopsy after dying with fatal and disseminated aspergillosis [31–36].

3 Conclusion

Fungal infection of the thyroid gland, while rare, is the second most common cause of infectious thyroiditis, with *Aspergillus* species being the most common agents. Most of the affected patients are immunosuppressed, frequently as a result of underlying conditions such as haematological malignancies, auto-immune diseases, and organ transplantation, along with therapy with steroids and cytotoxic drugs. In contrast to bacterial thyroiditis, pre-existing thyroid disease is not a frequent determinant of fungal infection to the thyroid gland. The pathology of *Aspergillus* thyroiditis is consistent with haematogenous spread of fungi, with focal abscesses, haemorrhagic lesions surrounding blood vessels or diffuse necrotising thyroiditis. Most patients are diagnosed at autopsy only – when local signs and symptoms are present, these are indistinguishable from other infectious conditions causing thyroiditis. Despite some patients having received systemic antifungal therapy, overall mortality is very high which is consistent with what is expected for disseminated aspergillosis. Also, it is worth noting that the vast majority of cases of *Aspergillus* thyroiditis were published in the 1990s of even before that, in a time where modern antifungal therapy was not available.

The thyroid seems to be a more common site of dissemination in systemic fungal infections such as invasive aspergillosis than previously thought. Therefore, a high index of suspicion is ultimately required for immunosuppressed patients manifesting local thyroid findings in the context of a disseminated infection. In an attempt to obtain an earlier diagnosis, fine-needle aspiration of the thyroid should be performed more frequently in these patients.

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Aspergillus Endophthalmitis

Philip A. Thomas

Abstract Several species of *Aspergillus*, particularly *A. fumigatus* and *A. flavus*, may cause endophthalmitis (intraocular inflammation involving the vitreous and anterior chamber). Exogenous *Aspergillus* endophthalmitis may follow ocular surgery, trauma to the eye or extension from *Aspergillus* keratitis while endogenous *Aspergillus* endophthalmitis may arise due to intravenous drug abuse, immunosuppression associated with organ transplants, valvular heart surgery, haematological malignancies and a human immunodeficiency virus-positive status. The endogenous variety usually presents acutely while the exogenous variety may occur early or late. Characteristic signs include iridocyclitis, yellow subretinal and retinal infiltrates with a predilection for the macula and inferior gravitational layering of inflammatory exudates. Diagnosis is best achieved by the performance of routine microbiological investigations (i.e., direct microscopy and culture) on specimens from the vitreous or aqueous. Molecular diagnosis, in particular the polymerase chain reaction (PCR), is increasingly being used. Treatment usually combines surgery (vitrectomy) and antifungal agents administered by different routes. Vitrectomy removes the bulk of inflammatory debris and microorganisms. Amphotericin B, the most widely used systemic agent in treatment of *Aspergillus* endophthalmitis, achieves relatively low concentrations in the aqueous and vitreous when used intravenously, hence intravitreal administration of amphotericin B is done at the time of vitrectomy. Voriconazole, given intravitreally and systemically, has recently yielded good results in some patients with *Aspergillus* endophthalmitis. However, the condition may not respond to these measures, and the patient may die of disseminated aspergillosis, lose the infected eyeball or, at best, lose vision. New antifungal agents may improve the outcome of *Aspergillus* endophthalmitis.

Keywords Amphotericin B · *Aspergillus* · Endophthalmitis · Eye infections · Vitrectomy

P.A. Thomas (✉)
Department of Microbiology, Institute of Ophthalmology, Joseph Eye Hospital, P.B. 138, Tiruchirapalli, 620001, India
e-mail: philipthomas@satyam.net.in

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Contents

1 Introduction

Intraocular fungal infection usually develops slowly, spreads to the eye through the blood stream, and consists of focal or multifocal lesions in the choroid and retina (chorioretinitis). The intensity of inflammation of the anterior segment of the eye varies from mild to severe, the severe variety resulting in the formation of a hypopyon [1]. When the initial focus of intraocular fungal infection in the choroid and retina extends into the vitreous to produce inflammation, which may involve the entire internal structure of the eye, endophthalmitis results. It may be difficult, from a practical viewpoint, to discuss fungal retinitis separately from fungal endophthalmitis [2]. Endophthalmitis, in turn, can be divided into endogenous endophthalmitis, which arises from haematogenous spread from a focus of infection elsewhere in the body, and exogenous endophthalmitis, resulting from primary inoculation of the eye following surgery or penetrating trauma.

2 Frequency of Occurrence of *Aspergillus* Endophthalmitis

Aspergillus species may cause exogenous or endogenous endophthalmitis. It is difficult to estimate the incidence or frequency of occurrence of *Aspergillus* endophthalmitis. In an evaluation of 29 eyes of 28 patients with culture-proven endogenous

endophthalmitis seen between 1996 and 2006 at the ophthalmology department of a university in Germany, it was reported that *Candida* species were isolated from 15 eyes whereas *Aspergillus* species were isolated from only 2 [3]. On the other hand, in an outbreak of exogenous endophthalmitis occurring after cataract surgery that was finally found to be associated with ongoing construction in an hospital in Saudi Arabia, *A. fumigatus* was isolated from all five patients [4], whilst in 27 patients with exogenous fungal endophthalmitis in an hospital in India, *Aspergillus* species were isolated from samples taken from 20 patients [5].

3 Aetiological Agents of *Aspergillus* Endophthalmitis

The *Aspergillus* species reported as causes of endophthalmitis since 2000 include *A. flavus* [5, 6], *A. fumigatus* [5, 7–15], *A. niger* [5, 16–19], *A. terreus* [20–22], *A. ustus* [23, 24] and *A. versicolor* [25, 26].

4 Risk Factors

4.1 Risk Factors for Exogenous Aspergillus *Endophthalmitis*

Risk factors for exogenous endophthalmitis due to *Aspergillus* species include ocular (usually cataract) surgery [24, 4–6, 27–35] and ocular trauma [36–38]. Bifrare and Wolfensberger [26] reported the occurrence of protracted endophthalmitis due to *A. versicolor*, which is commonly found in insulation materials and cables in dilapidated houses, in a 58-year-old man. This individual was hit in the eye by an old cable, in a dilapidated house, and suffered corneal microperforation. Moinfar et al. [22]. reported the occurrence of increasing inflammation and an hypopyon three weeks after primary repair of a corneal laceration. *A. terreus* was isolated from a vitrectomy sample taken from the eye.

4.2 Risk Factors for Endogenous Aspergillus *Endophthalmitis*

Endogenous *Aspergillus* endophthalmitis has been reported in patients who have received immunosuppressive agents or who have undergone organ (heart, lung or liver) transplants or valvular cardiac surgery [39–45] and in intravenous drug abusers [42, 46] (see specific chapter in this book about this subject). In general, intravenous drug abusers are at grave risk of developing endogenous fungal endophthalmitis, probably because they may use contaminated drugs, syringes, needles or cotton [47]. Schelenz and Goldsmith [7] described two patients who developed endogenous *Aspergillus* endophthalmitis with cerebral involvement 6 months following renal transplantation for polycystic kidney disease. In addition, they reviewed the literature from 1959 to 2002 and noted the occurrence of endogenous *Aspergillus* endophthalmitis in at least 8 patients who had undergone

renal transplantation. Their review led them to identify possible risk factors, including infection with cytomegalovirus, presence of diabetes mellitus (either naturally acquired or iatrogenic) and treatment for rejection with agents such as methyl prednisolone and mycophenolate mofitil. Augsten et al. [48] described a patient who developed bilateral endogenous endophthalmitis following renal transplantation. The patient was receiving immunosuppressive therapy and therapy for other systemic diseases. A bacterium was presumed to be the cause of the endophthalmitis in the right eye while *Aspergillus* was implicated as the cause of the endophthalmitis in the left eye.

Endogenous *Aspergillus* endophthalmitis has also been reported in individuals suffering from haematological malignancies, the species incriminated being *A. terreus* in a patient suffering from chronic lymphocytic leukaemia [49], *A. fumigatus* in a patient suffering from acute myeloid leukaemia [50] and bilateral endophthalmitis, suspected to be due to an *Aspergillus* species (based on histopathological characteristics), in a patient with chronic lymphocytic leukaemia [51]. Infection by the human immunodeficiency virus, even in the absence of disseminated aspergillosis [18] or immunosuppression [25], may also be a risk factor. Such patients, the patient may present with marked secondary glaucoma [18] or sudden visual loss [52], and the occurrence of endophthalmitis, either unilateral or bilateral, is an accidental finding.

Endogenous *Aspergillus* endophthalmitis has also been reported as a sequel to *Aspergillus* endocarditis of the systemic aortic root in an immunocompetent person [53]. Gupta et al. [54] reported the occurrence of culture-proven fungal endophthalmitis in 12 patients (12 eyes) who had been administered presumably contaminated intravenous infusion fluid in a rural setting – fungi were recovered from 12 of 72 bottles containing the same infusion fluid.

It is important to be aware of the occurrence of systemic conditions predisposing to endogenous endophthalmitis due to *Aspergillus* species since such conditions may modify the probability of dissemination, risk of mortality, and role for systemic treatment. In a retrospective analysis of clinical and histopathological features in 13 patients with morphologic features and/or positive culture for *Aspergillus* who had undergone enucleation, it was noted that 12 of the 13 patients had received immunosuppressive agents or had undergone organ transplants or valvular cardiac surgery. In contrast, in 12 patients with histological evidence and/or positive cultures for *Candida* spp., a similar clinical history was present in only one [44]. Thus, it might be possible clinically to suspect *Aspergillus* infection in those with drug-induced immune suppression or in patients who have had cardiac surgery. A review on cardiac aspergillosis is provided elsewhere in this book.

5 Clinical Features of *Aspergillus* Endophthalmitis

5.1 Symptoms of Aspergillus *Endophthalmitis*

Symptoms of fungal endophthalmitis (which are not specific for *Aspergillus* infection) include visual loss, the presence of a red eye, photophobia, pain and floaters.

It is important to remember that patients may be asymptomatic if the lesion is in the peripheral retina or if the patient is moribund.

5.2 Signs of Aspergillus *Endophthalmitis*

Endophthalmitis due to *Aspergillus* species may present with characteristic signs, which are of possible diagnostic utility. These include an iridocyclitis (with or without a hypopyon), yellow subretinal and retinal infiltrates that preferentially affect the macula, and inflammatory cells within the infiltrate [55]. An inferior gravitational layering of inflammatory exudate in either or both the subhyaloid and subretinal space may occur [42]. As the disease progresses, the vitreous becomes markedly involved, obscuring all details of the fundus. With time, scarring of the macula occurs. If the fungus invades the choroidal vessels, an exudative retinal detachment occurs. When the retinal vessels become involved, retinal necrosis may occur [55]. When data pertaining to 27 patients with exogenous fungal endophthalmitis (20 due to *Aspergillus* species) were evaluated, substantial corneal involvement was noted in 14 eyes, which multivariate analysis revealed was the single most important risk factor in determining final visual outcome [5]. In an outbreak of exogenous endophthalmitis due to *A. fumigatus* after cataract surgery in 5 patients, two patients exhibited superior limbal, scleral and corneal infiltration [4].

Endogenous *Aspergillus* endophthalmitis may be the presenting feature of disseminated aspergillosis [55]. Hence, the occurrence of endophthalmitis in a patient with symptoms and signs (e.g. pulmonary lesions) of disseminated aspergillosis should alert the clinician to a possible diagnosis of endogenous *Aspergillus* endophthalmitis. However, there are other features that should be looked for. A characteristic acute onset of intraocular inflammation, with a one or two day history of pain and marked loss of visual acuity, vitritis and, frequently, a chorioretinal lesion located in the macula, have been noted in patients with culture-proven endogenous endophthalmitis due to *Aspergillus* species [42]. In another analysis of patients with endogenous fungal endophthalmitis (seen over a 10-year period), it was observed that eyes with *Candida* endophthalmitis tended to present in an indolent fashion (the mean duration from onset of symptoms to initial treatment being 61 days), whereas those with endogenous *Aspergillus* endophthalmitis had a more acute presentation (mean duration of 5 days); in addition, all the eyes with *Aspergillus* infection had a poor visual outcome (worse than 20/400) due to direct infection of the macula or persistent retinal detachment [56]. Interestingly, the diagnosis of endogenous fungal endophthalmitis occurred in an outpatient setting for all the patients. In another review of culture-proven endogenous endophthalmitis seen over a 6-year period (1996–2002), eyes with *Aspergillus* endophthalmitis were found to have significantly worse visual outcomes than eyes with either *Candida* or bacterial endophthalmitis [57]. Poor visual outcome in patients with endogenous *Aspergillus* endophthalmitis has also been noted by other investigators [3, 11].

6 Diagnosis of *Aspergillus* Endophthalmitis

6.1 Imaging Studies

Since anterior chamber and/or vitreous inflammation may obscure visualization of the retina, echography may help to determine the anatomic status of the retina, the extent of inflammation, the presence of choroidal detachment, and the presence and location of intraocular foreign bodies.

6.2 Microbiological Investigations

Fungal chorioretinitis and endogenous fungal endophthalmitis are exceptions to the rule requiring isolation of a fungal strain from an ocular sample to confirm the diagnosis of an ocular fungal infection [58]. However, if non-specific findings are present, or if there is no positive culture from an extra-ocular site, a diagnostic vitrectomy can be performed. The diagnosis of exogenous fungal endophthalmitis is ultimately established by demonstrating fungi in samples from the vitreous or aqueous.

6.2.1 Collection of Samples

Samples of aqueous and vitreous should be obtained prior to instituting therapy. A vitrectomy specimen (where much of the vitreous is cut and removed by a vitreous cutter) is preferable to a vitreous aspirate (vitreous tap) sample (where only the putative focus of infection is sampled) since the latter may fail to sample the actual locus of infection. Although culture of an anterior chamber aspirate is a poor diagnostic technique and culture of a vitreous sample is more likely to yield positive results [15], both samples should be obtained whenever possible. A conjunctival swab is of relevance only if there is a leaking filtering bleb.

6.2.2 Direct Microscopic Examination and Culture

Fungal endophthalmitis can be confirmed by direct microscopic demonstration of fungal hyphae or yeast cells in 10% KOH wet mounts, or smears stained by calcofluor white and the Gram method [17]. Culture is performed on appropriate media, the best results being obtained if direct inoculation of vitreous and aqueous samples on the culture media is done immediately after collection. To facilitate recovery of fungi, vitrectomy samples can be concentrated in an ultracentrifuge prior to inoculation onto culture media, or can be passed through cellulose membrane filters which are then applied to the plates of culture media [55].

6.2.3 Microbiological Investigations in *Aspergillus* Endophthalmitis

Endophthalmitis due to *Aspergillus* species is comparatively more difficult to diagnose than that due to *Candida* species since skin and serological tests are unreliable,

pulmonary radiographic studies and echocardiograms may yield little information, isolation from blood cultures rarely occurs (even in patients who are immunosuppressed with definite dissemination), and systemic manifestations are often lacking in intravenous drug abusers harbouring this infection [42, 55, 59]. A diagnostic vitreous aspirate for cytology and culture isolation is usually necessary to identify this aetiological agent [59]. However, diagnosis of endogenous *Aspergillus* endophthalmitis by anterior chamber or vitreous aspirates/biopsies alone may be unreliable, since aspergillosis clinically presents with extensive areas of deep retinitis/choroiditis [44]. Culture of pars plana vitrectomy specimens, in conjunction with examination of Gram- or Giemsa-stained smears, appear to yield the highest percentage of positive results (almost 90% for previously untreated eyes) for *Aspergillus* species [42]. A review of the literature on endogenous *Aspergillus* endophthalmitis occurring in patients who had undergone renal transplantation revealed that in 70% of the patients, histology, microscopy or culture of vitreous fluid confirmed the diagnosis [7].

6.3 Molecular Methods for Diagnosis of Aspergillus *Endophthalmitis*

In recent years, the value of DNA-based technology in the diagnosis of fungal endophthalmitis has been evaluated [60], but there are few reports on the use of such technology specifically for the diagnosis of *Aspergillus* endophthalmitis. In one study on 27 intraocular specimens from 22 patients with suspected fungal endophthalmitis (which were proven to be non-bacterial in origin), four were positive for *Aspergillus* species by both culture and the polymerase chain reaction (PCR) and two were positive for *Aspergillus* species by PCR but negative by culture. PCR yielded results in a few hours whereas isolation and identification of *Aspergillus* by conventional culture methods took an average of 10 days [61]. Very recently, Biswas et al. [62] described the diagnosis of *A. fumigatus* endophthalmitis from formalin-fixed paraffin-embedded tissue by PCR-based restriction fragment length polymorphism. This was done by applying the technique to the paraffin section of an eyeball that had been enucleated from an 8-month-old child due to endogenous endophthalmitis. The use of such molecular methods for diagnosis of *Aspergillus* endophthalmitis is likely to increase in the future.

7 Histopathological Studies on *Aspergillus* Endophthalmitis

Histopathological studies have provided useful information about the extent and pathogenesis of *Aspergillus* endophthalmitis [44, 63]. The subretinal space or sub-retinal pigment epithelium appears to be the primary focus of infection in endogenous *Aspergillus* endophthalmitis, in contrast to endogenous *Candida* endophthalmitis where the vitreous appears to be the primary focus [44]. This may explain why vitreous biopsy may not yield positive results in *Aspergillus*

endophthalmitis. Another important finding in *Aspergillus* endophthalmitis, and not in *Candida* endophthalmitis, is the occurrence of invasion of retinal and choroidal vessel walls by fungal elements [44]. In one ultrastructural study of endogenous *Aspergillus* endophthalmitis [63], the spread of *A. fumigatus* along these two separate paths, that is via the retinal and choroidal vessels, resulted in separate, non-contiguous lesions. Moreover, while the fungi were found to have penetrated the blood vessel walls, Bruch's membrane and the internal limiting membrane, the retinal pigment epithelial layer appeared to have been spared this penetration. Interestingly, the retinal pigment epithelium appeared to act as a barrier, since the subretinal space was not invaded. However, phagocytosis of fungi by the retinal pigment epithelium was observed [63].

The severity of retinal involvement in endogenous *Aspergillus* endophthalmitis may range from subretinal or subhyaloid infiltrates to vascular occlusion and full-thickness retinal necrosis. Intraretinal haemorrhages are frequent [55]. Interestingly, histopathological examination of an eye with severe *Aspergillus* endophthalmitis that had been enucleated while the patient was receiving oral treatment with voriconazole revealed no fungal elements in choroidal or retinal vessels, the fungal hyphae mainly being restricted to the vitreal side of the preretinal inflammatory infiltrate [64]. Since the pathogenesis of endogenous *Aspergillus* endophthalmitis involves primary invasion of choroidal and retinal vessels, the authors suggested that their histological findings indicated the promising activity and ocular penetration of voriconazole.

8 Treatment of *Aspergillus* Endophthalmitis

8.1 General Principles of Treatment of Fungal Endophthalmitis

8.1.1 Vitrectomy

Vitrectomy is generally advised for the treatment of fungal endophthalmitis in all but very mild or exceptional cases, for example where the condition of the cornea does not allow this [5]. Vitrectomy allows for: acquisition of a large sample for laboratory study; concentration of the sample by centrifugation or filtration to give a better yield on culture; debulking of inflammatory and infectious material from the vitreous; removal of the scaffolding for vitreoretinal traction bands and epiretinal membranes that can contribute to late-developing macular pucker and retinal detachment; direct injection of antifungals into the vitreous cavity [55]. The indications for vitrectomy in patients with fungal chorioretinitis and endophthalmitis are advanced cases with extensive vitreous involvement and poor response to systemic antifungal therapy [55].

8.1.2 Antifungal Agents

In addition to vitrectomy, antifungals are administered orally, parenterally and, if the situation permits, by intravitreal or intracameral injection. Intravitreal injections

tend to improve the intraocular concentration of antifungal drugs, to concentrations greater than can be achieved by systemic preparations; however, toxicity may occur if the dilutions are not prepared properly. In the local treatment of endogenous fungal endophthalmitis, intravitreal amphotericin B is the usual recommended initial treatment, while intravitreal miconazole can be considered for those rare fungal cases resistant to amphotericin B [65, 66]. Periocular amphotericin B is rarely used because it has poor intravitreal penetration and frequently causes marked conjunctival necrosis. Since systemic (intravenous) amphotericin B may cause serious (sometimes irreversible) toxicity, its use is limited to very advanced endogenous fungal endophthalmitis, particularly if other non-ocular sites are involved [42, 65]. Less toxic azole compounds (fluconazole, itraconazole, ketoconazole, miconazole) can supplement intravitreal therapy and may contribute to a favourable clinical response without the risk of unwanted side-effects. Because of its broad spectrum of coverage, low minimum inhibitory concentration (MIC) levels for the organisms of concern, good tolerability, and excellent bioavailability with oral administration, voriconazole has recently emerged as a major advance in the prophylaxis or management of exogenous or endogenous fungal endophthalmitis [67]. Topically administered voriconazole achieves therapeutic concentrations in the aqueous of the non-inflamed human eye for many fungi and moulds, and may be a useful agent for prophylaxis against the development of fungal endophthalmitis [68].

Vitrectomy and intravitreal injections are better avoided in cases of neonatal endophthalmitis. The role of intravitreal corticosteroids remains controversial, but can be considered if appropriate antimicrobial coverage of the causative organism can be assured [65].

8.2 General Principles of Treatment of Exogenous Fungal Endophthalmitis

Recommendations for exogenous fungal endophthalmitis include surgical measures and medical therapy.

8.2.1 Recommended Measures for Post-Operative Fungal Endophthalmitis

Surgical measures include excision of clinically involved tissue, vitrectomy (if there is visible vitreous involvement) and retention of the intraocular lens (if the endophthalmitis is a sequel to cataract surgery), unless there is extensive infiltration around the lens or recurrent infection. Medical therapy consists of intravitreal, topical, subconjunctival and systemic antifungal therapy. For intravitreal therapy, amphotericin B (5–10 μg) is injected in regions of maximal involvement (these injections may be repeated if indicated). If there is no apparent response to amphotericin B, then miconazole (25–50 μg) can be injected intravitreally. Topical therapy is administered hourly; this may consist of topical natamycin (5%), topical amphotericin B (0.15%) and subconjunctival amphotericin B (500–1,000 μg) or topical miconazole (1%) and subconjunctival miconazole (5–10 mg). Systemic therapy, in the form of

oral ketoconazole (400–600 mg per day) is given if there is corneal, scleral or anterior chamber involvement [30].

8.2.2 Recommended Measures for Fungal Endophthalmitis Developing from Fungal Keratitis

Surgery is undertaken if there is deep keratitis with retrocorneal or anterior chamber involvement unresponsive to medical therapy. Surgical measures include penetrating keratoplasty with retention of the iris and lens (if possible), or iridectomy, lensectomy and vitrectomy if there is clinical evidence of infiltration of these structures, or if there is recurrence of the endophthalmitis. Medical therapy is similar to that given for post-operative or post-traumatic fungal endophthalmitis, and consists of: intravitreal amphotericin B (5–10 μg) if the anterior chamber or vitreous is involved; hourly administration of topical natamycin (5%), topical amphotericin B (0.15%) or topical miconazole (1%); subconjunctival miconazole or amphotericin B; and oral ketoconazole.

8.3 Treatment of Aspergillus *Endophthalmitis*

The outcome of treatment of *Aspergillus* endophthalmitis is highly variable, ranging from death of the patient due to disseminated aspergillosis [11, 44, 48] to enucleation or evisceration of the affected eye [13, 22–24, 35, 44, 45, 51, 64] to poor outcome [57] to improvement in the clinical condition [6, 10, 21, 25, 26, 35].

8.3.1 Treatment of Endogenous *Aspergillus* Endophthalmitis

The optimal treatment for endogenous *Aspergillus* endophthalmitis remains controversial. This is, in part, due to the fact that, in contrast to the outcome of endophthalmitis due to *Candida* spp., the mortality is high in patients with *Aspergillus* endophthalmitis, particularly those who have received organ transplants or who have undergone cardiac surgery [44]. Moreover, endogenous *Aspergillus* endophthalmitis during systemic aspergillosis usually requires more than systemic treatment to achieve resolution of the ocular infection. The visual outcome in these patients is influenced primarily by the propensity of *Aspergillus* for initial macular choroidal involvement.

The following treatment regimen was recommended in 1998 for endogenous *Aspergillus* endophthalmitis: pars plana vitrectomy to remove the focus of infection when vitreous seeding has occurred and to improve the likelihood of a positive culture, [may not be necessary in fellow eyes with isolated chorioretinal lesions and when the diagnosis can be made from evaluation of the first eye]; amphotericin B (5–10 μg) as intravitreal antifungal treatment; intravitreal dexamethasone 400 μg (this is a controversial recommendation) to reduce the marked intraocular inflammation in many of these eyes; intravenous amphotericin B to supplement the effectiveness of intravitreal amphotericin B therapy and to treat proven or clinically

suspected systemic aspergillosis; a well-tolerated and less toxic systemic medication (example, itraconazole) to supplement intravitreal amphotericin B in patients with only endogenous *Aspergillus* endophthalmitis and no systemic manifestations; repeat intravitreal amphotericin B (5–10 μg) and possibly repeat vitrectomy in patients with persistent vitreous infiltrates and suspected recurrent disease after initial treatment [42]. When this regimen was used to treat 12 eyes of 10 patients with endogenous *Aspergillus* endophthalmitis, a significant improvement in visual acuity was obtained in 3 eyes without central macular involvement, and an improvement in visual acuity in 8 eyes which had presented with initial central macular involvement; 2 eyes had to be enucleated due to intense pain and associated complications [42]. In addition to this case series, there is additional anecdotal evidence of resolution of lesions or marked improvement in the patient's ocular status in endogenous *Aspergillus* endophthalmitis by using a combined approach of surgery (usually pars plana vitrectomy) with an intravitreal antifungal (usually amphotericin B) and a systemic antifungal (usually amphotericin B). This is illustrated by the case of an intravenous drug abuser who developed endogenous endophthalmitis due to *A. flavus*. The patient recovered useful vision after resolution of the endophthalmitis. The lesions did not recur, in spite of extensive retinal and vitreous involvement, since vitrectomy and intravitreal injection of amphotericin B were performed within 48 h of presentation, followed by intravenous administration of amphotericin B several days later [59]. In another patient who developed bilateral endogenous endophthalmitis following renal transplantation, the infection being due to a bacterial cause in one eye and being due to *Aspergillus* in the other eye, pars plana vitrectomy and the administration of intensive antibacterial and antifungal therapy led to an improvement in the patient's ocular status. Unfortunately, the patient succumbed to disseminated *Aspergillus* infection [48]. In a centre in India, fungal endophthalmitis was found to have occurred in 12 eyes of 12 non-immunocompromised patients, each of whom had received a single intravenous administration of presumably contaminated dextrose infusion fluid [54]. All 12 eyes underwent initial vitreous tap with injection of intravitreal antibiotics, 11 eyes underwent pars plana vitrectomy and received oral fluconazole or itraconazole for 4–6 weeks, and one eye with panophthalmitis was treated with intravenous amphotericin B. Significant improvement in visual acuity was obtained in 8 of the 12 eyes, and *Aspergillus* species were isolated from 9 of the 12 eyes and from 9 of 72 samples from dextrose bottles [54]. *A. fumigatus* was isolated from the vitreous sample of 1 of 4 patients who developed endogenous fungal endophthalmitis following intravenous drug abuse. Treatment with itraconazole and pars plana vitrectomy led to a satisfactory outcome [46]. Pars plana vitrectomy and intravenous administration of liposomal amphotericin B brought about an improvement in visual acuity and a reduction of vitreal inflammation in a HIV-infected immunocompetent man who had culture-proven endogenous endophthalmitis due to *A. terreus*. Initial intravenous fluconazole administration, followed by intravenous administration of deoxycholate amphotericin B, had not brought about a significant improvement in the patient's condition [25]. Intravitreal injection of voriconazole (100 μg/0.1 ml) with pars plana vitrectomy effected a significant improvement in visual acuity and in ocular inflammatory reaction in a young

woman who had developed endogenous endophthalmitis due to *A. terreus* while receiving immunosuppressive agents 5 weeks after lung transplantation; previous treatment modalities, including vitrectomy with repeated intravitreal amphotericin B and systemic voriconazole, had failed to prevent deterioration [21]. The patient recovered with no evidence of systemic fungal infection. Thus, intravitreal voriconazole may be used as an adjunct to systemic treatment in patients with *Aspergillus* endophthalmitis, but further clinical studies are needed to determine how often this approach can safely treat this condition.

Unfortunately, other investigators have reported less favourable results when treating endogenous *Aspergillus* endophthalmitis. In two patients who developed culture-proven *A. flavus* retinitis following haematological stem cell transplantation, there was a poor response to antifungal medication [69]. When 20 eyes with culture-proven endogenous fungal endophthalmitis (17 due to *Candida* species, 3 due to *Aspergillus* species) were treated with pars plana vitrectomy, intravitreal amphotericin B and administration of appropriate systemic antifungals (oral fluconazole for *Candida* spp.), it was found that 13 (76%) of 17 eyes with *Candida* infection achieved visual acuities equal to or better than 20/400, while none of the 3 eyes with *Aspergillus* infection achieved such a visual acuity [56]. In another study, when 13 eyes with culture-proven endogenous fungal endophthalmitis received initial treatment consisting of vitreous tap/pars plana vitrectomy and injection of intravitreal medication, three eyes with *Aspergillus* endophthalmitis were found to have significantly worse visual outcomes than eyes with either *Candida* or bacterial endophthalmitis [57]. In endogenous *Aspergillus* endophthalmitis in a 38-year-old HIV-infected man who presented with sudden visual loss, high-dose systemic antifungal therapy could not prevent penetration of the posterior wall of the eye and infiltration of adjacent orbital structures; enucleation of the infected eyeball had to be performed [52]. A combination of intravenous and intravitreal amphotericin B therapy was not found to be effective in therapy of endogenous endophthalmitis due to *A. fumigatus* in a patient who developed the condition following induction chemotherapy for acute myeloid leukaemia [50]. Two patients who developed endogenous *Aspergillus* endophthalmitis following immunosuppression for renal transplantation were treated with combinations of different anti-fungal agents including liposomal amphotericin B, 5-flucytosine, itraconazole, voriconazole and terbinafine, prompting a review of the outcome of endogenous *Aspergillus* endophthalmitis in renal transplant patients [7]. It was found that the outcome in such individuals was generally poor, the mortality ranging from 70 to 100%, but that a combination of vitrectomy and intravitreal amphotericin B appeared to be the best way to manage such patients [7].

8.3.2 Treatment of Exogenous (Post-Operative) *Aspergillus* Endophthalmitis

The outcome of treatment of post-operative *Aspergillus* endophthalmitis is also varied, with successful outcomes being reported by a few investigators [10, 31] and treatment failures by others [4, 6, 23, 24, 32].

A middle-aged patient in India developed endophthalmitis due to *A. terreus* following intracapsular cataract extraction without implantation of an intraocular lens; the patient responded to vitrectomy and intravitreal amphotericin B [31]. Durand et al. [10] described exogenous (post-cataract surgery) endophthalmitis where *A. fumigatus* infection was diagnosed following three vitrectomies and removal of the intraocular lens. In this patient, there were early signs of recurrence one week after giving intravitreal amphotericin and systemic voriconazole, but the eye improved when intravenous caspofungin was added. Caspofungin was continued for 6 weeks and voriconazole for 6 months, resulting in good visual recovery. The authors concluded that caspofungin may act synergistically with voriconazole in treating *Aspergillus* endophthalmitis. Very recently, a successful outcome of therapy of post-operative endophthalmitis due to *A. flavus* was reported in an elderly diabetic male – the patient was treated with a combination of topical amphotericin B, intravenous liposomal amphotericin B and caspofungin following vitrectomy [6].

Failures in the treatment of post-operative *Aspergillus* endophthalmitis have been reported in different settings. In San Francisco, United States of America, a 94-year-old man who developed endophthalmitis due to an *Aspergillus* spp. following phacoemulsification and intraocular lens implantation failed to respond to therapy with vitrectomy and intravitreal amphotericin B [32]. Tabbara and Al-Jabarti [4] reported on an outbreak of culture-proven exogenous endophthalmitis due to *A. fumigatus* in 5 patients who had undergone cataract extraction during hospital construction in an hospital in Jeddah, Saudi Arabia. One patient received only antifungals (intravitreal and subconjunctival miconazole, intravenous and intravitreal amphotericin B), two patients had pars plana vitrectomy and intravitreal amphotericin B, while one patient had only pars plana vitrectomy. Unfortunately, evisceration or enucleation had to be ultimately performed for all 5 patients [4]. Brar et al. [17] reported the occurrence of *A. niger* endophthalmitis 9 weeks after cataract surgery, with extension to the cornea; the outcome of the infection was unknown due to loss to follow-up. *A. ustus* appears to cause a particularly severe form of postoperative endophthalmitis. Two recent papers have described the occurrence of post-operative *A. ustus* endophthalmitis in four patients in Turkey which failed to respond to treatment with vigorous systemic (itraconazole and caspofungin) and intravitreal (amphotericin B and caspofungin) antifungal therapy and complete vitrectomy; enucleation had to be performed for all four patients.

8.3.3 Treatment of Exogenous (Post-traumatic) *Aspergillus* Endophthalmitis

Bifrare and Wolfensberger [26] treated a patient with protracted endophthalmitis due to *A. versicolor* following corneal microperforation due to ocular trauma by vitrectomy with dissection of a protuberant epiretinal filamentous tissue in the temporal fundus and intravitreal injection of antibacterials and amphothericin B. There was an improvement in visual acuity after treatment with topical corticosteroids and oral antifungal therapy.

In an experimental rabbit model of *A. fumigatus* keratitis, an intravitreal injection of the echinocandin, micafungin, was found to be as effective as intravitreally

administered amphotericin B and slightly more effective than voriconazole in preserving the amplitude of the b-wave in electroretinogram readings; a significant reduction in the b-wave amplitude was noted in infected eyes receiving only intravitreal saline [70].

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Urinary Tract Infections Caused by *Aspergillus* Species

Geetha Sivasubramanian and Jack D. Sobel

Abstract Urinary tract aspergillosis is uncommon even in the era of increased frequency of invasive mycoses. Experience is largely a function of isolated case reports and rare case series or reviews. The majority of cases involve transplant recipients predominantly following renal transplantation but is also reported in other immunocompromised states such as AIDS and uncontrolled diabetes mellitus. Most cases represent haematogenous spread to renal parenchyma, often in the absence of recognized disseminated infection, presenting as small or large abscesses, infarcts, renal insufficiency or urinary drainage system fungal balls with obstructive uropathy. Diagnosis is usually made on the basis of renal tissue aspiration and urine cultures. Newer anti-*Aspergillus* drugs notably voriconazole offer less toxic therapeutic options and are quite successful in combination with drainage measures to relieve urinary stasis.

Keywords Bezoars · Fungal balls · Prostate · Renal abscess · Urinary aspergillosis

Contents

J.D. Sobel (✉)
Division of Infectious Diseases, Wayne State University School of Medicine, Harper University Hospital, 3990 John R, Detroit, MI 48201, USA
e-mail: jsobel@med.wayne.edu

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1 Introduction

Opportunistic infections due to filamentous fungi such as aspergillosis have increased in incidence in the past decades due to higher number of immunocompromised patients such as transplant recipients and AIDS. However, *Aspergillus* infection of the genitourinary tract still remains a very rare entity. In majority of these cases, renal infection results from dissemination of primary pulmonary infection in an immunocompromised host and is most frequently seen in renal transplant recipients [1, 2].

2 Pathogenesis

Genitourinary tract involvement in aspergillosis is very uncommon. When infection does occur, it most commonly involves the kidney and occasionally the prostate. Sporadic cases of other anatomic sites such adrenals and testes have been reported [3]. *Aspergillus fumigatus and Aspergillus flavus* are the most common causative species reported [2].

Defective phagocytic function, both qualitative and quantitative and prolonged neutropenia due to various causes such as metabolic dysfunction, chronic disease, and steroid or immunosuppressive therapy predisposes to this opportunistic mycotic infection. Underlying clinical conditions include uncontrolled diabetes mellitus, intravenous drug use, HIV, malignancy, steroid therapy and immunosuppressive therapy [4–10]. Other predisposing factors include antibiotic therapy, renal stones, obstruction and rarely instrumentation during ureteroscopy and stent placement [2, 11].

The urinary system may be affected in any of the following three patterns [12]:

√ Disseminated aspergillosis with renal parenchymal involvement
√ *Aspergillus* cast of the renal pelvis leading to formation of fungal ball/bezoar
√ Ascending pan-urothelial aspergillosis.

Renal involvement in disseminated aspergillosis results from haematogenous spread of the fungi to the kidneys. Angioinvasion by the fungus leads to vascular occlusion producing parenchymal necrosis, multiple infarcts and abscesses. This is typically seen in immunocompromised hosts, usually post-transplant, bone marrow or solid organ with no post-renal disease or obstruction. Renal aspergillosis is most frequently described in renal transplant patients as part of disseminated disease [10]. However, cases of pseudoaneurysm of iliac artery and isolated renal aspergillomas leading to ureteric obstruction from within few days to several months post-transplant have been described [1, 7, 13–16]. The concern in such cases is either a donor-transmitted infection from an immunocompromised, sub-clinically infected donor or contamination during the preservation process.

Aspergillus spp. colonisation of the urinary tract is rare but has been reported in patients with malignancy, diabetes, intravenous drug use and immunosuppressive

therapy. Uncontrolled diabetes leads to defective phagocytic function, glycosuria and renal injury, all promoting antegrade fungal colonization of the renal pelvis. Many such patients have more than one risk factor [17]. Other contributing factors may include renal stones and retrograde introduction of contaminated instruments [2]. However, a critical factor in such cases is pre-existing urinary stasis. Fungal balls or bezoars which are masses of tangled hyphal elements, fibrin and mucus develop in the urinary drainage system and block the ureters leading to further urinary obstruction and worsening renal function.

Ascending pan-urothelial aspergillosis refers to ascending infection involving the urethra, bladder, ureters and kidney. This pattern is the least commonly reported [12].

3 Pathological Features

The histopathologic findings in disseminated aspergillosis involving the kidneys include multiple microabscesses, vasculitis, infarction and papillary necrosis with sloughing. The papillary necrosis with the inflammatory infiltrate may extend in cords from renal papillae to the adjacent medulla sparing the cortex [1]. Microscopic examination of excreted fungal balls and sometimes the urine specimen may show large non-pigmented acutely branching septate hyphae [18].

4 Clinical Manifestations

Clinical symptoms vary based on whether the underlying pathology is an *Aspergillus* abscess or if there is formation of fungal bezoar. Patients with parenchymal involvement and abscess formation present with symptoms such as fever, flank or abdominal pain, fatigue and weight loss [6, 19, 20]. The duration of symptoms may vary from a few days to several months. Presenting features in patients with fungal bezoars include flank pain, fever, dysuria, back pain and passage of brownish-grey soft irregular masses [2, 17] (see Fig. 1). The flank pain may worsen prior to passage of the soft masses [2]. If the fungal balls block the ureters, the patient may develop ureteric colic and present with acute onset fever, flank pain, nausea, vomiting, dysuria and haematuria [4, 7]. Cases of sepsis, hydronephrosis, anuria and acute renal failure due to bilateral ureteral obstruction from *Aspergillus* bezoars have been described [9, 21]. In renal transplant patients, parenchymal involvement or ureteric obstruction is associated with a high risk of graft failure, dissemination and sepsis [1, 10]. Formation of iliac artery pseudoaneurysm may cause paresthesias, sciatica and lower limb ischemia [13, 14]. Physical findings in most cases may be normal except for costovertebral angle tenderness. Initial laboratory evaluation may show increased creatinine, elevated white blood cell count, haematuria and pyuria. Renal failure occurs infrequently in cases of renal transplant recipients as well as in ureteral obstruction [1, 21].

Fig. 1 *Aspergillus* fungal ball or cast passed in urine in a male presenting with ureteric colic and passage of soft particulate matter in urine. Culture was positive for *A. fumigatus.* Fungal cast likely developed following retrograde ureteric procedure, lithotomy of urinary calculi

5 Diagnosis

A high index of suspicion is required to make the diagnosis. Positive urine or tissue culture for *Aspergillus* spp. usually initiates the consideration for aspergillosis in the diagnostic workup. Fungal infection of the upper urinary tract should be considered in the differential diagnosis of any immunocompromised patient presenting with focal renal findings, filling defects on excretory urography or has obstructive uropathy of unknown aetiology. In *Aspergillus* invasive parenchymal disease and abscess formation, imaging study, ultrasound or contrast enhanced computed tomography scan may demonstrate heterogeneous mass-like hypodense lesions in the involved kidney [6, 20]. Urine fungal culture may yield the organism. Percutaneous aspiration of the abscess fluid must be performed if urine cultures are negative. Histopathologic examination of renal tissue may demonstrate the fungal hyphal elements with inflammatory infiltrate and areas of infarction and necrosis.

Microscopic analysis of fungal balls excreted in the urine reveals a mesh of fungal hyphae and *Aspergillus* may be cultured. Routine cultures may miss the diagnosis if careful attention is not paid to the expelled masses [17]. Computed tomography scan or ultrasound in such cases usually reveals pelvicalyceal dilatation [9]. Cystoscopy and retrograde pyelogram may reveal filling defects [21]. Occasionally, the fungal balls may be visualized at the ureteral orifice as a whitish friable mass on cystoscopy [9].

The role of serodiagnosis with galactomannan, β-glucan studies in urinary tract aspergillosis is unknown, including the use of urinary samples.

6 Management

Renal parenchymal or other urinary tract *Aspergillus* disease is treated with systemic antifungal therapy. Lipid formulations of amphotericin B are not recommended due to the limit concentration of these drugs in the renal parenchyma. Underlying renal dysfunction especially in renal transplant recipients frequently limits the use of amphotericin in these situations making voriconazole an attractive alternative choice. Even though parenteral voriconazole may have limitations in impaired renal function, oral therapy is quite effective. None of the azole drugs achieve effective level in the urine with the exception of fluconazole, a drug with no activity against *Aspergillus* species [22]. While fungal bezoars are found in the ureters, the disease originates in or involves the renal parenchyma. Local irrigation with amphotericin B has also been used for urinary bladder and renal pelvic infections [22]. However, systemic anti-fungal therapy should also be administered simultaneously.

Treatment of *Aspergillus* abscess in the kidney is a challenge especially in view of underlying immunosuppressed state. Management requires surgery, in the past invariably nephrectomy, reinforced by post-operative systemic antifungal therapy. Outcome in HIV positive patients with renal *Aspergillus* abscess were unsatisfactory in patients treated with medical therapy alone [20].

Successful treatment of *Aspergillus* fungal bezoars requires a combination of systemic and local antifungal therapy and surgical drainage when necessary [12]. Evacuation of the obstructing hyphal mass is of utmost importance in patients with acute ureteric obstruction and hydronephrosis [4]. This may be achieved by percutaneous nephrostomy, endoscopic or open surgical routes [23]. Temporary percutaneous nephrostomy or ureteral stenting must be done in patients with bilateral obstruction and azotemia [7, 9, 10, 15, 21]. In patients with incomplete obstruction and no evidence of hydroureteronephrosis medical management with antifungals alone may be attempted [17]. Prolonged duration of therapy with initial few weeks of systemic therapy followed by oral voriconazole may be required. Clinical parameters that guide therapy include improvement in symptoms such as fever, passage of fungal balls, resolution of renal failure, sterilisation of previously positive urine cultures and resolution of imaging changes. However, duration of antifungal therapy in most cases is determined by the underlying immunosuppression.

7 Outcome

Despite surgery and adequate therapy, prognosis of patients with renal abscess and parenchymal disease is variable [20]. Early diagnosis and appropriate drainage of ureteric obstruction may improve survival.

8 Prostatic *Aspergillosis*

Involvement of the prostate in aspergillosis is very uncommon with less than 10 cases reported in the medical literature [3, 11]. The patients may present with outflow obstructive symptoms that are indistinguishable from those due to benign prostatic hyperplasia [3]. Other symptoms may include fever, dysuria and perineal discomfort. Patients usually have underlying predisposing conditions such as diabetes, steroids and multiple antibiotic courses [11]. Diagnosis is usually made by unexpected identification of *Aspergillus* on tissue sections in concert with inflammation [11]. Occasionally, *Aspergillus* may be cultured from prostatic fluid. Prostatic aspergillosis should be treated with systemic antifungal therapy similar to therapy directed against renal aspergillosis. In the past, surgery was invariably needed in most cases. However, with the currently available effective antifungal drugs, surgery should only be considered in patients with refractory obstructive symptoms or in the presence of an abscess.

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Cutaneous and Wound Aspergillosis

Arunaloke Chakrabarti, Shivsekhar Chatterjee, and Bishan D. Radotra

Abstract *Aspergillus* infections of skin and wound have been increasingly reported in recent years. These diseases can range from non-invasive colonisation of wounds to invasive lesions that may serve as the aspergillosis portal of entry in immunosuppressed patients. Cutaneous aspergillosis can be broadly divided into: primary infections (starting from skin) and secondary infections (extending to skin from deeper tissues either contiguously or haematogenously). There has been marked increase in number of primary cutaneous aspergillosis cases in conjugation with prematurity, burn, HIV infection, organ transplantation, and malignancy. Wound aspergillosis affects mainly solid organ transplant recipients and those submitted to open-heart surgery. Small outbreaks of primary cutaneous aspergillosis have been reported in neonatal and surgical wards, and intensive care units in association with a contaminated environment. Simultaneous breach of skin and neutropenia predisposes a patient for cutaneous aspergillosis, though rare infection in healthy hosts has been reported. Demonstration of septate hyphae in tissue and isolation of *Aspergilli* help in diagnosis. Early diagnosis depends on increased awareness. These diseases can be of considerable morbidity and mortality if the patients are not managed aggressively. Combined surgery and antifungal therapy prevents the progression to disseminated aspergillosis, though surgery may be difficult in neonates and in HIV-infected patients.

Keywords Aspergillosis · Burn · Skin · Diagnosis · Management · Wound

A. Chakrabarti (✉)
Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh 160012, India
e-mail: arunaloke@hotmail.com

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Contents

1 Introduction

The skin is the largest and most superficial organ of the human body. Being so, it is affected by most systemic diseases, which includes invasive aspergillosis (IA). In rare occasions, cutaneous lesions may be the first recognized manifestation of disseminated IA [1]. Extension of deep-seated *Aspergillus* infection onto skin by contiguous spread has been recognized occasionally [2]. On the other end of the spectrum, a primary cutaneous aspergillosis (PCA) may act as a source of fun-

gal dissemination to various organs of the body, including the lungs, the heart and the central nervous system [3]. The presence of non-infectious (mainly allergic type reactions) satellite lesions affecting the skin of IA patients have also been reported [4]. Simple fungal colonisation on the outermost layers of the stratified squamous epithelium and its adventitious structures may lead to a range of non-invasive infections, including onychomycosis, dermatomycosis and dacrocystitis [5]. For instance, *Aspergillus* infection on the skin of the external auditory canal may lead to simple colonisation on the wax or end up in malignant external otitis [5]. Thus manifestations of *Aspergillus* infection on human skin and wound are varied.

Aspergillus conidia are ubiquitously present in air. Therefore, conidia are in constant contact with patient's wound surfaces. Most immunocompetent hosts are able to ward off these fungi [6]. However, *Aspergillus* spp. do infect wounds specially burn wounds and surgical wounds in transplant recipients, or rarely even accidental wounds. Infections in wounds, though occasional, can lead to severe morbidity and even mortality [7].

IA is typically related to progresses obtained in modern medical science and practices. Newer immunosuppressive therapies, longer longevity of cancer and immunocompromised patients, massive misuse of systemic steroids and broad-spectrum antimicrobial agents, invasive devices and catheters are contributory to the ever-growing incidence of IA [6]. The same is true for cutaneous aspergillosis, whether primary (direct skin inoculation) or secondary (resulting from disseminated IA or contiguous spread). In fact, PCA was rarely reported before 1970 [8]. Literature and case series of cutaneous aspergillosis are being reported with increasing frequency, especially since 1990s and this exponential growth in the number of cases seem unstoppable. Cutaneous and wound aspergillosis can no longer be disregarded as rare and must be kept in the differential diagnosis of all appropriate lesions.

2 History

Cutaneous aspergillosis is a rather new entity. Possibly, the first ever case of primary cutaneous infection was reported in 1930, in a 37-year-old farmer who presented with an indolent ulcerative lesion caused by *A. terreus* on the back of his hand [9]. *Aspergillus* spp. was first recognized as a cause of post-operative wound infection in 1933 [10]. Additional cases of cutaneous aspergillosis were reported in 1947 [11, 12] and in 1967 [5], both in patients with disseminated aspergillosis. Between 1970 and 1984 only 10 such cases of PCA were reported in English literature [8]. Cases of burn wound aspergillosis were published from the USA Army Institute for Surgical Research in 1971 [13]. Granstein et al. in 1980 reported PCA in a premature neonate [14]. Hui et al. in 1984 reported the first case of PCA in AIDS patients at the very beginning of the AIDS pandemic [15]. The second such case occurred only in the early 1990s [16]. Non-invasive cutaneous infections due to *Aspergilli*, though uncommon, have been known for long time and are not related to the advent of modern medical practices. Onychomycosis due to *Aspergillus* species was reported way back in 1919 [17] and 1920 [18].

3 Classification of the Disease

Cutaneous aspergillosis can be subdivided as (modified from references [2, 7, 19]):

I. Non-invasive cutaneous aspergillosis (including onychomycosis, dermatomycosis and otitis externa, dacrocystitis, blepharitis) [11]
II. Immune mediated skin lesions (though only rarely reported, these may be due to immune complex diseases and may present as dermatitis in areas far away from the site of primary aspergillosis) [4]
III. Invasive cutaneous aspergillosis (which may divided in two main groups and several categories, as below):

a. Primary cutaneous aspergillosis (PCA), where route of infection is transcutaneous. These cases may again be divided into:

i. Infections at sites of injury, including intravenous accesses, central venous catheters, traumatic inoculation, adhesive tapes, arm boards, and occlusive dressings
ii. Burn wound infections
iii. Post-operative surgical wound infections [20]

b. Secondary cutaneous aspergillosis (SCA), resulting from an *Aspergillus* infection spreading to the skin (these infections might also be grouped based on the mechanism leading to skin involvement, as below):

i. Haematogenously (as in cases of disseminated aspergillosis)
ii. Contiguously (from underlying diseases like *Aspergillus* rhinosinusitis)

Invasive PCA is known to affect certain patient subgroups. Depending on the underlying risk factors, various authors have clustered the disease into a few groups [19, 21]:

1. HIV-related PCA
2. PCA in burn patients
3. PCA in cancer patients
4. Haematopoietic stem cell transplantation (HSCT) patients with PCA
5. PCA in solid organ transplant recipients
6. PCA in neonates

However, these epidemiological niches do not cover all infections and invasive cutaneous aspergillosis may rarely present even in “healthy” individuals. Post-operative wound infections can be considered another subset of PCA [20, 22], and this is discussed in more detail in a separate chapter. Though the number of cases is still limited (maybe due to the infection being unrecognised), surgical wound aspergillosis is increasing in frequency especially in association with open-heart surgery [20, 23–28].

4 Epidemiology

PCA has been documented in both tropical and temperate climates. Due to the lack of proper medical and diagnostic facilities, the number of cases appears to be low in Africa and parts of Asia. Children (especially neonates) are affected more commonly than adults. In adults the disease is confined to special risk groups like burn victims, HIV-infected patients, and transplant recipients. No special age-related predisposition is seen in adults. Male sex appears to be relatively common sufferers though the number of cases is too small to reach any conclusion.

SCA has been recorded in very few cases. Findlay et al. estimated that SCA accounts for < 5% of cases of disseminated aspergillosis [29]. No other special predisposing factor has been identified for the development of SCA, apart from the well known variables predisposing patients to disseminated aspergillosis (i.e., persistent granulocytopenia, severe and ongoing immunosuppression, progressive underlying malignancy, and long term high dose therapy with steroids). SCA has also been documented in advanced AIDS cases. Considering cases of cutaneous aspergillosis, PCA is more frequent in burn victims, neonates and solid organ transplant recipients, while HSCT recipients tend to develop SCA [30]. Exception to this general rule is also documented in case reports like renal and liver transplant recipients developing SCA [1, 31]. Patients with malignancy particularly leukaemia are prone to both primary and secondary *Aspergillus* infections [19]. The main risk factors for developing PCA are listed as follows.

4.1 Neonates

Aspergillus skin infection is more frequent in the first three months of life, particularly in age group of 5 days to 1 month. The majority of these infections have been recorded in premature infants and very low birth weight babies (< 1,000 g). Term infants have been affected occasionally. All reported cases had a history of skin breach due to adhesive tapes (sometimes in association with mechanical ventilation), chest tubes, occlusion under a pulse oximetry sensor or prolonged placement in supine position [19, 32]. Lying supine for prolonged period leads to maceration of skin of the back and in turn predisposes to PCA [33]. In fact, PCA seems to be the most common type of IA in neonates [34]. Particularities about IA in children are discussed in another chapter in this book.

4.2 Systemic Steroid Therapy

Corticosteroids act in number of ways that increase the risk for IA [19, 35]:

a. Steroid exposure increases conidial germination rate. Linear growth of conidia increases by 30–40% and cellular synthesis by more than 150% in presence of steroids.
b. Steroids decrease the oxidative killing of conidia by macrophages.
c. Steroids are responsible for a state of functional neutropenia.

4.3 Other Immunosuppressive Agents

Important risk factors include the use of antimyeloblastic agents like cyclophosphamide, monoclonal antibodies, antithymocyte globulin, mycophenolate mofetil, and tacrolimus [19].

4.4 Cancers Including Haematological Malignancies

PCA have been reported in patients with leukaemia, multiple myeloma, lymphoma, and astrocytoma [36–39]. Bone marrow in these patients enters in a state of aplasia by overcrowded malignant cells. Aplastic anaemia patients have also been reported to develop PCA. Combination of denuded or macerated skin and haematological malignancy greatly increases the chances of developing PCA. Intravenous catheters (including Hickman catheters), arm boards, or adhesive tapes have all been implicated as risk factors in these cases. Breaks in epithelium while using vaginal clotimazole troches and phlebotomy may also predispose to the disease [19].

4.5 HSCT Recipients

Recent neutropenia is the most important factor in these patients that predisposes to PCA. However, pulmonary and disseminated infections are more common presentations of IA in these patients than PCA. Skin lesions may be multiple. The infection can be acquired from airborne exposure or contaminated material used for treatment purposes [19, 40].

4.6 Solid Organ Transplant Recipients

PCA has been reported in liver and renal transplant recipients [41, 42]. Infection can occur directly on the surgical wound or at a site different from the primary surgical transplantation wound. The latter more frequently occurs in cardiac transplant recipients. Cases are associated with adhesive tapes, pressure sores from a shoe, rigid brace and venous access catheters. Nosocomial spread of *Aspergillus* conidia has been documented in many of these cases [19, 20].

4.7 HIV Infection

At least 24 cases of PCA had been identified in association with HIV by the year 2000, mostly in patients with advanced disease [43]. As expected, fluconazole prophylaxis does not prevent the infection to occur. CD4 counts of such patients are usually < 50 cells/mm^3. However, few cases have been documented with CD4 count

between 50 and 150 cells/mm^3. Cases have been reported in both children and adults. Neutropenia is an additional risk factor and was present in majority of these cases. Roilides et al. found low antifungal activity of neutrophils from paediatric patients infected with the HIV [44]. Poorly functioning neutrophils act synergistically with the low absolute counts to enhance the chances of acquiring PCA. Unlike other groups, *A. fumigatus* is the most common cause of cutaneous infection in these individuals. Trauma, intravenous catheters, adhesive tapes, and occlusive skin dressings have all been recorded as additional risk factors leading to the disease [19]. Murakawa et al. [16] suggested that cutaneous aspergillosis in HIV-patients is usually related to *Aspergillus* colonisation in one of the following sites: burn or other wounds, site of trauma, and at the site of intravenous infusion.

4.8 Burn Victims

Burn is associated with decreased phagocytic activity of neutrophils and macrophages [45–48]. Bacterial microbiota disturbances due to use of broad spectrum antibiotics adds to the risk of *Aspergillus* infection. Locally applied agents on burn wounds like silver sulfadiazine or mafenide acetate have no action against fungi. Barrier function of the skin is obviously lost in burn patients. Most patients with cutaneous aspergillosis associated with such injuries had > 70% of the body surface burned, and the infection usually occurred within 10–35 days of burns. Occasional infection may occur even at 50–60 days after burn. Diabetes mellitus and hyperglycaemia from hyperalimentation are the additional risk factors. Though the rate of infection is low (ranging from 1.0 to 2.4%), these infections may be relatively common after extensive use of topical antimicrobial agents and systemic antibiotics. Construction work in hospitals and poor servicing of air conditioning ducts and filters have been implicated as the source of *Aspergillus* infection in burn unit.

4.9 Placement of Hickman Catheters

Many cases of cutaneous aspergillosis in the early late 1980s and 1990s were due to use of these catheters [8, 19, 32, 49]. The primary mechanism of infection involves making a tunnel through the skin, allowing for a direct inoculation with *Aspergillus* species.

4.10 Maceration of Skin

This may result from prolonged use of contaminated fomites such as contaminated arm boards [19, 32, 50], adhesive tapes [19, 32, 51], and occlusive dressing used for burn patients [52].

4.11 Construction Work in Hospitals

At least four cases of neonatal PCA have been associated with such activities [32].

4.12 Improper Cleaning of Air Conditioning Ducts, Contaminated Ventilating Systems

This has been reviewed in detail by Drs Woodruf et al. [32].

4.13 Post-Surgical Wound Infections

Environment and inanimate objects in the surgical theatres have been found to be contaminated with same *Aspergillus* species in such circumstances [24]. This topic is reviewed in detail in another chapter by Dr Pasqualotto.

4.14 Other Recognised Risk Factors

Intensive care unit (ICU) stay and use of broad-spectrum antibiotics are other important factors in the development of PCA. The plethora of gadgets, some of which may be contaminated with *Aspergillus* conidia, recurrent breakage of cutaneous barrier due to multiple invasive procedures, and the associated immobility contribute to the risk of PCA in ICU patients. Many of such patients have multiple risk factors and the effects are synergistic. Broad-spectrum antibiotics change the commensal skin microbiota that would have otherwise resisted to fungal invasion. PCA has also been documented in patients of chronic granulomatous disease, a congenital immunodeficiency disorder [53] (a discussion on IA in patients with chronic granulomatous disease is presented in another chapter by Dr Segal).

Most *Aspergillus* skin infections are nosocomially-acquired, especially in children, although some of the PCA cases in AIDS patients were acquired in the community. *Aspergillus* conidia usually contaminate nosocomial fomites in close contact with the patients like chest tubes, adhesive tapes, and arm boards. Nosocomial infection may also leads to outbreaks of IA.

5 The Fungi

Most cases of cutaneous aspergillosis involve *A. flavus*, followed in frequency by *A. fumigatus* [54]. *A. flavus* is particularly frequent as an agent of cutaneous aspergillosis in places like Sudan and North India, which is probably influenced by climate conditions [35, 48, 54–56]. But case reports for SCA from such those areas are scant. Considering cases of SCA, a third of cases are caused by *A. fumigatus* and another

third by *A. flavus* [19]. Burn infections, post-operative surgical wound infections, and infections in HIV-infected patients are most commonly caused by *A. fumigatus* [19]. Also in neonates the incidence of *A. fumigatus* infections seems to be higher than *A. flavus* in 2:1 ratio [34]. Apart from these two species, which cause the bulk of diseases, *A. niger, A. terreus* and *A. ustus* have also been reported to cause cutaneous aspergillosis [32, 57–61]. *A. terreus* was noted to cause an outbreak related to arm boards [50]. Rare *Aspergillus* species causing skin infections include *A. versicolor, A. chevalieri, A. sydowii, A. amstelodami, A. oryzae, A. restrictus, A. glaucus, A. candidus* and *A. tamarii* [32]. In some situations the fungus has been misidentified [46, 62].

6 Pathogenesis

The skin is exposed to a continuous shower of *Aspergillus* conidia, which are present in abundance in the air even in normal circumstances. Skin integrity is protected mechanically by a layer of keratin and epidermis. Disruption of this mechanical barrier permits the *Aspergillus* conidia and hyphae to gain access to the epidermal tissue. In immunocompetent host polymorphonuclear leukocytes, peripheral blood monocytes, and monocyte-derived macrophages are present in the epidermal tissue. These cells challenge the fungal elements through phagocytosis and oxidative and non-oxidative mechanisms [7]. Fungi can overcome this defence when the patient becomes immunocompromised. In rare situations, fungi may cause diseases even in immunocompetent hosts [63], although the mechanisms for this are not clear. A review on immunity and fungal infections are presented by Dr Romani in this book.

It is widely believed that only when both the mechanical and the immunological barriers are broken, PCA may develop. As long as skin is intact, PCA is rare even in the setting of profound immunosuppression. Various reports suggest that > 50% of skin surface in burn patients become colonised by fungi, especially *Aspergillus* species. Colonisation alone does not pose any threat unless fungi invade tissues to cause infection [13, 48]. However, traumatic inoculation of *Aspergillus* conidia rarely causes PCA in the apparently healthy host. The epidermis may be broken down by minor injuries (including surgical, burn and accidental ones) or due to maceration in a semi-occlusive environment. Maceration may be produced due to coexistence of heat and moisture, usually occurring under circumstances such as pressure dressings, adhesive tapes, and arm boards. Premature and very low birth weight babies have a very thin skin, just like parchment, and these structures can be easily damaged, especially in settings where maceration is common due to gadgets in the ICU [64].

As already mentioned, the incidence of PCA was very low before 1970. But with the expanding spectrum of immunosuppression and longer life of immunocompetent patients, cases have become relatively frequent since the 1990s. If a wound contaminates with *Aspergillus* conidia in a patient with neutropenia, the paucity of effective local phagocytic host defences permits conidia to germinate into hyphal

forms. The hyphae then invade deep dermal and subcutaneous structures, although rarely reaching deep tissues such as muscles, fascias and bones. Hyphal invasion in such patients may lead to angioinvasion, tissue infarction and necrosis. Occasionally angioinvasion may progress to disseminated infection, affecting the lungs, the heart, the liver and brain. Thus the sequence of conidia germination, hyphal growth with subsequent angioinvasion and tissue infarction, necrosis and dissemination parallels that of pulmonary IA. The main difference between these syndromes is the portal of entry; in this case, the skin [7].

Situations that decrease host phagocyte response might also increase the risk for cutaneous aspergillosis. These include functional deficiencies of immature neutrophils in preterm babies, very low birth weight babies, high dose treatment with systemic steroids, malnutrition, burns, diabetes mellitus, HIV infection, organ transplant, graft versus host disease, uraemia, liver diseases and autoimmune conditions. Patients with advanced AIDS with a < 25% expected CD_4 count have shown to have poor neutrophil and macrophage phagocytic responses [44]. Even patients with a CD_4 count of > 25% expected are found to have intermediate levels of response. In addition, many patients with advanced AIDS have also profound neutropenia, a well-known risk factor for IA. This neutropenia may be related to increase expression of interleukin (IL)-4 and IL-10 or suppressed expression of T helper (Th)1-related cytokines. Such neutropenia predisposing to PCA is classically seen in patients with haematological malignancy, those receiving cytotoxic chemotherapy and HSCT recipients.

PCA may occur in non-neutropenic hosts with granulomatous diseases, severe burns, and autoimmune conditions. High dose of steroid is a major predisposing factor in those patients [7].

In addition to host factors, *Aspergilli* themselves have a plethora of putative virulence factors directed at the cutaneous structures. Conidia express receptors for laminin, fibrinogen, collagen I and IV and fibronectin, all of which are found in abundance in damaged cutaneous tissue. In addition, these conidia bind complement cascade inhibitor molecules like factor H, factor H-like proteins, factor I, and plasminogen. These in turn cleave C3b and hinder opsonization of the conidia. Conidial pigment molecules may also protect against deposition of complement [7].

7 Pathology

The pathological lesions of cutaneous aspergillosis vary from erythematous papules, infiltrated plaques, ulcerative nodules to deep subcutaneous erythematous nodules [65, 66]. The infection may also be manifested by small skin infarcts and burn wound infections. The gross appearance of the skin lesions may vary from firm to soft necrotic lesions depending on the nature of inflammatory reaction and presence or absence of necrosis. In embolic lesions in which vasculitis occurs, the lesions are soft and haemorrhagic (erythematous). In deeper disseminated lesions, the subcutaneous fat may become necrotic with yellowish central areas surrounded by fibrous

tissue and normal lobules of adipose tissue [67]. The histopathological reactions in cutaneous aspergillosis vary to a greater extent. In surgical biopsies, the overlying epidermis is normal in most cases except for ulcerated lesions. However, focally acanthotic and hyperkeratotic epidermis is sometimes seen. In some reports pseudoepitheliomatous hyperplasia secondary to cutaneous aspergillosis have also been described [68]. In our experience variable histological reactions in the dermis have been seen in cutaneous aspergillosis. It may consist of heavy lymphomononuclear cell infiltration admixed with multinucleate giant cells and occasional eosinophils with a few neutrophils (Fig. 1). The multinucleate giant cells on careful examination would contain occasional septate fungal hyphae. These are difficult to identify when cut in transverse sections. The high index of suspicion in these cases followed by demonstration of fungi on histochemical stains and growth by culture confirms the diagnosis of cutaneous aspergillosis. In other cases a well-formed granulomatous response consisting of discrete epithelioid cell granuloma with Langhans type of giant cells is noted (Fig. 2). The branching fungal profiles are found within giant cells or in the accompanying fibrous tissue. The distribution of fungal hyphae may be sparse and therefore other skin conditions associated with granulomatous reactions need exclusion. However, the presence of collections of neutrophils within granuloma is a good clue to the diagnosis of a fungal aetiology. In order to confirm the presence of fungal hyphae appropriate stains such as the periodic acid-Schiff stain (PAS) and Grocott modification of Gomori methenamine silver stain (GMS) are ultimately necessary. The adjacent dermal areas may show dense perivascular lymphomononuclear infiltrate. The third type of histological reaction consists of dense necrotising inflammation with focal accumulation of multinucleate or Langhans type of giant cells. These lesions show a large number of septate dichotomously branching fungal hyphae in necrotic areas and within giant cells. In addition, vascular thrombosis and nuclear debris are noted in these lesions. This is particularly true for the disseminated form of the disease, which involves deeper parts of the

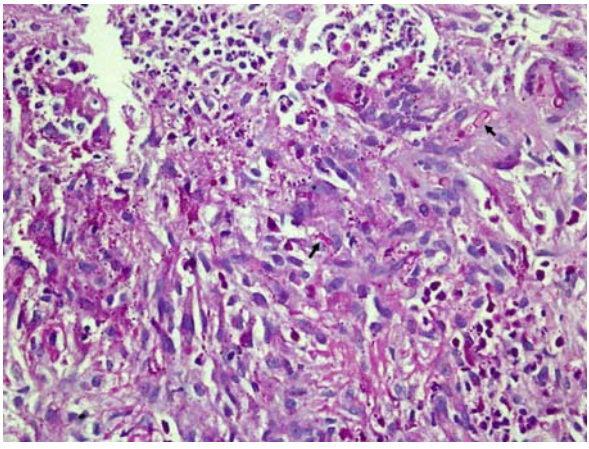

Fig. 1 Mixed inflammatory reaction with a few multinucleate giant cells containing septate fungal hyphae (*arrows*). The centre of the lesion contains collections of neutrophils (PAS)

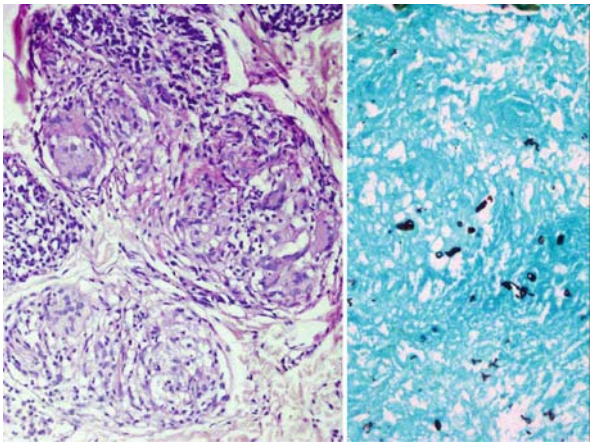

Fig. 2 Multiple epithelioid cell granulomas with lymphocyte and Langhans type of giant cells (H&E) (*left side*). GMS stain (*right side*) shows fungal profiles in giant cells

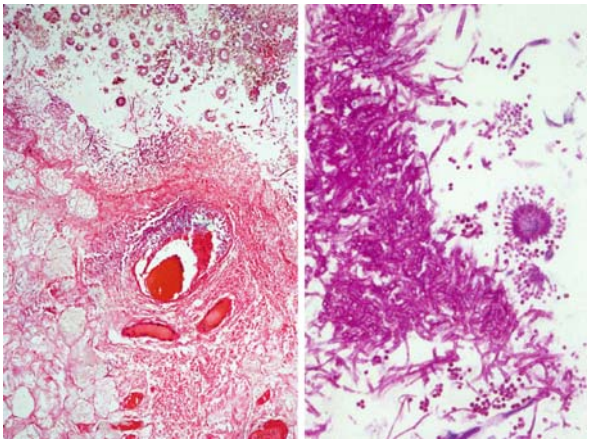

Fig. 3 Acute necrotising inflammation involving subcutaneous fat. Vasculitis and conidiophores of *Aspergillus* are noted (H&E) (picture on the *left*). Numerous fungal hyphae and conidiophores are seen on PAS stain in other area (*right*)

dermis and subcutaneous tissues. Such cases are clinically very similar to pyoderma gangrenosum. In superficial wound infection like surgical wounds, besides large number of hyphae, characteristic *Aspergillus* head may be observed (Fig. 3).

8 Clinical Features

Findlay et al. in 1971 first classified cutaneous aspergillosis (including both primary and secondary) into five categories [29]:

1. Solitary necrotising dermal plaques: characterised by the presence of a solitary, firm, red, cellulitic lesion, which is followed by ulceration and the presence of a black crusting. It occurs usually at the site of intravenous infusion or direct inoculation or by selective settling of blood borne fungi at the damaged site.

2. Subcutaneous granulomas and abscesses: these are usually associated with metastatic lesions.
3. Persistent eruptive dermal maculopapules: 2–15 mm reddish purple dermal granulomata. These lesions start out deceptively as a macular rash mainly on trunk in the course of pulmonary aspergillosis. There is a tendency for suppuration and necrosis formation. Parts of the rash persist and evolve into chronic granulomata with overlying epidermal necrobiosis and hyperkeratosis.
4. Miscellaneous erythemas and toxicodermas: the evanescent parts of the above-mentioned rash are characteristic of these lesions. These represent mainly allergic reactions.
5. Progressive confluent granulomata: a variety of changes may be observed in these lesions. The overlying epidermis may be smooth, scaly, crusted or oozing. The granuloma itself may remain solid or transformed into a necrotic or exudative lesion.

Apart from these, SCA may also present as ecthyma gangrenosum. Embolic skin lesions similar to those found in disseminated candidiasis may also appear in up to 10% of disseminated IA cases.

It is worth noting that the above classification by Findlay [29] was mainly created to group cases of SCA, since PCA was rarely encountered in those times. In comparison to SCA lesions, PCA may present as macules, papules, nodules, or plaques [19, 32]. In some patients just a rash is seen. PCA is an important differential diagnosis in the setting of purpuric rash in a preterm neonate – simple eruptions on preterm VLBW infants in a hospital setting should be considered seriously. Pustules and lesions with purulent discharge are primarily seen in neonates. Haemorrhagic bulla is one more type of lesion not described by Findlay [29], but commonly found in *Aspergillus* infections associated with arm boards, adhesive tape and occlusive dressings. Lesions related to arm boards are mostly erythematous macules and papules, which progress to haemorrhagic ulcers and eschars that in neonates are usually restricted to palms. These may become confluent and involve the whole of the palm [50]. The appearance of these lesions is similar to that of ecthyma gangrenosum. Lesions located on the back in neonates are not usually associated with trauma. In adults with organ transplants, isolated erythematous fluctuant swellings with multiple pustules may be present. In patients with diseases related to adhesive tapes, the lesion usually starts below the tape and mostly present as black necrotic ulcers with eschar and haemorrhagic bulla formation. Lesions associated with Hickman catheters may be located at exit sites (most commonly), entrance or in the tunnel [8]. Multiple large and small tender nodules on face, limbs and trunk along with infiltrated papules on nose, forehead, cheek and trunk have been recorded. Slowly progressive multiple painful nodules over the extremities and trunk of a farmer (who was on systemic steroids) has also been recorded [35]. Contiguous spread from paranasal sinuses leads to haemorrhagic or blistering lesions on the forehead, cheek and eyelids [2].

Aspergillus lesions on the face have been documented in adults with no underlying risk factors [5]. These may range from necrotic ulcers on the cheeks to pruritic

papules and nodules. Lesions may remain stable and progress locally to cause extensive facial deformities in such immunocompetent patients. The skin may become thick, oedematous and nodular. Serum and mucopurulent material may be seen to ooze out from the base of multiple confluent nodules. Differential diagnosis in such circumstances includes lepromatous leprosy.

Fungal lesions on burn wounds usually occur on second-degree burns or greater. Most patients have > 50% burns although a few have ∼30% burns. Fever is the most common clinical sign, followed by wound swelling, "conversion" of the wound, ulceration, induration, oedema, and tenderness with early separation of eschar and muscle necrosis. The earliest work on *Aspergillus* burn wounds by Bruck et al. in 1971 [13] carries the most vivid and lucid description of the lesions. Fungi may penetrate deep from the burn wound to cause osteomyelitis. Our own case series documents necrosis and eschar formation and separation as the commonest clinical presentation [48]. Appearance of dark brown alteration in a burn wound signifies haemorrhage and is a marker of severe *Pseudomonas aeruginosa* or *Zygomycetes* infection. But occasionally these lesions may be associated with infection by *Aspergillus* spp.. Multifocal involvement may be seen in some individuals. These may occur in several sites simultaneously or serially. Koebner's phenomenon on healed scars may also be noted. Overall patients with PCA present with significantly less necrosis and systemic toxicity than wounds infected with *Zygomycetes.*

Surgical wounds infected with *Aspergillus* species show increased local inflammation, non-healing, graft rejection, fistula formation, and pus collection [20]. Lesion may extend into the bones causing osteitis and chondritis. In case of open-heart surgery patients, most wounds are located on the sternum. Mediastinitis and post-operative spondylodiscitis have also been described as post-surgical *Aspergillus* wound infections. These lesions usually develop in the 2nd post-operative week. Apart from the anterior chest, wounds over abdomen, ear, spine and exenterated orbit are also reported. Some cases are associated with systemic dissemination and death.

8.1 Contiguous Spread of Deep Aspergillus Infection

Cases have been described in fungal rhinosinusitis where cheek, eyelids, and nose are affected by necrotic ulcers. A case of bronchopleural fistula resulting from underlying lung and pleural *Aspergillus* infection has also been described [69].

8.2 Onychomycosis

Onychomycosis due to *Aspergilli* was considered rare in the past. Many thought these to be as mere contaminants. To meet diagnostic criteria one must isolate the same *Aspergillus* spp. in multiple samples from the same patient. However many

recent reports document it to be the most common cause of non-dermatophytic mould infections of the nails. The incidence seems to be high (5–15%) in the tropics [70, 71]. Increased incidence is also seen in HIV-infected individuals. Some cases may be due to co-infection with dermatophytes, where the later may be the primary invader, lowering the tissue resistance for a second invader like *Aspergillus* spp.. However, the majority of *Aspergillus*-related onychomycosis are unimicrobial. In these cases, there may be a general or local lowered resistance or even a nutritional or metabolic dysfunction that predispose hosts to the infection. Lesions may be divided into mainly three varieties: distal and lateral subungual, proximal subungual and white superficial disease. Total dystrophic and paronychia are not usually observed, except in HIV-associated disease. A deep-seated greenish discoloration of the nails may be seen in a few cases. Bereston et al. first made this observation in 1941 [72]. Associated areas of the skin may be affected by *Aspergilli*. Scaling, vesicles and patches of superficial exfoliation are seen on the affected nearby skin. For additional detail on *Aspergillus* onychomycosis please refer to the chapter written by Dr Negroni in this book.

9 Laboratory Diagnosis

Early clinical suspicion is crucial and tissue specimens are the best samples for a proper diagnosis. Superficial swabs are not recommended. Both histopathology and isolation of fungi should be attempted from the sample. Calcofluor white stain may help in the detection of *Aspergilli* on direct microscopy. In rare cases of superficial wound colonisation *Aspergillus* heads or even sclerotium [22] can be seen in the clinical sample (Fig. 3). Special fungal stains like PAS and GMS should be used in the histopathology laboratory to detect the fungus. These allow for a better discrimination of the narrow (3–5 μm wide large), acute angled branching septate hyphae in tissue. Surrounding tissue may show acute or chronic inflammation – chronic reactions are commonly seen and made up of lymphocytes, giant cells and epithelioid cells. The Splendore-Hoeppli phenomenon (eosinophilic pseudomycotic structures composed of necrotic debris and immunoglobulin) may be seen on haematoxylin and eosin (H&E) staining. Fungi are stained negative on H&E, black on GMS and red on PAS staining. The differential diagnosis should be made on histopathological examination include with infections caused by *Fusarium* spp., *Penicillium* spp., *Scedosporium* spp., and other hyalohyphomycetes. Culture helps in accurately identifying *Aspergillus* species.

Galactomannan antigen detection does not usually add in the diagnosis of cutaneous aspergillosis due to false-negative results, except in disseminated cases. If a positive result is encountered, one should search for other foci of infection like lungs. Other serological tests also do not seem to help in the diagnosis of cutaneous aspergillosis. One of the major diagnostic criteria for the diagnosis of PCA is the exclusion of *Aspergillus* infection in other organs, since the skin has to be the primary site of infection.

10 Differential Diagnosis

Table 1 summarises the main conditions to be considered in the differential diagnosis of cutaneous aspergillosis.

Table 1 Differential diagnosis of cutaneous aspergillosis

Infectious	Non-infectious
Cutaneous zygomycosis	Pyoderma gangrenosum
Cutaneous fusariosis	Polyarteritis nodosa
Cutaneous infections due to dematiaceous moulds (black fungi)	Cryoglobulinemia
Cutaneous infections due to *Pseudomonas aeruginosa* (ecthyma gangrenosum)	Insect and spider bites
Soft tissue infections due to *Aeromonas* spp.	Transilluminator burns in babies at neonatal intensive care units [73]
Cutaneous cryptococcal infections	Dermatitis

11 Management

The three-pronged treatment strategies for PCA include surgery, systemic and topical antifungal agents, and reversal of underlying immunosuppression. Many authors have suggested the use of immunomodulator agents like granulocyte colony stimulating factor (G-CSF) to reverse immunosuppression but there is still controversy about this. PCA has been recorded in AIDS patients despite prophylaxis with G-CSF. Surgery is not well-tolerated in neonates. SCA cases should be treated as disseminated aspergillosis with systemic antifungal agents. Removal of offending adhesive tapes, arm boards, and dressing is mandatory. Reversal of underlying immunosuppression may help in managing SCA.

11.1 Surgery

Surgical debridement of lesion depends on the extent and depth of invasion, the host capacity of wound healing, and clinical stability of the patient to undergo a surgical procedure. Removal of cutaneous aspergillosis by surgery may be accomplished with less risk if the diagnosis is made early [7]. Some reports have shown that complete excision of the lesion alone may result in cure [7, 14]. However, this may be a risky proposition if the patient has some sort of immunosuppression. Wound healing depends on the underlying neutropenic state. With reversal of immunosuppression, good healing usually occurs. Slow healing is observed in newborn infants and patients on steroids. Burn wounds may be excised and kept open since wounds treated with occlusive dressings are more frequently infected by fungi. Skin grafting may be required in some cases. Removal of sternal wires, curettage and wide

debridement of bone and cartilage may be required. Amputation may be the last resort for some rare and advanced cases. Most authors recommend concomitant use of systemic antifungal agents.

11.2 Systemic Antifungal Agents

A deeper discussion on the treatment of IA has already been presented in other chapters. In the past amphotericin B deoxycholate was the drug of choice for all cases of IA. The recommended dosage is 1.0–1.5 mg/kg/day intravenously for at least 3 weeks so that the total dose is more than 1.0 g. However many authors would prefer achieving higher total doses, in the range of 3.0–5.0 g, which is associated with considerably toxicity. Currently the lipid preparations of amphotericin B are marketed: liposomal amphotericin B, amphotericin B lipid complex, and colloidal amphotericin B. These are known to cause less severe renal toxicity than amphotericin B deoxycholate, which is particularly true for liposomal amphotericin B. Accordingly, doses of can be raised to 3–5 mg/kg/day. However, these preparations are very costly.

Voriconazole has been the drug of choice against *Aspergillus* species since 2002 [74]. This drug has recently been tried against PCA [28, 75, 76]. Skin sensitivity (i.e., photoaging and phototoxicity) and retinal lesions are frequent side effects with its use [77]. Limited studies demonstrated good success with the use of voriconazole especially in cases refractory to liposomal amphotericin B. Itraconazole has also been tried successfully in some patients [78]. Itraconazole at a dose of 200 mg twice daily is recommended after completion of amphotericin B therapy in stable patients.

Echinocandins are also newer agents active against *Aspergillus* species. Echinocandins act irreversibly inhibiting glucan synthesis in the cell wall. Caspofungin and micafungin have both been used, in treatment of PCA especially as combination therapy [28, 79]. However, approval to use in neonates is still not available.

Many authors recommend the use of more than one antifungal agent for the treatment of this devastating disease, especially in neonates. Even three antifungal drugs have been combined for treatment of PCA (liposomal amphotericin B, itraconazole, and caspofungin) in an extremely low-birth-weight preterm infant [28]. Evidence however is still pending for the benefits of combining antifungal therapy in patients with IA. In spite of all efforts mortality is between 30 and 70% in cases of PCA.

11.3 Topical Therapy

Rarely topical therapy can be used in limited lesions of immunocompetent patients. It is however advisable to add to systemic antifungal agent and/or surgery. Agents that have been used with some success include nystatin, amphotericin B, voriconazole and terbinafine [40].

11.4 Onychomycosis

Most *Aspergillus* onychomycosis infections are misdiagnosed and targeted with fluconazole. Hence most lesions do not respond well to therapy and may become chronic. Therapy with itraconazole 200 mg once daily for 6 months (toe nails) or 3 months (finger nails) is recommended. However there is no conclusive proof of a better efficacy compared to other drugs in these cases. Terbinafine may also be used and it is preferred by some authors.

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Onychomycosis Due to *Aspergillus* Species

Ricardo Negroni

Abstract Fungal infections of the nail plate accounts for 50% of nail diseases. The prevalence of onychomycosis varies between 2 and 8% of the general population, being more frequent amongst adults aged 60 years or more. Several fungi have been implicated, including dermatophytes, yeasts and non-dermatophyte moulds. Non-dermatophyte filamentous fungi are the most heterogeneous groups of organisms which may cause onychomycosis. These are environmental fungi which may behave as contaminants or colonisers of a previously damaged nail or pathogens. Several *Aspergillus* species have been isolated from nails, including *A. fumigatus, A. flavus, A. terreus, A. sclerotiorum*, and *A. nidulans.* These are not common conditions – only ∼2% of onychomycosis cases are caused by *Aspergillus* species. Diabetes, peripheral vascular disease, orthopaedic trauma and advanced age are the most important underlying conditions, tough in half of the cases no risk factor is evident. *Aspergillus* onychomycosis may present with distinct clinical forms. In the majority of the patients lesions resemble those produced by dermatophytes – proximal subungueal onychomycosis with painful paronychia without pus is the most characteristic clinical manifestation. The results of treatments are less predictable than in dermatophyte nail infections. Itraconazole and terbinafine by oral route are effective against *Aspergillus* onychomycosis. Additionaly, nail antifungal lacquers with ciclopirox or amorolfine, as well as partial nail avulsion with 40% urea ointment usually improve the clinical response of systemic treatment.

Keywords *Aspergillus* spp · Non-dermatophyte fungal nail infection · Onychomycosis · Superficial mycoses

R. Negroni (✉)
Hospital Francisco Javier Muniz, Buenos Aires, Argentinas
e-mail: ricardonegroni@intramed.net

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Contents

1 Introduction

Onychomycosis refers to a fungal infection that affects the nail plate. These may be caused by three different groups of fungi: dermatophytes (named *Tinea Unguium*); yeast-like fungi and non-dermatophyte filamentous fungi [1–5]. Onychomycosis are responsible for 50% of total nail diseases and its prevalence in general population varies between 2 and 8%, according to different authors. The frequency of onychomycosis is lower in children and higher in persons above 60 years of age (> 25%), affecting both sexes [1, 3, 4, 6–9]. Nail fungal infection are usually asymptomatic conditions that have an important impact on the quality of life of affected patients, interfering in interpersonal relationships and the work environment, particularly when fingernails are affected.

There are several local and general predisposing factors which may increase the prevalence of onychomycosis [10, 11]. These are summarized in Table 1.

Dermatophytes are responsible for > 90% of onychomycosis cases located in toenails. The most prevalent species in this group is *Trichophyton rubrum,* followed by *T. mentagrophytes, T. tonsurans*, *Epidermophyton floccosum* and *T. schoenleinii*.

Table 1 Predisposing factors for *Aspergillus* onychomycosis

General predisposing factors
Age: prevalence is greater in elder people (slow nail growth)
Peripheral vascular diseases
Genetics: *T. rubrum* infection shows a familiar pattern of autosomal dominant inheritance
Diabetes mellitus
Hypothyroidism and other endocrine diseases
Cell-mediated immunity impairment, HIV-infection, steroids, anti-blastic therapy
Local factors
Orthopaedic trauma
Trauma caused by sports or non-proper foot wears (e.g., football, rugby, tennis, jogging, female's foot wears)
Hyperhydrosis
Poor hygiene
For fingernail onychomycosis: chronic contact with water, solvents, detergents and chemical products

These are all anthropophilic species and the presentation of onychomycosis caused by dermatophytes is influenced by genetic predisposing factors [3, 4, 7, 10, 12, 13].

Yeast-like fungi frequently attack fingernails – in this location, yeasts are more prevalent than dermatophytes as agents of onychomycosis, which is often associated with onycholysis and paronychia [1, 3, 12].

Non-dermatophytic filamentous fungi are responsible for 2–20% of the nail fungal infections. These are the most heterogeneous group of fungi which may cause onychomycosis, and include different environmental fungal species which live in the soil or on rotting vegetation. Some of these fungi are often found as primary pathogens of the nails, including *Scopulariopsis brevicaulis, Scytalidium hyalinum, Scytalidium dimidiatum,* and *Onychocola canadensis*; but a great variety of other fungi may also cause onychomycosis or are isolated from nail clinical samples, such as *Aspergillus* spp., *Fusarium* spp., *Acremonium* spp., *Chaetomium globosum,* and species of *Alternaria* and *Curvularia* [12–22].

The interpretation of a positive nail culture for non-dermatophyte moulds is always difficult. The recovery of some of these fungi from a nail sample culture does not represent unequivocal evidence linking the aetiological agent to the nail disorder. For instance, fungi may contaminate the nail surface or behave as a saprobe, transiently colonising the nail without causing invasion. Alternatively, fungi might persistent colonise a nail that was previously damaged by trauma or other causes. Mixed infections in which a dermatophyte and a non-dermatophyte mould are associated have also been described [2, 6, 7, 16, 17, 23, 24]. Onychomycosis caused by non-dermatophyte moulds is often observed in adults of both sexes and is more prevalent in elder persons, suffering orthopaedics or/and peripheral vascular alterations [5, 10, 11, 21, 25, 26].

2 Clinical Forms

Clinical characteristics of onychomycosis correlate with the infection's route of entry. The most prevalent clinical form is distal or lateral subungueal onychomycosis (DLSO), in which the hyphae invade the hyponichium and/or the nail bed from the distal part and then spread proximally causing thickening of the horny layer. Onycholysis is often observed and the nail becomes yellow-brown and opaque. This type of onychomycosis is commonly observed in toenails and they may be caused by both dermatophytes and moulds [1, 3, 7, 12, 23].

White superficial onychomycosis (WSO) is observed in fingernails and toenails. The infection begins at the superficial layer of the nail plate, invading progressively deeper layers, appears as small white patches on the nail dorsal surface. It is often produced by *T. mentagrophytes* var. *interdigitale*, but some moulds such as *Aspergillus terreus, Fusarium oxysporum* and *Acremonium* spp. have also been implicated [1, 3, 4, 12]. Black superficial onychomycosis is a very rare form of nail infection; some cases due to *T. rubrum* and *Scytalidium dimidiatum* have been published [1].

Proximal subungueal onychomycosis (PSO) is produced by hyphae invasion of the proximal nail fold and spreads distally under the nail plate. Clinically it appears as a white patch from the cuticle to 2–6 mm distally. Its most frequent aetiological agent is *T. rubrum*. PSO may affect toenails and less commonly fingernails; the majority of the patients suffer from cell-mediated defects in immunity. Rarely this type of onychomycosis is associated with proximal nail destruction and chronic paronychia without pus. This type of lesions is often caused by *Fusarium* spp. and less frequently by *Aspergillus* species [4, 6, 25–29].

"Endonyx" onychomycosis (EO) is produced by the distal penetration of the fungi to the nail plate without nail bed invasion. This type of onychomycosis causes white patches without subungueal hyperkeratosis or onycholysis. This has been related to *T. violaceum* or *T. soudanensis*. Non-dermatophyte mycelial fungi have not been isolated from this clinical form [1, 7].

Total dystrophy (TDO) is final status of any type of fungal nail infection. In these cases, the nail bed appears hyperkeratotic and no normal nail structure is detected. The nail plate looks thickened, opaque and brown-yellow in colour. The primary total dystrophy is observed in patients suffering from chronic mucocutaneous candidosis [1, 30].

Paronychia of the fingernails is usually produced by yeasts, mostly affecting adult women who work handling carbohydrates-containing foods, water, detergents or chemical active substances. It is also detected in patients suffering from hyperhydrosis, maceration and acrocyanosis. Initially the cuticle is damaged followed by a chronic inflammation of the proximal nail fold near to the matrix, which compromise leading to a nail plate dystrophy as irregular transverse grooves. *Candida* spp. and bacteria are frequently isolated from paronychia [1, 2, 7, 12].

3 Mycological Diagnosis

The diagnosis of onychomycosis is based on the finding of fungal elements such as hyphae, pseudohyphae and budding yeast in direct microscopic examination with KOH and the isolation of fungi in cultures. Histopathological study of nail clippings using Periodic Acid-Schiff (PAS) staining is a very important step in the diagnosis, especially in onychomycosis due to non-dermatophyte moulds. In a study carried out in Italy, histopathology presented positive results in more than 82% of the cases [31].

A proper specimen should be obtained by a professional with experience in the field. It is very important to advise patients in advance not to use any powder, cream or nail lacquer as well as systemic or topical antifungal drugs. In order to prevent dust contamination, patients should wear socks and closed shoes. Nail samples should be taken from the nail bed or from ventral nail plate in DLSO or by scraping the nail dorsum in cases of WSO, EO and PSO. These nail specimens are usually collected in small Petri dishes. Before microscopic examination the sample is divided into small fragments [6, 12, 1–3].

For microscopic examination one half of the clinical sample is put on a glass slide with a drop of 20–40% KOH, then a cover ship is applied and the preparation is gently heated in a Bunsen flame. The slide is observed at 200× or 400× using a light microscope, searching for hyphae, pseudohyphae, yeast cells or spores.

Other type of solutions may be used for clearing of the keratin and allowing the visualization of fungal elements include 30% KOH plus 10% glycerine, to avoid dessecation; 20% KOH in 60% water plus 40% dimethyl sulphoxide; 30% KOH with a drop of chlorazol black E or a fluorochrome named calcofluor white, which requires a fluorescence microscope (Fig. 2) [1, 3, 5, 7, 9, 12].

Cultures are the second step in the mycological examination. It is very important to inoculate the nail sample into different culture media such as Sabouraud dextrose agar with 0.05% chloramphenicol; Borelliś lactrimel with the same antibiotic con-

Fig. 1 Onychomycosis of the big toenail due to *Aspergillus terreus* following orthopaedic trauma in a 56-year-old woman

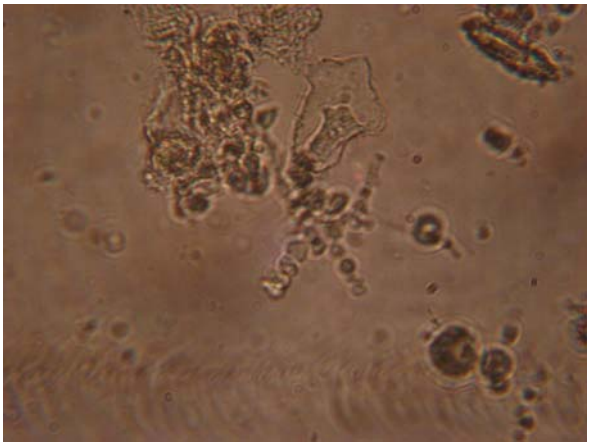

Fig. 2 Direct microscopic examination with 40% KOH of a nail sample shown in Fig. 1 revealing hyaline septated hyphae of a non-dermatophyte mould, 400×

Fig. 3 Nail culture from Fig. 1 showing several colonies of *Aspergillus terreus*

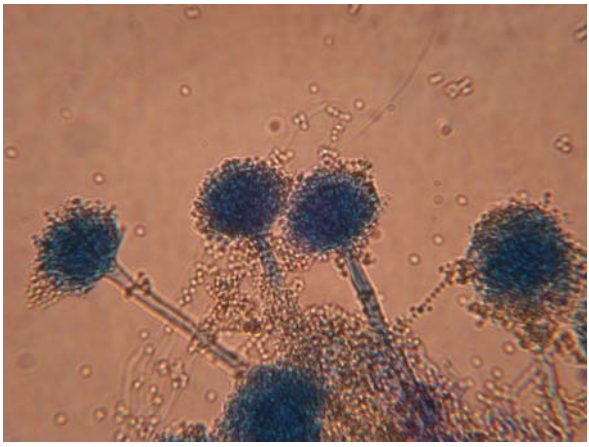

Fig. 4 Microscopic examination with lactophenol cotton blue, 400× (same as Figs. 1–3)

centration; and Sabouraud dextrose agar with chloramphenicol and cycloheximide (Actidione^{R}). The latter inhibits growth of bacteria, some yeast and most of the non-dermatophyte moulds. The nail sample is seeded with sterile needle in several points on the medium surface and the specimen is gently pushed into de medium. Cultures are incubated for 4 weeks at 26–28°C (Fig. 3 and 4) [1, 3, 12].

A molecular biology kit for the diagnosis of dermatophyte infections has been presented recently, but it is still not widely used in clinical laboratory [32].

4 Special Characteristics of Onychomycosis Due to *Aspergillus* Species

Several *Aspergillus* species have been isolated from nails, including *A. fumigatus, A. flavus, A. versicolor, A. niger, A. terreus, A. sclerotiorum, A. nidulans* and *Emericella quadrilineata* (which is the teleomorphic state of *A. tetrazonus*) [15, 17, 22, 25, 26, 29, 31, 33, 34].

The prevalence of onychomycosis due to *Aspergillus* spp. seems to be variable in different parts of the world. Apparently it is more often observed in Mexico, Europe, Pakistan and India, and less common in South America (where *Fusarium* spp. and *Acremonium* spp. are the non-dermatophyte moulds most frequently involved). In Italy *Aspergillus* spp. were responsible for 2.6% of all onychomycosis and in Pakistan, *Aspergillus* species were isolated from 2% of cases [4, 10, 12–15, 17, 19, 20, 26–28, 31, 35, 36].

In Mexico, Bonifaz et al. described that 37.1% (29/78) of onychomycosis cases due to non-dermatophyte moulds were caused by *Aspergillus* spp. The following *Aspergillus* species were identified: *A. niger* ($n = 13$), *A. terreus* ($n = 8$), *A. fumigatus* ($n = 7$), and *A. flavus* ($n = 1$). Predisposing factors for *Aspergillus* onychomycosis include peripheral vascular diseases, diabetes, orthopaedic trauma, and advanced age – however, none of these are found in 50% of the cases [11, 15].

From the clinical point of view *Aspergillus* onychomycosis may produce different clinical forms: onycholysis, DLSO, WSO or PSO with painful paronychia without pus (Fig. 5,6 and 7). The latter clinical form is the most characteristic of this type of nail infection, but it is also observed in onychomycosis due to *Fusarium* spp. [16, 25, 26, 29, 31]. Other types of nail invasion are rarely detected.

5 Treatment

Onychomycosis is one of the most difficult fungal infections to treat, due to the slow growth of the nails, the hardness of the nail plate and the location of the infectious process between the nail bed and plate. Although systemic antifungal drugs as terbinafine and itraconazole are effective in the treatment of onychomycosis caused by *Aspergillus* spp., fungi are difficult to eradicate and these drugs should always be associated with nail avulsion using a 40% urea ointment and antifungal nail lacquers containing amorolfine or ciclopirox olamine [1, 31, 3–5].

Itraconazole is a first generation triazole active against dermatophytes, yeasts and non-dermatophytes moulds including *Aspergillus* species. It is usually administered

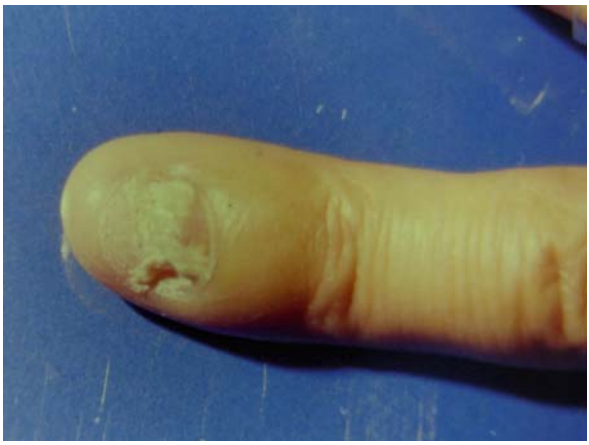

Fig. 5 Total dystrophic onychomycosis with chronic paronychia due to *Aspergillus flavus*. This was located in the little finger of the right hand of a 65-year-old woman suffering from chronic bacterial endocarditis who was submitted to a long-term treatment with antibacterial antibiotics

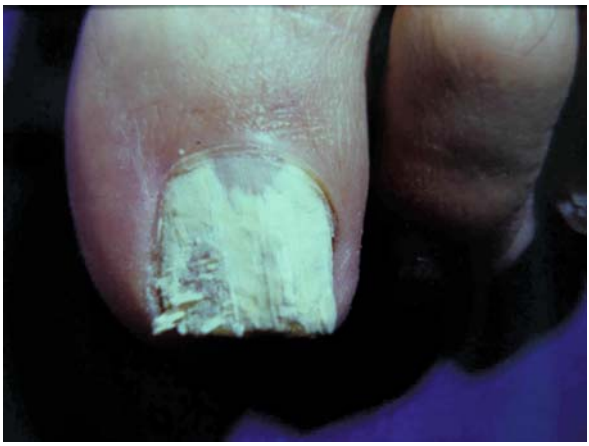

Fig. 6 White total dystrophy of the big toenail due to *Aspergillus terreus* in a 71-year-old man with peripheral vascular insufficiency

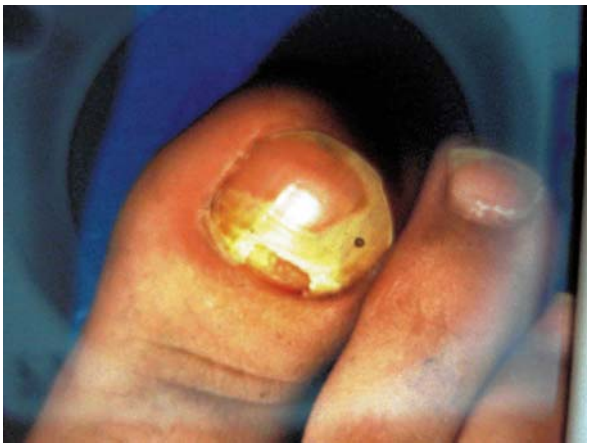

Fig. 7 Proximal subungueal onychomycosis in a 46-year-old woman following orthopaedic trauma. There was onychomadesis (spontaneous separation of the nail plate from the matrix area) and chronic paronychia but no signs of pus. The infection was caused by *Aspergillus nidulans* and located in the big toenail

in capsules, either as a continuous treatment (200 mg once daily for 3–4 months) or as a pulse therapy (200 mg 12 hourly for 1 week each month, over 3–4 months for toenail and shorted period for fingernails). Both regimens seem to be equally effective [37, 38]. Monitoring itraconazole blood levels has been recommended to ensure proper drug absorption – this is discussed in more detail elsewhere in this book. A pharmacokinetic comparison study [37] showed that intermittent therapy with itraconazole resulted in higher maximum itraconazole plasma concentrations but lower total drug exposure, and hence lower itraconazole nail tip concentrations, than continuous therapy. However, the intermittent schedule was not associated with a lower cure rate, suggesting that itraconazole nail concentrations remained within the therapeutic range. Headache and gastrointestinal tract upset are the most frequent side effects of itraconazole. Asymptomatic rise of hepatic enzymes is observed in less than 3% of patients. The drug should not be given to patients with evidence of

ventricular dysfunction and a wide range of drug interactions should be considered when itraconazole is indicated in patients who are receiving other treatments [1, 39, 40].

Terbinafine is the first orally active allylamine, an antifungal drug especially active against dermatophytes and moulds. The drug is fungicidal and remains at therapeutic levels in keratinized tissues, but with a short plasma half-life of 36 h. For the treatment of onychomycosis terbinafine is given at 250 mg daily for 3–4 months for toenails and during 2 months for fingernails. Although several studies have been performed with pulsed therapy it is not clear if it is as effective as continuous treatment. Gastrointestinal alteration and rash are the most common side effects in patients treated with terbinafine. Interactions with rifampin, cimetidine, cyclosporine, tricyclic antidepressants and other psychotropic drugs should be taken in consideration when terbinafine is indicated [1, 3, 39, 40]. More detail on terbinafine are given in the chapter by Drs. Cuenca-Estrella and Rodriguez-Tudela elsewhere in this book.

Nail lacquers should always be applied in those patients with WSO, nail partial avulsion with 40% urea ointment is indicated in those cases with deep onycholysis, in LSO or when a remarkably nail plate thickening is detected.

Combination therapy using topical and systemic treatment has shown to be more effective than monotherapy with orally active drugs. The association of itraconazole or terbinafine by oral route with nail lacquers containing 8% ciclopirox olamine or 5% amorolfine has been successfully used.

Oral sequential therapy using two pulses of itraconazole 200 mg every 12 hours for 1 week per month for 2 months followed by one pulse of terbinafine 250 mg every 12 hours for 1 week and if it is necessary a second pulse of terbinafine 3–4 months later proved to be more efficient than monotherapy with itraconazole or terbinafine [1, 31, 40].

The results oftreatment in *Aspergillus* onychomycosis are less predictable than in *Tinea Unguium* due to the lack of a wide clinical experience. According to Italian authors itraconazole and terbinafine are very effective in the treatment of this fungal infection and the pulsed regimen is more economical and less demanding [26, 31].

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Aspergillus Keratitis

Philip A. Thomas

Abstract The cornea must be perfectly transparent to allow an individual to visualize his/her environment. Keratitis, an inflammation of the cornea that frequently arises due to infection, is a threat to corneal transparency. Species of *Aspergillus* may cause keratitis, especially in outdoor workers in agricultural communities in the developing world and in tropical and subtropical areas. *Aspergillus* keratitis frequently occurs following traumatic inoculation of *Aspergillus* conidia into the cornea through injury or surgical procedures, but other factors may also predispose to the infection. *Aspergillus* keratitis is a medical emergency, since the patient frequently presents with extreme pain and loss of vision, and needs to be recognized and treated promptly. A presumptive diagnosis of *Aspergillus* keratitis can be established by a careful clinical examination and by direct microscopic examination of stained smears of corneal scrape material. The diagnosis can be confirmed by culture. Other, more rapid methods of diagnosis such as confocal microscopy and molecular methods have also been described. For therapy, topical application of natamycin (5%) or amphotericin B (0.15%) drops is most widely used. Intracameral injection of amphotericin B is a recently described alternative to topical use. Itraconazole, as an oral preparation or ophthalmic solution, has also been successfully used. Recently, voriconazole, administered by different routes, has shown considerable promise for therapy. Surgical intervention includes debridement, lamellar keratectomy/keratoplasty, or a conjunctival flap and may be required in patients with deep lesions. Therapeutic penetrating keratoplasty may be the final resort for uncontrolled infection. A better understanding of the pathogenesis of *Aspergillus* keratitis may improve its outcome.

Keywords *Aspergillus* · Keratectomy · Keratitis · Keratomycosis · Keratoplasty · Voriconazole

P.A. Thomas (✉)
Department of Microbiology, Institute of Ophthalmology, Joseph Eye Hospital, P.B. 138, Tiruchirapalli 620001, India
e-mail: philipthomas@satyam.net.in

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Contents

1 Introduction to Fungal Keratitis and *Aspergillus* Keratitis

The cornea, which is the anterior, transparent, projecting part of the external coat of the eyeball, allows rays of light to pass into the eye. Clear images of external structures can be formed on the retina only if the cornea is transparent. In other words, corneal transparency is essential if a person is to visualise his/her surroundings. Blindness resulting from corneal pathology is the second most common cause of preventable blindness in the world. Keratitis, an inflammation of the cornea, may be caused by bacteria, fungi, protozoa or parasites. If not suitably treated, keratitis can result in serious ocular morbidity or even blindness [1]. Keratitis of fungal aetiology (mycotic or fungal keratitis, keratomycosis) is a suppurative, usually ulcerative, infection of the cornea that was once considered to be an uncommon ophthalmological problem. However, it probably occurs more commonly than realised, particularly in tropical and subtropical environments. This fact is highlighted by the observation that fungal keratitis occurred in 139 patients over a 3-month period in one eye centre in southern India [2] but in just 24 such patients over a 9-year period at an eye centre in the United States of America (USA) [3]. Fungal keratitis may account for almost 90% of all fungal infections of the eye [4, 5] and for from 10% [6, 7] to almost 50% [8, 9] of infections of the cornea (microbial keratitis). In terms of occurrence, risk factors and therapeutic approaches, there are two different presentations of this condition, namely, keratitis due to filamentous fungi, such as species of *Fusarium*, *Aspergillus* and *Curvularia*, and keratitis due to yeast-like and related fungi, such as *Candida albicans.* The proportion of keratitis caused by filamentous fungi tends to increase towards tropical latitudes, whereas in more

temperate climates, fungal keratitis appears to be uncommon and to be more frequently associated with *Candida* species than filamentous fungi [10, 11]. The extent of urbanisation may also influence the incidence of fungal keratitis in countries with similar annual rainfall and temperature range [12]. Risk factors for fungal keratitis may be present in 95% of affected patients [5]. Ocular trauma appears to be the most important risk factor for filamentous fungal keratitis [5, 13] whilst previous ocular (particularly corneal) surgery, ocular and systemic diseases, and application of steroids appear to be the key risk factors for yeast keratitis [5, 14]. Contact lens wear may predispose to both forms of fungal keratitis [11, 15, 16]. However, any of these factors may predispose to any type of fungal keratitis and even to bacterial and other forms of infectious keratitis. Culture remains the cornerstone of diagnosis. Direct microscopic detection of fungal structures in corneal scrapes or biopsies permits a rapid presumptive diagnosis [17]. In recent years, molecular techniques are being increasingly used for diagnosis of the condition [18, 19]. Various antifungals have been evaluated in therapy of this condition. Natamycin can only be given topically, whilst amphotericin B, miconazole, ketoconazole, itraconazole, and fluconazole can be administered by various routes [17]. Topical amphotericin B (0.1–0.3%) is frequently the treatment of choice for infections due to *Candida* and related fungi, whilst topical natamycin (5%) is the choice for keratitis due to filamentous fungi [17]. In recent years, voriconazole and caspofungin have been used for the treatment of fungal keratitis [20]. Medical therapy may fail, necessitating surgical intervention.

Aspergillus keratitis represents an important example of keratitis due to filamentous fungi. *Aspergillus* species are frequently implicated as aetiological agents of fungal keratitis, particularly in tropical countries such as India and China but also in more temperate climates (reviewed by Leck et al. 2002) [10]. Gingrich [21] noted that out of 109 cases of fungal keratitis from all parts of the world reported in the literature up to 1962, *Aspergillus* species were isolated in 38 patients. In recent years, *Aspergillus* species have been reported as the commonest causes of fungal keratitis in different parts of northern India [8, 16, 22–24] and in eastern Nepal [9], and as the second or third most common causes of fungal keratitis in southern Australia [25], different parts of Brazil [5, 26], different parts of China [4, 6], France [14], southern India [10, 13], Malaysia [7], Paraguay [27], Thailand [6] and the USA [28]. The high proportion of corneal infections caused by *Aspergillus* spp. in drier climates may be because conidia of *Aspergillus* spp. can tolerate hot, dry weather conditions [29]. In Taiwan, concentrations of airborne conidia of *Aspergillus* species and other filamentous fungi were found to be significantly increased during harvesting, therein predisposing to fungal eye infections [30].

2 Aetiological Agents of *Aspergillus* Keratitis

The *Aspergillus* species reported as causes of keratitis since 2000 include *A. flavus* [10, 13, 24, 31–37], *A. fumigatus* [10, 13, 23–25, 27, 31, 34, 38–47], *A. niger* [15, 16, 48], *A. tamarii* [49], *A. terreus* [35] and other unspecified *Aspergillus* species [35, 50–52].

3 Risk Factors for *Aspergillus* Keratitis

It is important to note that no factor has been identified as specifically predisposing to *Aspergillus* keratitis, rather than to other types of fungal keratitis or even bacterial keratitis. The usual risk factors reported (described above) are applicable to different types of fungal keratitis and even to bacterial keratitis. For example, with reference to ocular trauma, Dunlop et al. [53], in Bangladesh, observed that 52% of patients with bacterial keratitis as opposed to 35% of patients with fungal keratitis reported antecedent ocular trauma whereas Wong et al. [54], in Singapore, reported that fungal keratitis (principally due to species of *Fusarium* and *Aspergillus*) was more frequently associated with mechanical ocular trauma than was bacterial keratitis (principally due to *Pseudomonas aeruginosa*). Moreover, although antecedent topical steroid therapy is traditionally believed to be a specific risk factor for fungal keratitis, this appeared to predispose more frequently to bacterial than to fungal keratitis in a study in Singapore [54].

Filamentous fungal keratitis, especially that due to species of *Fusarium* or *Aspergillus*, appears to occur most commonly in healthy young men engaged in agricultural work or outdoor occupations [10, 13, 55]. Trauma is the principal risk factor in 44–55% of such patients. Various traumatising agents have been reported, including vegetable matter, mud or dust particles, paddy grain, the swish of a cow's tail, tree branches, and metallic foreign bodies. Prolonged use of topical steroids or antibacterials, diabetes mellitus, pre-existing ocular diseases and contact lens wear, pre-existing allergic conjunctivitis or vernal keratoconjunctivitis, and the occupation of onion harvesting, are less frequently encountered factors in filamentous fungal keratitis [13, 16, 17, 33, 55–57]. Seasonal variations that have been observed in the incidence of filamentous fungal keratitis, and in the predominant genera of fungi isolated from such cases, have been linked to environmental factors, such as humidity, rainfall and wind, and also to the harvest [30, 58–60].

In addition to the predisposing factors mentioned above, culture-proven Aspergillus keratitis has been reported to occur following ocular surgical procedures such as amniotic membrane transplantation for pseudophakic bullous keratopathy [52] and laser- in situ-keratomileusis (LASIK) for correction of refractive errors. The species implicated in post-LASIK infections are *A. fumigatus* [38, 40, 42, 45] and *A. flavus* [32], whilst presumed (based on corneal biopsy findings) *Aspergillus* keratitis has been reported following a non-penetrating glaucoma surgery (viscocanalostomy) [51] and an epicorneal aspergilloma observed in a patient who had undergone keratoplasty and was wearing a bandage soft contact lens [61]. Interestingly, a retrospective review of fungal infections occurring following a sutureless self-sealing incision for cataract surgery revealed that *Aspergillus* species accounted for 6 of the 7 fungal organisms isolated [35], whilst a retrospective analysis of 8 patients who developed fungal keratitis soon after cataract surgery revealed that all 8 developed culture-proven *Aspergillus* infection, 7 due to *A. fumigatus* and 1 due to *A. flavus* [31]. *A. niger* keratitis as a consequence of contact lens wear [15] and in the context of uncontrolled ocular cicatricial pemphigoid [48] and *A. flavus* keratitis associated with vernal keratoconjunctivitis (a type of allergic disorder of

the cornea and conjunctiva) [33] have also been reported. Interestingly, in England, where filamentous fungal keratitis is an infrequent occurrence, *A. fumigatus* keratitis was observed in 3 males, each of whom had sustained a corneal injury by a metallic foreign body [43], whilst the combined bacterial and *A. fumigatus* keratitis in another patient was thought to have been acquired due to contamination from fungal conidia released during demolition work at the hospital in which the patient was being treated [62].

4 Diagnosis of *Aspergillus* Keratitis

4.1 History and Clinical Features

A rapid and accurate diagnosis of fungal keratitis due to *Aspergillus* species improves the chances of a complete recovery. A comprehensive clinical history, including details of possible predisposing factors, should first be obtained. Ocular and systemic defects that may have predisposed to the keratitis should then be looked for since these require correction. Symptoms are usually as in any other type of keratitis, but, perhaps, more prolonged in duration (5–10 days).

Keratitis due to filamentous fungi (including *Aspergillus* keratitis) may involve any area of the cornea and usually presents with firm (sometimes dry) elevated necrotic slough, "hyphate" lines that extend beyond the edge of the ulcer into the normal cornea, multifocal granular (or feathery) gray-white "satellite" infiltrates in the corneal stroma and folds in the Descemet's membrane (Fig. 1) – additional features that occur less frequently include an "immune ring", minimal cellular infiltration in the adjacent stroma, mild iritis and an endothelial plaque and hypopyon [63]. There may be variations from this pattern depending on the aetiological agent, and the clinical presentation of *Aspergillus* keratitis may simulate that of another form of microbial keratitis. For example, *A. fumigatus* keratitis with "wreath pattern" infiltrates (usually associated with keratitis due to *Nocardia* species) has been

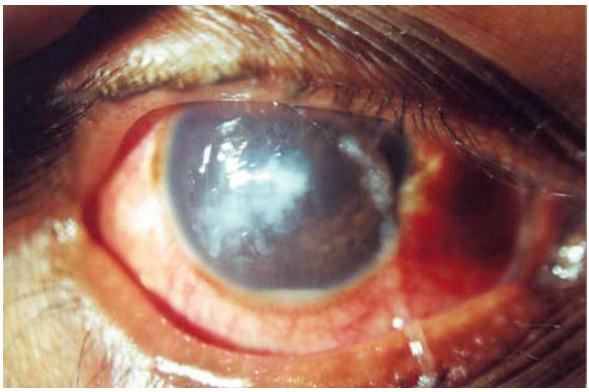

Fig. 1 Clinical photograph of *Aspergillus* keratitis

reported [39]. Keratitis due to *Aspergillus* spp. is generally believed to be less severe than keratitis due to *Fusarium* spp. [64], although one group of workers [65] noted that a high percentage of patients with *Aspergillus* keratitis ultimately required therapeutic penetrating keratoplasty due to non-responsiveness to medical therapy.

4.2 Non-invasive Methods of Diagnosis

The clinical suspicion of *Aspergillus* keratitis may be confirmed by non-invasive means using confocal microscopy, an imaging technique that allows optical sectioning of almost any material, with increased axial and lateral spatial resolution, and better image contrast than conventional slit-lamp microscopy. In an experimental model of *A. fumigatus* stromal keratitis in New Zealand white rabbits, confocal microscopy appeared to be more sensitive than culture in fluconazole-treated and untreated rabbits at 14 and 22 days after the start of the experiment [66]. In clinical keratitis due to *Aspergillus* species, fungal hyphae may be imaged as high-contrast filaments, 60–400 μm long and 6 μm in width [67]. In vivo confocal microscopy imaging using the Heidelberg Retina Tomograph II-Rostock Cornea Module has been found useful in the early diagnosis of fungal, including *A. fumigatus*, keratitis since the fungal hyphae appear as numerous high-contrast elements in the anterior corneal stroma [68]. Thus, confocal microscopy appears to be a rapid and sensitive diagnostic tool for both the early diagnosis, as well as the non-invasive follow up, of *Aspergillus* keratitis, although limited resolution of some types of confocal microscope and difficulty in performing serial examinations are potential limitations.

4.3 Microbiological Diagnosis of Aspergillus *Keratitis*

4.3.1 Sampling

For microbiological diagnosis, the best specimen is material from the affected part of the cornea. The material may be collected in the form of scrapings (possible when the affected part is ulcerated) or biopsy material (necessary when the corneal epithelium is intact and the infection is within the corneal stroma). Corneal scrapings are obtained by using an instrument (platinum spatula, Beaver blade, Bard Parker knife No.15, blunt cataract knife) to debride material from the base and edges of the ulcerated part of the cornea. If done several times, a good amount of material can be obtained for processing. If the material has been streaked onto a sterile medium, the same blade or spatula can be used again to collect additional material. However, if the material has made contact with a non-sterile microscope slide, the blade must be changed whilst the spatula can be flamed, cooled and then reused [69]. Cotton-tipped swabs are no longer preferred for debriding the necrotic corneal slough. However, when calcium alginate swabs (pre-moistened with tryptone soy

broth) are used for the debridement, they may facilitate recovery of fungi in culture [70].

If corneal scrapings do not yield positive results, a corneal biopsy may aid diagnosis since a greater quantum of tissue can be obtained from a greater depth of the cornea. Whilst free dissection of the corneal lamellae by a sharp surgical knife can easily be done, corneal perforation is a distinct risk. It is probably better to first remove the epithelium and necrotic debris overlying the suppurated area and then to incise the corneal stroma by a Bard-Parker No.15 blade and corneal forceps to about one half the corneal thickness [71]. Another method is to use a corneal trephine to define the precise diameter and depth (0.2–0.3 mm) of corneal tissue that is to be removed [72]. The biopsies may be relatively superficial (keratectomy) or deep. The biopsied tissue may then be stained by ink-potassium hydroxide [71] or lactophenol cotton blue [72] to visualise fungal structures. Although several experimental studies have clearly demonstrated that corneal biopsy is a valuable diagnostic tool for fungal, including *A. fumigatus*, keratitis [71], the results obtained in a clinical setting are less clear-cut.

4.3.2 Direct Microscopic Examination

Whilst direct microscopic examination of corneal scrapes or corneal biopsy samples permits a rapid presumptive diagnosis of fungal keratitis, it may not be possible to conclude that one is specifically dealing with *Aspergillus* keratitis, even if dichotomously branching hyphae are present, since other fungi, such as *Scedosporium apiospermum*, may also exhibit this pattern of branching in ocular samples [73]. Examination of a wet preparation stained by 10% potassium hydroxide [55], ink-potassium hydroxide [74] or lactophenol cotton blue [75, 76], a smear stained by the Gram method [75, 76] or Giemsa method [55], and a smear stained by special fungal stains, such as methenamine silver, periodic acid Schiff, calcofluor white [55] or chlorazol black E [75] may yield valuable results. The corneal material should be spread out as thinly as possible on the microscope slides to allow the easy detection of fungal structures. In culture-proven clinical *Aspergillus* keratitis, examination of lactophenol cotton blue mounts (Fig. 2) and Gram-stained smears of corneal scrapings yielded positive results in 82 and 76%, respectively [76].

4.3.3 Culture

Corneal scrape or biopsy material is inoculated onto the surface of solid media by making rows of "C" streaks (two rows from each scraping) or by immersing the tip of the spatula, loop or swab into a broth (liquid) medium. Commonly used culture media and incubation temperatures include Sabouraud glucose neopeptone agar (Emmons' modification, neutral pH) incubated at 25°C, 5% (preferably sheep) blood agar (25°C and 37°C) and brain heart infusion broth (25°C). *Aspergillus* species usually grow out within 2–4 days, but culture media may need to be kept for up to 3 weeks if growth is not obtained earlier. Antibacterial compounds, such

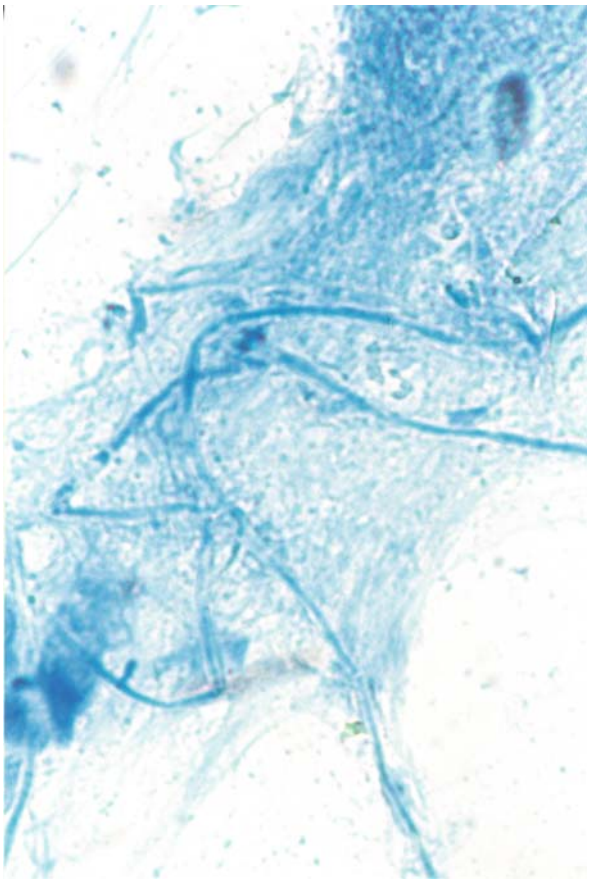

Fig. 2 Photomicrograph of lactophenol cotton blue stained wet film of corneal scrape material showing *Aspergillus* hyphae

as chloramphenicol (40 μg/ml), gentamicin (50 μg/ml) or a penicillin-streptomycin combination, are usually incorporated in the media to suppress bacterial growth, but cycloheximide must never be used since it may suppress the growth of *Aspergillus*. "Sham cultures" are maintained to ensure that there is no contamination from the environment, in which *Aspergillus* conidia abound, during collection of the sample. Growth of an *Aspergillus* species in culture media inoculated with corneal material is considered significant if the same *Aspergillus* species is isolated more than once, or in two or more agar media, or on multiple "C" streaks of one agar medium (Fig. 3) or in one liquid medium with fungal hyphae or fungal conidia detected by direct microscopic examination of the corneal material.

4.4 Histopathological Diagnosis of Aspergillus *Keratitis*

These offer certain advantages over culture, when used in the diagnosis of *Aspergillus* keratitis, since contamination is avoided, tissue penetration by the fungi can be gauged and the outcome of surgical procedures can be anticipated. In one

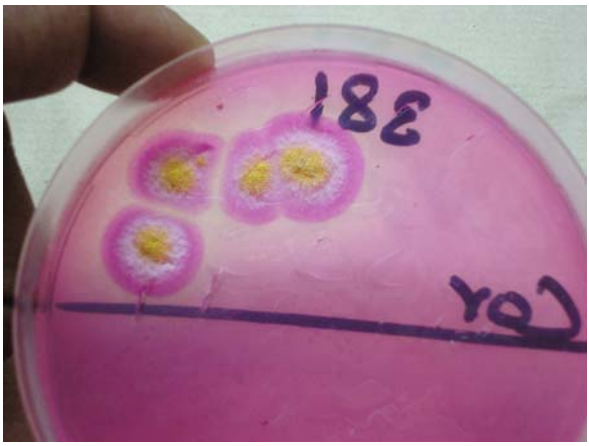

Fig. 3 Photograph of rose bengal agar plate showing growth of *Aspergillus flavus* on "C" streaks made whilst inoculating corneal scrape material

histopathological study of corneal buttons from patients with fungal keratitis about to undergo keratoplasty, *Aspergillus* infections were identified in 33% [65]. Corneal material for histopathology is obtained as a biopsy or button following penetrating keratoplasty. *Aspergillus* species can be satisfactorily stained and studied in tissue sections by light microscopy [71]. Lectins (concanavalin A, wheat germ agglutinin) are proteins that are commonly found in plant seeds that bind specifically to carbohydrates. Fluorescein-conjugated concanavalin A provided consistently bright staining of fungal structures in corneal scrapes from 18 patients with culture-proven fungal keratitis [77]. More recently, when a peroxidase labelled-wheat germ agglutinin staining method was used for the diagnosis of experimental *A. fumigatus* keratitis in a rabbit model, the sensitivity and specificity of the test were found to be 96.4 and 100%, respectively [78]. There was also a high degree of test-retest and inter-rater concordance. Such a sensitive and specific technique could prove useful in the clinical setting without the need for microscopes with attachments for fluorescence microscopy.

4.5 Polymerase Chain Reaction (PCR) in Diagnosis of Aspergillus *Keratitis*

This technique, which achieves enzymatic amplification of a specific sequence of nucleic acids, can be used to rapidly detect and amplify fungal DNA even in minute quantities of ocular samples, thus allowing early initiation of specific antifungal therapy. However, the patient's response to treatment cannot be monitored by this technique, nor can a differentiation be made between viable and non-viable organisms. In one study, PCR and typing of fungal internal transcribed spacer (ITS)2 and 5.8S ribosomal DNA (rDNA) allowed the rapid diagnosis of *A. fumigatus* keratitis in one patient (culture and direct microscopic examination also yielded positive results) and *A. niger* keratitis in another patient (culture and direct microscopic

examination yielded negative results in this patient) [79]. In another study, when PCR was applied to the study of corneal scrapes from 30 patients with presumed microbial keratitis, using primers to target fungal 18S rDNA, PCR and culture yielded positive results for *Aspergillus* organisms in 2 patients [80]. In yet another study, when PCR was used to amplify 6 different regions of the rDNA gene followed by analysis of the amplified products by single-stranded conformation polymorphism, the PCR products obtained with primer pair ITS1 and ITS2 were found to be the best for the identification of fungi causing keratitis. The results were confirmed by sequencing of the PCR products, and the approach was successfully tested for diagnosis of experimental *A. fumigatus* keratitis [19]. In a very recent clinical study, PCR amplification and sequencing of the ITS region of rDNA permitted rapid identification of *A. flavus* as the aetiological agent in 2 patients with keratitis. Culture also yielded positive results, but after 4 days [18]. Thus, these molecular techniques hold much promise for a rapid, specific diagnosis of *Aspergillus* keratitis in the clinical setting.

4.6 Antifungal Susceptibility Testing

The in vitro susceptibility of fungi isolated from patients with keratitis in China was tested using itraconazole, fluconazole, and amphotericin B in the Etest® commercial antifungal susceptibility testing system (AB BIODISK, Solna, Sweden); 60.6% of *Fusarium* isolates were susceptible to amphotericin B and all were resistant to itraconazole and fluconazole whilst all isolates of *Aspergillus* were susceptible to itraconazole, 44.4% to amphotericin B, and 22.2% to fluconazole [81]. A more recent study sought to compare the minimal inhibitory concentration (MIC) of natamycin, amphotericin B, caspofungin, itraconazole, voriconazole, and posaconazole against 41 *Aspergillus* strains isolated from ocular specimens. The triazoles and caspofungin had the lowest MIC against *Aspergillus* species. The MIC of natamycin, amphotericin B and voriconazole against *Aspergillus* species were 32 μg/ml, 2–4 μg/ml and 0.25–0.5 μg/ml, respectively [82]. Although in vitro antifungal susceptibility testing may not accurately predict the clinical response of individual patients of *Aspergillus* keratitis, these data are valuable in guiding the clinician in selecting antifungal agents that would probably be useful in treating *Aspergillus* keratitis.

5 Pathogenesis of *Aspergillus* Keratitis

The pathogenesis of *Aspergillus* keratitis probably arises out of an interplay of various agent (fungal) and host (corneal tissue) factors. Agent factors include invasiveness of the infecting fungus, ability to adhere to host tissue and ability to secrete toxins and enzymes (toxigenicity). Host factors include the activities of infiltrating inflammatory cells and corneal tissue cells (keratocytes) and enzymes.

5.1 *Putative Agent Factors in the Pathogenesis of* Aspergillus *Keratitis*

5.1.1 Adherence to Corneal Tissue and Invasiveness by *Aspergillus*

The first step in the pathogenesis of fungal keratitis appears to be the binding of fungal conidia to the corneal epithelium (the outermost layer of the cornea which is in direct contact with the environment) and subsequent formation of fungal hyphae. In an experimental rabbit cornea model, the binding of *A. fumigatus* conidia and *C. albicans* blastoconidia to corneal epithelial basement membrane was found to be initiated earlier than that of conidia of *F. solani* and *Penicillium citreo-viridé* [83]. Binding of *A. fumigatus* conidia to immortalised corneal epithelial cells has recently been shown to stimulate a corneal inflammatory response through a pathway dependent on toll-like receptor (TLR)2 and TLR4 signalling [84].

After penetrating the corneal epithelium, the fungal hyphae enter the corneal stroma. In an experimental model, the hyphae of *A. fumigatus* and (pseudohyphae of) *C. albicans* were found to traverse the cornea in a plane perpendicular to the stromal lamellae, whereas the hyphae of *F. solani* and *P. citreo-viridé* were found to lie parallel to the corneal lamellae [83]. The significance of this finding is that it may predict recurrence of infection following lamellar keratoplasty (a form of corneal transplantation). In a study of corneal tissue samples from patients, it was observed that 91.2% of *Fusarium* hyphae lay parallel to the corneal stromal lamellae, whereas 90.9% of *Aspergillus* hyphae grew vertically. Moreover, in cases of recurrence of fungal infection, 80% of the hyphae were found to grow vertically, and there was a significantly higher recurrence rate in patients with vertically growing hyphae (46.2%) than in those with horizontally growing hyphae (2%) as well as in those with *Aspergillus* keratitis (36.4%) versus those with *Fusarium* keratitis (7.4%) [85].

In some instances, the fungal organism causing keratitis may be so virulent that it traverses all the layers of the cornea and then enters the underlying anterior chamber of the eye. This may sometimes result in a condition called “keratomycotic malignant glaucoma”, particularly if a *Fusarium* species is causing the infection [64, 86]. This “malignant glaucoma” occurs because the fungal strain forms a lens-iris mass at the angle of the anterior chamber which blocks the drainage of the aqueous humour, leading to a marked increase in the intraocular pressure of the affected eye. Recently, “keratomycotic malignant glaucoma” was reported in a patient suffering from *A. flavus* keratitis [37], with the use of topical steroids being the factor that possibly triggered increased fungal penetration, with the formation of a lens-iris fungal mass and subsequent malignant glaucoma.

5.1.2 Toxigenicity of Ocular *Aspergillus* Isolates

Some studies have examined the possible role of fungal proteinases in *Aspergillus* keratitis. Clearly, isolates of *A. flavus* from patients with keratitis possess the ability

to secrete proteinases [87, 88]. However, it is not clear whether these fungi actually secrete these proteinases when infecting human corneal tissue and whether these proteinases appreciably influence the outcome of such infections. In one study that sought to examine this aspect, corneal isolates of *A. flavus*, when grown in vitro, were found to exhibit predominantly serine proteinase and little metalloproteinase activity whereas homogenates of rabbit corneas that had been infected with the same strains of *A. flavus* exhibited only metalloproteinase activity (derived from innate corneal tissue enzymes and from infiltrating polymorphonuclear leucocytes, PMN) and no serine proteinase activity. These findings suggested that although the *A. flavus* strains could secrete proteinases in vitro, they did not do so whilst infecting corneal tissue [87].

5.2 *Putative Host (Corneal Tissue) Factors in the Pathogenesis of* Aspergillus *Keratitis*

5.2.1 Matrix Metalloproteinases and Polymorphonuclear Leucocytes

It is becoming increasingly clear that in the pathogenesis of fungal keratitis, host (corneal tissue) factors play a vital role. The key factors appear to be the corneal matrix metalloproteinases (MMP) and the infiltrating PMN. In one experimental study, basal proteolytic activity (65 kDa) in uninfected rabbit corneas was found to reside in MMP2 whereas when the rabbit corneas were experimentally infected with *A. flavus*, additional proteolytic activity (92 and 200 kDa) was detected, with the 92 kDa activity residing in MMP9. The expression of 92 and 200 kDa gelatinases was found to correlate positively with the number of PMN in infected corneas [87]. The results of another study [83] suggest that the activity of corneal MMP and the intensity of corneal infiltration by PMN in experimental fungal keratitis are dependent on the fungal species causing the infection. When the growth patterns of *A. fumigatus*, *C. albicans, F. solani* and *Penicillium citreo-viridé* in experimental keratitis were compared, destruction of basement membrane was found to begin at 1–3 days in all the groups but, histopathologically, infiltration of inflammatory cells was more evident in the *A. fumigatus* and *C. albicans* groups than the other 2 groups at 3 days. Moreover, although MMP-9 and MMP-2 were found in all infected corneas (with proteolysis being most active at 3 days) the level of MMP-9 was higher in the *A. fumigatus* and *C. albicans* groups than in the other two groups. In this study, positive correlations were observed among the number of binding conidia, degree of inflammation and level of MMP-9, suggesting the interplay of these factors in pathogenesis of fungal keratitis [83].

5.2.2 TLR and Corneal Immune Responses to *A. fumigatus*

Corneal epithelial cells play early and crucial roles in the initiation of ocular surface responses to pathogens. Participation of TLR2 and TLR4, which are major forms of fungal receptors, may be involved in *A. fumigatus*-induced immune responses. Zhao and Wu [84] found that *A. fumigatus* conidia elicited the expression of TLR2,

TLR4, tumour necrosis factor (TNF)-α and interleukin (IL)-8 mRNA in cultured immortalised human corneal epithelial cells. NFκB was also activated and increased at 30 min and then steadily up to 4 h after challenge. Concomitant with NFκB activation, secretion of TNF-α and IL-8 in conidia-challenged cells was increased in a time-dependent manner. Incubation of the corneal epithelial cells with antibody against TLR2 or TLR4 before exposure to the *A. fumigatus* conidia resulted in subsequent inhibition of conidia-induced secretion of TNF-α and IL-8 secretion. These findings have potential implications for therapy of *Aspergillus* keratitis in the future.

5.2.3 The Plasminogen Activator-Plasmin System in Pathogenesis

The plasminogen activator-plasmin system has been implicated in the evolution of corneal ulceration, although not necessarily that of infectious keratitis. Aprotinin, being an antiplasmin agent, could possibly inhibit corneal ulcer progression. One study sought to determine whether aprotinin exhibited adjuvant activity when given along with natamycin and fluconazole in therapy of experimental *A. fumigatus* keratitis [89]. However, the use of aprotinin with either fluconazole or natamycin did not significantly reduce the average healing time compared to the use of the antifungal agents and placebo. Possibly, the plasminogen activator-plasmin system does not play a major role in pathogenesis of *Aspergillus* keratitis.

5.3 Molecular Pathogenesis of Aspergillus *Keratitis*

In a recent study, a microarray system was used to compare the gene profiles in control murine corneas and in murine corneas challenged with heat-inactivated *A. fumigatus* conidia [90]. Of a total of 18,335 genes detected, 61 exhibited a greater than one-fold increase in RNA expression whilst 48 showed a decrease of over 50%. The regulated genes included IL-3, mannose binding lectin A, and prostaglandin D2 synthase (genes known to be related to host defence) and dopachrome tautomerase (a gene hitherto not known to be correlated to ocular or host defence). Another 33 changed genes either had unknown functions or encoded hypothetical proteins. Whilst the changes in gene expression were confirmed by real time PCR, mannose binding lectin A expression regulation was further confirmed by using immunohistochemistry in both in vitro and in vivo challenged murine corneas, suggesting that this is among the primary responding genes during the onset of fungal keratitis.

6 Management of *Aspergillus* Keratitis

In general, fungal keratitis is managed by medical and/or surgical means.

6.1 Medical Therapy of Aspergillus *Keratitis*

This consists of non-specific measures, and the use of specific antifungal agents.

6.1.1 Non-specific Measures in the Medical Therapy of *Aspergillus* Keratitis

Cycloplegics are used to relieve the iridocyclitis (anterior uveitis) that usually accompanies fungal keratitis whilst broad-spectrum antibacterials may be needed to combat secondary bacterial infection. Where access to specific antifungal drugs is limited, various antiseptics, such as chlorhexidine, polyhexamethylene biguanide (PHMB), povidone-iodine and silver sulfadiazine, have been evaluated in the therapy of fungal, including *Aspergillus*, keratitis (reviewed by Thomas in 2003) [17].

6.1.2 Specific Compounds in the Medical Therapy of *Aspergillus* Keratitis

Evaluation of Specific Antifungal Compounds in Experimental *Aspergillus* Keratitis

The response of experimental *Aspergillus* keratitis to ketoconazole appears to be species-specific. Topical 1% ketoconazole (in arachis oil) applied every hour for 10 h for 16 days was effective in clearing *A. flavus* keratitis in a rabbit model whereas oral or topical ketoconazole therapy was not successful in experimental *A. fumigatus* keratitis [91]. Oral itraconazole therapy was found to be effective in therapy of experimental keratitis due to *Aspergillus* species [92]. In an experimental study in Dutch-belted rabbits, oral fluconazole therapy, given for 1 day pre-inoculation and then for 5 days post-inoculation, resulted in a significant decrease in isolate recovery and in clinical disease in experimental *A. fumigatus* keratitis. More recently, Avunduk et al. [93] observed that topical (2 mg/ml) and oral (37.5 mg/kg twice daily) fluconazole treatment were effective in lessening the severity of fungal keratitis in a rabbit model of *A. fumigatus* keratitis. No significant differences were observed between topical and oral treated groups at any examination point. A recent experimental study on *A. fumigatus* keratitis in rabbits reported that the average healing times and number of perforations of the ulcerated area of the cornea were 21.5 $\pm$3.08 days and no perforations when 5% natamycin was applied, 27.8 $\pm$2.28 days and one perforation with 0.02% polyhexamethylene biguanide, 36.4 $\pm$2.57 days and three perforations with 1% povidone iodine, and 38.2 $\pm$ 4.74 days and five perforations with 0.5% hydroxypropylmethylcellulose (controls) [94]. Thus, polyhexamethylene biguanide (0.02%) may be a moderately effective drug for experimental *Aspergillus* keratitis, but 1% povidone iodine does not appear to be effective.

Evaluation of Specific Antifungal Compounds in Clinical *Aspergillus* Keratitis

Medical therapy of mycotic keratitis is usually protracted since the effective concentrations achieved by most specific antifungal agents in the cornea, with the possible exception of amphotericin B, only inhibit growth of the fungus, and host defence mechanisms must eradicate the organism.

Amphotericin B

For many years, amphotericin B was the treatment of choice for *Aspergillus* keratitis. It continues to be so in countries where natamycin is not available, or is

used when there is no response to natamycin [54]. Current treatment consists of 0.15% (w/v) amphotericin B eye drops prepared from the intravenous preparation (Fungizone®), containing deoxycholate, which is irritant for the cornea. Since such irritation may reduce patient compliance, an ophthalmic preparation of 0.5% (w/v) liposomal amphotericin B has been suggested as a convenient alternative [95]. However, it is the 0.15% solution prepared from the commercially available intravenous preparation that is still widely used and this appears to be well-tolerated [3].

Topical hourly applications of amphotericin B (0.15%) resulted in resolution of lesions in 56% of Indian patients with *Fusarium* keratitis and 27% of those with *Aspergillus* keratitis [96], whilst use of amphotericin B ointment resulted in resolution of *Aspergillus* keratitis [97]. In a patient who developed *Aspergillus* keratitis following non-penetrating glaucoma surgery [51], the keratitis responded to amphotericin B treatment.

In addition to topical drop therapy, other methods of administering amphotericin B for treatment of *Aspergillus* keratitis have also been tried. Collagen shields that had been soaked in 0.5% amphotericin B for 2 h at 25°C before application were used in conjunction with amphotericin B 0.25% eye drops (applied every 2 h) to treat the eyes of 3 patients with *Aspergillus* keratitis. Cultures from the eyes of all three patients became negative within 15 days of treatment, although 2 patients subsequently required keratoplasty [98]. More recently, the same group of authors [31] treated 8 patients with post-cataract surgery *Aspergillus* keratitis (7 due to *A. fumigatus* and 1 due to *A. flavus*). After antifungal therapy, the infection resolved in 6 patients but there was no response in the other 2, necessitating evisceration. Intracameral (i.e., into the anterior chamber) injection of amphotericin B may be a useful modality in the treatment of severe fungal keratitis not responding to topical natamycin since it ensures adequate drug delivery into the anterior chamber and may be especially useful to avoid surgical intervention in the acute stage of the disease. When 3 patients with culture- proven *A. flavus* keratitis with hypopyon unresponsive to topical natamycin 5%, topical amphotericin B 0.15%, and oral itraconazole were administered intracameral amphotericin B (the concentrations being 7.5 μg or 10 μg in 0.1 ml and the number of doses varying from 1 to 3), all 3 patients responded favourably, with the ulcer and hypopyon clearing completely, without clinical evidence of corneal or lenticular toxicity in any patient [34]. In another study, 14 eyes of 12 patients (including four eyes with direct microscopy and culture evidence of *Aspergillus* keratitis) who had not responded to oral itraconazole and oral and topical fluconazole received intracameral amphotericin B (5 μg). Although twelve eyes responded to amphotericin B therapy (details were not provided regarding the species-wise response), a drawback of intracameral administration was the development of anterior subcapsular cataract in 4 eyes [99].

Natamycin

Natamycin (pimaricin) was the first antifungal specifically developed for topical ophthalmic use. Where commercially available as a 5% topical preparation, it is used as primary therapy for fungal keratitis, particularly that due to filamentous

fungi. However, interpretation of data relating to response of *Aspergillus* keratitis to natamycin therapy has been difficult. When the clinical efficacy of hourly topical applications of a 1% itraconazole ophthalmic preparation was compared with that of hourly topical 5% natamycin eyedrops in monotherapy of fungal keratitis, natamycin was found to be significantly more effective than itraconazole in therapy of *Fusarium* keratitis. However, in *Aspergillus* keratitis, favourable responses were noted in 6 (54.5%) of 11 patients receiving natamycin and 5 (50%) of 10 patients receiving itraconazole [100]. These authors concluded that although topical natamycin should continue to be considered as the treatment of choice for filamentous fungal keratitis, topical itraconazole therapy could be used if the infection is due to *Aspergillus*, when natamycin is unavailable.

Miconazole

Intravenous administration of miconazole in a dose of 600–3,600 mg/day was reported to be effective in treatment of clinical *A. fumigatus* keratitis [101]. Topical (1%) and subconjunctival (10 mg) miconazole therapy resulted in resolution of all lesions, including endothelial plaques, in 2 patients with *A. fumigatus* keratitis and 1 with *A.flavus* keratitis. The *Aspergillus* strains were all susceptible in vitro to miconazole [102].

Ketoconazole

In a clinical setting, oral ketoconazole therapy was not found effective in 1 patient with *A. fumigatus* keratitis, perhaps because of intraocular invasion [103]. Topical 1% ketoconazole, given for 7 weeks, was reported to be effective in the treatment of 6 patients with mycotic keratitis, principally caused by *Aspergillus* and *Fusarium* [104]. A comprehensive assessment of data from two studies conducted in India using oral ketoconazole (200 mg 8 hourly) or a topical 1% ketoconazole suspension or both revealed that ketoconazole therapy effected complete or partial resolution of the keratitis in 60% of patients, including 11 of 22 patients with *Aspergillus* keratitis [105, 106]. However, treatment with oral ketoconazole could not halt progressive infection due to the thermotolerant mould *Neosartorya fischeri* var. *fischeri,* the teleomorph of *Aspergillus fischerianus* [107]. Kredics et al. [49] reported a patient with *Aspergillus tamarii* keratitis in whom topical natamycin and econazole treatment and subsequent systemic ketoconazole treatment proved effective. Side effects, drug-drug interactions and poor absorption in patients with achlorhydria are additional limitations to ketoconazole use.

Itraconazole

A comprehensive assessment of data from two studies conducted in India using oral itraconazole (200 mg once daily) or a topical 1% itraconazole suspension or both revealed that itraconazole therapy effected complete or partial resolution of the keratitis in 76% of patients with *Aspergillus* keratitis [96, 108, 109]. A more

recent paper has confirmed the efficacy of a commercial 1% itraconazole ophthalmic preparation, in relation to topical natamycin, in therapy of *Aspergillus* keratitis [100].

Voriconazole

Voriconazole, administered topically, systemically, or via intracameral injection has been successfully used in *Aspergillus* keratitis [36, 38, 47, 110]. In one patient with *A. flavus* keratitis who did not respond to conventional antifungals, the infection was successfully controlled with oral voriconazole and penetrating keratoplasty [36]. One patient with *A. fumigatus* keratitis responded to combined topical (1%) and oral (400 mg every 12 hours) voriconazole. There was exacerbation of stromal keratitis when only topical voriconazole was given [47]. The patient received oral voriconazole continuously for 7 months, the rationale for selecting the relatively high maintenance dose of 400 mg of voriconazole 12 hourly being that with this dose, aqueous humour concentrations of voriconazole exceeding the MIC for *Aspergillus* species have been reported [111]. A high risk of drug-related toxicities will occur however with this dosage.

In a patient who developed *A. fumigatus* keratitis following LASIK, a combination of topical and oral voriconazole and topical natamycin helped to control the infection [38]. Mehta et al. [110] reported the occurrence of *A. fumigatus* keratitis in a diabetic female in whom the corneal infiltrate continued to progress in spite of therapy with topical natamycin and topical amphotericin B and systemic ketoconazole. Initiation of topical and systemic voriconazole therapy led to a steady resolution of the infiltrate.

All these observations suggest that voriconazole holds great promise in the treatment of *Aspergillus* keratitis which is unresponsive to conventional antifungal therapy.

Analysis of Data Pertaining to Use of Specific Antifungals to Treat Clinical *Aspergillus* Keratitis

The data pertaining to outcome of therapy was analysed [17] for 61 patients with *Aspergillus* keratitis (Fig. 1) reported in 13 studies in the literature. The data suggested that more than 80% of patients with superficial keratitis due to *A. flavus*, *A. fumigatus* and other *Aspergillus* spp. responded to medical therapy with a variety of topical (amphotericin B, natamycin, ketoconazole,itraconazole) or systemic (oral ketoconazole, oral itraconazole) antifungals, with surgery not being required. However, in the presence of deep corneal lesions, almost 60% of patients did not respond to medical therapy alone, particularly if natamycin was not used, and surgery was required to control the infection. More recently, a prospective, nonrandomized, interventional, comparative study on 115 consecutive patients with fungal keratitis treated at one centre during a 6-month period concluded that in patients with fungal keratitis treated with 5% natamycin monotherapy, the presence of an ulcer that exceeded 14 mm with hypopyon and infection with *Aspergillus* species were predictors of a poor outcome [112].

6.2 *Surgery in the Management of* Aspergillus *Keratitis*

Surgery may be required in those patients with *Aspergillus* keratitis whose response to conventional antifungal therapy is not satisfactory, or where perforation or descemetocele formation is imminent. Importantly, antifungal agents need to be given for as long as possible prior to surgery, the rationale being that this renders the infecting fungus non-viable and therein improves the surgical outcome. The types of surgical procedures used range from simple removal of the corneal epithelium (debridement) through anterior lamellar keratectomy, conjunctival flaps and tissue adhesives to therapeutic penetrating keratoplasty. These techniques augment the effects of medical therapy by removing infected corneal tissue (therein reducing or eliminating the fungal load) and increasing drug penetration, by bringing in blood vessels (when conjunctival flaps are formed), by stabilising the corneal epithelial surface itself or by providing tectonic support to the entire globe when its integrity is threatened by thinning or perforation of the cornea [69].

6.2.1 Debridement in *Aspergillus* Keratitis

In *Aspergillus* keratitis, regular debridement of necrotic material from the base of the corneal ulcer aids elimination of fungi and also facilitates penetration of antifungal drugs into the corneal stroma. Debridement is usually performed, under topical anaesthesia, with a Bard-Parker blade No.15, ensuring that a margin of 1–2 mm is left at the limbus [69].

6.2.2 Lamellar Keratectomy/Keratoplasty in *Aspergillus* Keratitis

This procedure helps to remove the thick mat of fungal filaments that may have formed on the cornea, therein facilitating increased drug penetration and resolution of the corneal lesions in patients with superficial fungal keratitis [113, 114]. Until a few years ago, the technique of lamellar keratoplasty itself (where only the superficial 50% of the corneal thickness is removed and replaced by a 50% thickness donor graft) was contraindicated in management of fungal keratitis due to fears of recurrence of infection. However, with advances in corneal surgical procedures, new views have emerged about the efficacy of this procedure, particularly in patients with superficial fungal keratitis who have not responded satisfactorily to antifungal therapy. This procedure can provide useful vision with few complications and the corneal tissue used in this procedure is more easily obtained than the healthy tissue used in therapeutic penetrating keratoplasty [115]. In one study, 6 patients with culture-proven *Aspergillus* keratitis who had not responded to topical and oral antifungal medication underwent lamellar keratoplasty followed by topical antifungal treatment for 2 weeks with gradual tapering of the drugs. Therapeutically beneficial results were achieved in 5 of the 6 patients, with recurrence of infection in one patient [115]. Interestingly, when factors predisposing to treatment failure after lamellar keratoplasty in patients with fungal keratitis were recently analysed, infection due to *Aspergillus* species was found to be one of the major risk factors [85].

Anterior (superficial) stromal corneal infiltrates (involving less than half the corneal thickness) in *Aspergillus* keratitis can also be ablated by the excimer laser. Recently, phototherapeutic keratectomy with a 193 nm excimer laser was successfully used to eradicate the infiltrates and facilitate antifungal therapy in a patient with culture-proven *Aspergillus* keratitis [114].

6.2.3 Conjunctival Flap in *Aspergillus* Keratitis

Fashioning a conjunctival flap aids the achievement of a stable conjunctival surface in patients who have persistent or recurrent epithelial defects and progressive ulceration. Such flaps have been found especially beneficial in patients with peripheral fungal corneal ulcers, since the flap will not encroach onto the visual axis and blood vessels present in the flap aid healing of peripheral fungal corneal ulcers [69].

A superficial lamellar keratectomy should first be done to remove the necrotic stroma, and then a thin conjunctival flap should be anchored over the ulcerated site. There have been no studies evaluating the specific use of conjunctival flaps in *Aspergillus* keratitis.

6.2.4 Amniotic Membrane Transplantation in *Aspergillus* Keratitis

Permanent or temporary amniotic membrane transplantation may be a useful adjunctive surgical procedure for the management of microbial (including *Aspergillus*) keratitis by promoting wound healing and reducing inflammation [116]. This procedure was used in the management of persistent corneal ulcers and corneal perforations in 23 patients with acute fungal keratitis, including three patients with active, culture-proven *Aspergillus* keratitis. The active infection was controlled and there was no recurrence of infection in 2 of the 3 patients with *Aspergillus* keratitis [117]. A larger number of patients will need to be studied to confirm the utility of amniotic membrane transplantation in management of *Aspergillus* keratitis. It is also not clear whether this technique will be useful in patients who have extensive corneal epithelial ulceration and stromal infiltration, and whether the infecting *Aspergillus* is completely eradicated in this procedure, or whether foci of viable fungi persist in the corneal tissue.

6.2.5 Therapeutic Penetrating Keratoplasty

Therapeutic penetrating keratoplasty (corneal transplantation using entire thickness corneal grafts) may be the last resort to preserve or to restore useful vision in the infected eye in patients with fungal keratitis. This is especially true when there is a poor response to antifungal therapy. This procedure may have to be performed in about 53.9% of eyes with filamentous fungal keratitis (particularly when caused by species of *Fusarium* or *Aspergillus*) and in 41% of eyes with yeast keratitis (particularly when caused by species of *Candida*) [11]. Fungal keratitis is reported to be associated with a 5–6-fold higher risk of subsequent perforation and need for penetrating keratoplasty than bacterial keratitis [54]. To decrease the incidence of

recurrence, at least 0.5 mm of clear tissue all around the infected area is to be excised. Although antifungal therapy should be continued post-operatively, topical steroids should be used with caution. When donor grafts 8 mm or less in diameter are used for penetrating keratoplasty in fungal corneal ulcers, the outcome is better than when larger grafts are used. Topical cyclosporin A (5 mg/ml) has been used to prevent allograft rejection in patients with culture-proven fungal keratitis [56].

Although there have been no studies that have specifically tried to correlate the type of fungal aetiological agent and the ultimate need for penetrating keratoplasty, two groups of workers [63, 65] noted that a relatively higher percentage of patients with *Aspergillus* keratitis, compared to patients with other types of fungal keratitis, ultimately required therapeutic penetrating keratoplasty due to non-responsiveness to medical therapy. Again, there have been no studies that have specifically tried to examine the outcome of therapeutic penetrating keratoplasy for *Aspergillus* keratitis *versus* the outcome for other types of fungal keratitis. Both successful outcomes [32, 36, 56] and poor outcomes [56] have been reported following therapeutic penetrating keratoplasty for *Aspergillus* keratitis.

Keratomycotic malignant glaucoma may be one of the complications of *Aspergillus* keratitis [37]. In such patients, therapeutic penetrating keratoplasty with cataract extraction alone might not be sufficient for management of such cases, and a limited pars plana vitrectomy might be needed.

Complications may occur subsequent to therapeutic penetrating keratoplasty, such as allograft rejection, especially when the donor graft is greater than 8.5 mm in diameter, refractive errors and other visual problems [56]. The unusual occurrence of an epicorneal aspergilloma was reported following penetrating keratoplasty and the wearing of a bandage contact lens in one patient [61].

6.2.6 Anterior Chamber Tap

An anterior chamber tap is an extremely useful procedure in the management of ocular infections confined to the anterior segment but needs to be performed under strict aseptic conditions. If the infection involves the anterior capsule of the lens, care should be taken to avoid injury to the lens. An anterior chamber tap was performed for a patient with *Aspergillus* keratitis who had not been responding to topical natamycin and systemic ketoconazole, and who had a persistent infiltrate and thick hypopyon. Performing the tap allowed the hypopyon to be evacuated and amphotericin B (5 μg) to be injected into the anterior chamber [50]. The infection resolved following the tap.

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Aspergillus Otitis

Alexandro Bonifaz, Rogelio Chavolla-Magaña and Javier Araiza

Abstract *Aspergillus* otitis is a superficial mycosis that usually affects the external auditory canal. It is the most frequent aetiology of otomycosis, with *Aspergillus niger* and *Aspergillus flavus* being the most frequent agents. It is a cosmopolitan condition that occurs more often in warm and humid climates, usually affecting young adults. Major predisposing factors include: humidity, poor hygiene and treatment with antibacterial drugs and steroids. Clinically the infection may present with oedema, scabs, erythema and epithelial exfoliation, with symptoms like itching and hearing loss. The diagnosis is established by the observation of fungal forms in the direct exam (hyphae, conidia and *Aspergillus* heads), and with repeated cultures in special media. Topical treatment is effective and includes cleaning of the outer auditory canal to remove the cell debris, sebum and fungal growth. Topical antifungal agents are useful and may be used as concomitant therapy with systemic antibiotics to reduce relapses.

Keywords *Aspergillus* · *Aspergillus niger* · Otitis externa · Otomycosis · Swimmer's ear

Contents

A. Bonifaz (✉)
Dermatology Service & Mycology Department, Hospital General de México OD,
México CP 03020, Mexico
e-mail: a_bonifaz@yahoo.com.mx

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1 Introduction

Otitis externa is one of the most frequent ear infections. Most cases are caused by bacteria, with fungi representing only a small proportion, around 5–10%. However, according to some authors, during certain periods fungi may account for as much as 30% of the cases of otitis externa [1–5]. Otomycosis or fungal otitis externa is a superficial mycosis caused by various opportunistic fungi, particularly those belonging to the *Aspergillus* genus, which affect primarily the outer auditory canal. These conditions are also known as *Aspergillus* otitis, myringomycosis, fungal ear infection and "swimmer's ear" [6–9].

2 Epidemiology

Aspergillus otitis is a cosmopolitan condition that occurs more often in warm and humid climates. It affects both genders in the same proportion, with a slight predominance of females – actually, *Aspergillus* species may be part of the habitual microbiota of the external auditory canal. *Aspergillus* otitis has been reported in all age groups. It is almost always seen in people who practice water sports but rarely affects children. It is more frequent among adolescents and this has been related to growth spurts. However, the highest frequency is observed amongst young adults aging 20–30 years-old [5, 9, 10, 11]. The disease is frequently related to patients' profession, affecting individuals whose ears remain wet for long periods of time, i.e., swimmers, divers or individuals living in very humid regions. The incubation period is indefinite and is rather dependent on patient-related factors [5, 6, 12, 13]. The major predisposing factors are humidity and poor hygiene of the outer auditory canal. However, the disease has also been associated with topical use of antibacterial drugs and steroids, since both of these allow the growth of fungi that are part of the normal microbiota. *Aspergillus* otitis has also been reported in patients with diabetes, leukaemia, lymphoma, and after ear surgery, particularly canal wall down mastoidectomy [3, 6, 10, 14].

3 Aetiology and Pathogenesis

In general, otomycosis may be caused by a variety of agents, with more than 60 fungal species being reported in the literature. Polluting filamentous fungi, also known as hyphomycetes, predominate. Most of these are light-coloured (hyalohyphomycetes), with some infections also being caused by darkly pigmented fungi (phaeohyphomycetes) [6, 15, 16].

Most reports indicate that *Aspergillus* species are involved in ∼70% of fungal otitis cases, reinforcing the importance of *Aspergillus* otitis. Although *Aspergillus niger* is the globally most frequently recovered species, *A. flavus* occurs in equal frequency in Mexico. Otitis caused by *A. fumigatus* and *A. terreus* has also been

described – exceptionally, infections are associated with *A. clavatus, A. candidus,* and *A. nidulans*. Several other hyalohyphomycetes (*Scopulariopsis* spp., *Penicillium* spp., and *Fusarium* spp.) and phaeohyphomycetes (*Alternaria* spp., *Cladosporium* spp.) have also been isolated from patients with otomycosis, as well as *Candida* species, especially *C. albicans*. Given that the aetiology of *Aspergillus* otitis consists of common environmental pollutant fungi, its frequency may vary depending on the geographical area and even on the season of the year [6, 13, 17, 18].

The initial step in *Aspergillus* otitis is colonization of the outer auditory canal with *Aspergillus* species. As mentioned before, *Aspergillus* spp. can be part of the habitual microbiota – alternatively, these airborne organisms maybe introduced into the ear by an object. It may also follow a bacterial otitis [8, 9, 10, 19]. *Aspergillus* spp. growth is usually limited to the stratum corneum and produces a mild inflammatory process. In patients with excessive cerumen, the fungal filamentous masses intermingle to form a plug that may mechanically impair hearing. According to many authors, given that this is a very superficial process, it does not constitute an actual parasitic infection, but rather a saprotrophic condition to the auditory canal. This is supported by the fact – unlike most other fungi – *Aspergillus* spp. growth is not reduced in these cases. Accordingly, all ornamentations and reproductive forms of *Aspergillus* spp. are seen in cases of *Aspergillus* otitis, in a similar proportion to what occurs in the environment and on culture media [6, 11].

4 Clinical Aspects

Aspergillus otitis is a local process that remains restricted almost exclusively to the external auditory canal. In most cases only the stratum corneum is involved, with exceptional cases involving the pinna or spreading farther to perforate the tympanic membrane and thus involving the middle ear [2, 4, 19–21]. Clinically, the ear or auditory canal shows oedema, scabs, erythema and exfoliation of the superficial epithelium. Once the disease becomes chronic, the subepithelial layers may be parasitized. The most frequent symptoms include fullness and, mainly, intense itching. That may stimulate patients to introduce cotton swabs or other objects into the auditory canal, which may cause lesions to become more eczematous, with skin lichenification. In chronic cases, usually associated with poor outer auditory canal hygiene, the latter may become obstructed by a mass composed of cerumen, epithelial debris and abundant fungal structures that result from the growth of mould consisting of filamentous masses. Moderate hearing loss can occur at this stage. Filamentous masses are easily identified during otoscopic examination – these are covered by a creamy fluid, resulting in a "wet blotter paper" appearance. In some chronic cases myringitis granulosa may develop, characterized by an erythematous to pink membrane, showing streaks and granulations, as mentioned above. Perforation occurs seldom [8, 14, 19, 22] (Fig. 1). This condition is often associated or may follow bacterial infections, leading to a more acute presentation that includes persistent and intense pain and abundant otorrhoea. Suppuration of different colours may be seen, depending

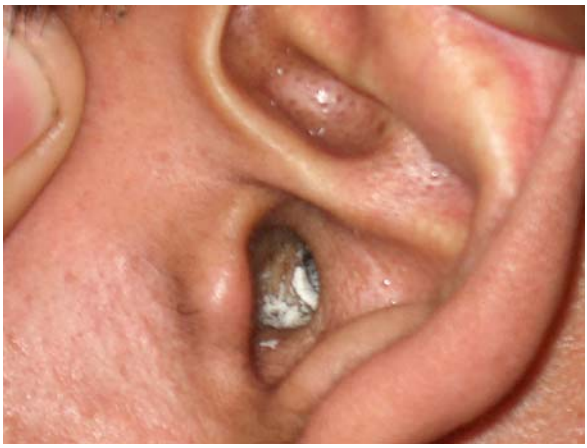

Fig. 1 Fungal otitis externa caused by *Aspergillus flavus*

mainly on the causative agent, e.g., greenish (*A. fumigatus* or *Pseudomonas* spp.), black (*A. niger* or *Rhizopus* spp.), or whitish (due to *Candida* spp.) [6, 7, 20].

Exceptionally, otomycosis may spread to the cartilaginous ear structures and the ear drum, particularly in immunosuppressed patients. It is important to mention that the involvement of the ear drum may cause permanent deafness and the involvement of the middle ear may allow the infection to spread to the brain. Fungal balls (aspergillomas) and involvement of various ear structures have also been reported [22–25]. In a recent literature review [20], 25 cases of invasive otitis externa were described. Most of these patients were imunocompromised by conditions such as AIDS (28%) [27–31], diabetes mellitus (24%) [26, 32–36], and acute leukaemia (20%) [37–41]. All but one case were monomicrobial, usually caused by *A. fumigatus* ($n = 9$) or *A. flavus* ($n = 8$).

Clinically the differential diagnosis of *Aspergillus* otitis should include bacterial otitis, especially those caused by *Pseudomonas aeruginosa, Staphylococcus aureus, Proteus* spp., and *Micrococcus* spp., as well as mycobacterial otitis externa and otomycosis caused by other filamentous fungi and yeasts. Other conditions to be considered include seborrhoeic dermatitis, impetigo, and contact dermatitis [1, 3, 7].

5 Laboratory Diagnosis

Proper specimen collection is essential for a successful diagnosis. Debris of epithelial exfoliation (scales) and the cerumen should be collected using a curette. If an exudate is present, a cotton swab may be preferred. The specimen is then divided in two parts, for microscopy and culture [8, 20, 26, 27]. For microscopy, the collected

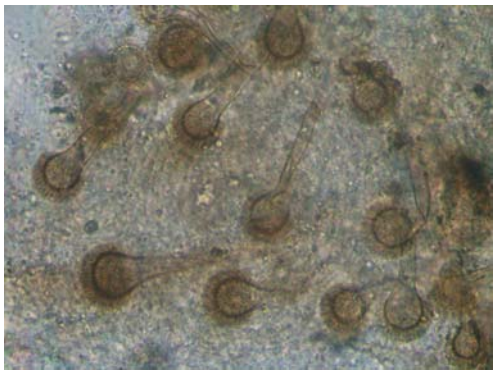

Fig. 2 Microscopy showing multiple *Aspergillus* heads of *Aspergillus fumigatus* (KOH, 10×)

Table 1 Microscopic observations according to the etiology of otomycosis

Aetiology	Microscopic Observations
Aspergillus (70%) *A. niger*, *A. flavus* and *A. fumigatus*	Abundant hyphae, microconidia, and *Aspergillus* heads may be observed even in chronic cases. (Fig. 2)
Candida spp. (10%) *Candida albicans*	Clusters of blastoconidia plus pseudohyphae, which indicate the parasitic status.
Hyalohyphomycetes *Penicillum spp.*, *Acremonium spp.*, *Scopulariopsis spp.*	Septate hyaline hyphae or, at times, their reproductive structures (phialides and sterigmata)
Phaeohyphomycetes *Alternaria spp.*, *Cladosporium spp.*	Septate dark hyphae or, at times, their reproductive structures (microconidia and dictioconidia)
Mucorales Mucor spp., *Rhizopus spp.*, *Absidia spp.*	Coenocytic hyphae (no septum)

material is placed between two microscope slides adding one drop of 10–20% KOH or a cotton blue solution. Abundant hyphae are usually observed, in addition to specific fungal structures, including microconidia – in chronic cases, one may even see *Aspergillus* heads [6, 26]. It is important to stress that depending on the type, certain otomycoses may produce a microscopic image that are highly suggestive of the causative agent. (Table 1) (Fig. 2).

Fungal cultures should be done repeatedly in antibiotic-free media, such as the Sabouraud agar, potato dextrose agar and the Czapek agar. Incubation must be performed at room temperature for 3 to 5 days. Fungi are identified at the species level based on their micro- and macro-morphologic features. Histopathology is also useful: a mild inflammatory with multiple fungal structures on the stratum corneum is the usual finding [2, 6, 10, 20, 26, 28].

6 Treatment and Prophylaxis

Appropriate cleaning of the auditory canal is essential by mechanically removing the remaining cerumen and cell debris. Several solutions may be used for that purpose, including Burrow's solution (5% aluminium acetate), 2% acetyl salicylic acid in a 75% alcohol base, and mercurochrome (1% thimerosal). Afterwards, washings with hypertonic saline solution are usually recommended [10, 29]. Topical antifungal agents are very helpful and should be started after proper cleaning of the auditory canal is obtained. The most widely used agents are amphotericin B (1 mg/ml) (which is active against most fungi but causes irritation), nystatin (100,000 U/ml bid), and tolciclate (1%). These agents are extremely effective in the treatment of *Aspergillus* otitis, while imidazole derivatives such as clotrimazole, miconazole and ketoconazole are less useful options [14, 15, 24]. Some authors have also used terbinafine for this purpose [9, 19, 29]. Other treatments that have been used with variable results include mercurochrome and hyperbaric oxygen, and drops of acid solutions [25, 30, 31].

Most patients with invasive *Aspergillus* otitis require long-term therapy with systemic antifungal drugs, which is usually associated with a complete recovery. A literature review showed that most cases were treated with amphotericin B, followed by itraconazole. Voriconazole is an attractive option for that purpose, based on its favourable bone penetration [20]. Oral antifungal might be indicated to reduce the number of relapses and to prevent the spread of the infection. The most widely recommended agent is itraconazole at 100–200 mg/day for 15–30 days. Recently, in vitro resistance to itraconazole was described in isolates of *A. niger* causing otomycosis, while susceptibility was retained to voriconazole [32]. As for topical antifungal drugs, systemic antifungal agents are of limited efficacy in absence of proper local cleaning.

Finally, it is important to mention that the cleaning of the canal and the radical cavity must be performed with the assistance of the surgical microscope and aspiration. The most appropriate prophylactic measure is hygiene, particularly in individuals whose ears are permanently wet (swimmers) or who are on therapy with antibiotics or steroids. Prevention depends crucially on keeping the auditory canal dry and clean [5, 10, 19].

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