Exposure, Sensitization, and Mechanisms of Fungus-induced Asthma

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Current Allergy and Asthma Reports 2003, **3:**430–437
Current Science Inc. ISSN 1529-7322

Healthy individuals are continuously exposed to fungal biomass, which includes live and dead spores and fungal debris that is entrapped in the airways. In patients with asthma and/or atopy, exposure to fungal biomass might result in age-dependent sensitization and asthmatic reactions. Interaction with Toll-like receptors (TLRs) of the innate immune defense (alveolar macrophages and epithelial cells) and protease-activated receptors (PARs) determine the effectiveness of elimination of fungal material. The association of sensitization to *Alternaria* with severe asthma is discussed in relation to the age-dependent sensitization, rate of release of allergens from spores, and activity of its proteases. A model is described concerning the influence of polymorphic genes for airway hyperresponsiveness (AHR) and atopy, showing a cumulating influence on susceptibility for allergen-induced asthma, and explaining that fungus-induced airway obstruction is mainly associated with more severe asthma.

Introduction

Airways are continuously exposed to a diversity of both pathogenic and nonpathogenic microorganisms—*eg*, bacteria, fungi, and viruses. Although fungal spores are inhaled in large quantities, they are recognized by the innate defense system, resulting in the effective elimination of microorganisms from the airways, without extensive activation of the cognate immune system. In allergic patients, inhalation of fungal biomass might induce immunologic responses, resulting in allergic manifestations of the airways, such as asthma and/or rhinitis. In asthmatic patients, at least two genetically determined characteristics interact with each other in the final clinical outcome of atopic asthma. Initially, the patient has the genetic propensity to mount an IgE response to inhaled allergens. Airway hyperresponsiveness (AHR) is an inherited property that determines the tendency of airways to narrow too easily to environmental stimuli. The immune response to fungi is composed of an innate and a cognate part, which might result in IgE antibody formation against fungal components. Before being presented to the immune system, allergens must pass the barriers of the innate defense system of alveolar macrophages and the epithelial cell layer, which actively eliminates particulates of biologic origin (pollen, fungi, animal dander) [1,2]. Presentation of fungal antigens to the immune system is dependent on the nature and quantity of fungal biomass that is inhaled, depth of deposition, and the rate of clearance and elimination by phagocytes. Furthermore, characteristics of the fungal wall and biologic active components excreted by different fungi (*eg*, proteolytic enzymes, toxins) determine the effectiveness of elimination, activation, and/or damage of the mesenchymal (epithelial-fibroblast) cell layer and final passage of fungal allergens through the airway wall.

Interest for fungus-induced asthma is currently increasing after recognition that severity of asthma, both in children and adults, is associated with sensitization to certain fungi, but not to pollens [3]. Furthermore, sensitization or exposure to fungi was found to be associated with hospital admissions for asthma, life-threatening exacerbations of asthma, and death from asthma [3]. However, an understanding of how and why fungi are associated with manifestations of severe asthma is poorly understood. In this review, we only shortly describe the exposure to the various fungal spores, because several excellent reviews have been published recently [4,5]. We discuss the association of asthma with sensitization to fungi and the limited data describing sensitization to fungi in relation to corresponding exposure in the same environment. Furthermore, this review is focused on aspects of the interaction of fungal components with cells of the airway wall and how genetic variation underlying both the atopic constitution and airway reactivity might interact in the final outcome of fungus-induced asthma.

Exposure to Fungal Biomass

Exposure to fungal biomass consists of exposure to living fungal spores and fungal debris after death of fungal spores and mycelium. Fungal spores generally do not easily release their allergen content, owing to the rigidity of the spore wall, and release of allergens will mainly occur during germination of the spores [6–8] or after cell death. Germination times of spores differ largely, showing a short germination time of 2 hours for *Alternaria* compared with the germination time of other fungal spores (germination time 5–10 hours)

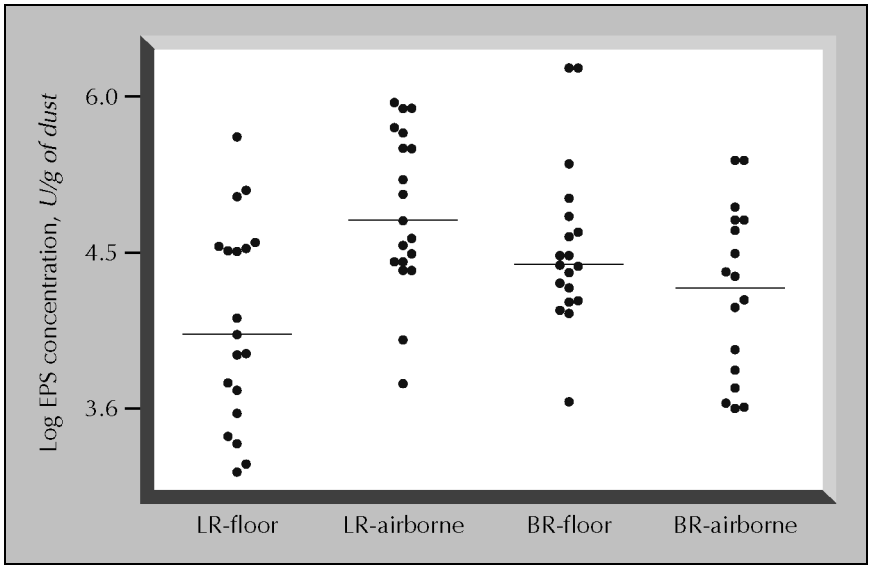

Figure 1. Levels of *Aspergillus*- and *Penicillium*-specific extracellular polysaccharides (EPS) in samples from floor dust and airborne dust. Filters from air cleaners were extracted with 0.01 M NH_4HCO_3 buffer during a 2-hour period, as described previously [10]. An enzyme-linked immunosorbent assay protocol, using polyclonal antibodies [11•], was used for measuring EPS concentrations. LR—living room; BR—bedroom. (*Antibodies provided by* G Doekes.)

[9]. Aeroallergens in death fungal masses are more soluble and readily available for contact with the airway system, which most likely explains the rate of asthmatic attacks (often within 10–15 minutes) after inhalation of mold-contaminated air (*eg*, in barns) by sensitized patients. However, the quantity and composition of this fungal dust is not fully known. Currently, number and genera of fungal spores measured in the air is commonly used, assuming that:

1. Numbers of spores will reflect exposure to the total amount of inhaled fungal biomass
2. Availability and release of soluble fungal allergens will be similar for all fungi

Although the first assumption might be valid, the second assumption is doubtful. The ratio of life over death fungal mass for different fungi is unknown, but will most likely be variable for different fungal species dependent on, for example, differences in heat-resistance and/or size of spores. Therefore, numbers of spores are not necessarily related to bioavailability for the immune response. New techniques, such as extraction of the filters used in air cleaners, followed by immunologic determination of specific allergen [10], can be used for the study of airborne fungal allergens. Figure 1 demonstrates measurement of extracellular polysaccharides (EPS) specific to *Aspergillus* and *Penicillium* in both floor dust and filter extracts from air cleaners [11•]. Aerobiologic studies have shown that fungal spores are continuously present in the indoor and outdoor milieu and are inhaled in relatively large quantities. Genera that are most commonly identified as a possible cause of fungus-related respiratory-tract disorders belong to separate fungal groups—Ascomycetes, Basidiomycetes, and Deuteromycetes [5,12,13]. Observations in European countries show remarkable similarities in prevalence of outdoor fungi. *Cladosporium* is found most abundantly and is often responsible for the majority of the spores that are inhaled [12]. It is interesting to note that *Alternaria* is found in lower numbers compared with other fungi and much less than *Cladosporium* (50 to 100 times less) [14•,15]. In the United States, these quantitative differences between *Alternaria* and *Cladosporium* are similar but less impressive [4,5,16]. Exposure to fungal spores inside houses is dependent on the quality of the building, the furniture, the bedding, the humidity, and the presence of pets as important determinants for growth of a diversity of fungal species [5,17,18] and on numbers of outdoor fungi [4].

Exposure, Sensitization, and Age

Although it is assumed that fungal species found in the greatest numbers will be associated with disease states in humans [19], this concept is challenged by the observations that spore numbers and sensitization in patients living in the same area are only weakly or even not related to one another. A study of the prevalence of spores of individual species and corresponding sensitization shows that *Cladosporium* is the least sensitizing fungus, comparable with yeasts, followed by increasing skin test/spore count ratio for *Penicillium* and *Botrytis*, whereas *Alternaria* and *Aspergillus* show a high score for sensitization compared with corresponding spore counts [14•]. Additionally, IgE determination in a pooled serum of patients of our department who were multiply sensitized to fungi showed high titers for IgE to *Alternaria*, followed by *Aspergillus* and *Penicillium*, whereas *Cladosporium* showed the lowest titer [20]. Similar observations were reported for sensitization to fungi and corresponding data of exposure in lawn cutters [21].

Epidemiologic data concerning sensitization to fungi have been studied in different locations [4,5,19,22–24], showing that sensitization to fungi is highly variable from place to place and is dependent on local exposure.

Age-dependent sensitization to fungal allergens has been studied in a limited number of cases, suggesting an age-dependent distribution [25,26]. A first study, in a

group of adult allergic patients (14 to older than 50 years), demonstrated that sensitization to different fungi, as measured by skin testing, showed lower sensitization at older ages [27]. Additionally, prevalence for *Alternaria* and *Cladosporium* ranked first (18%–20%) in the 14- to 19-year-old group, with a lower prevalence (less than 5%) in older-aged groups. In a second cross-sectional study, in atopic children (aged 1–14 years), using prevalence of IgE to different fungi, an age-dependent sensitization was found, followed by lower IgE titers with increasing age [28•]. IgE to these fungi started around age 1, reaching a maximum probability for IgE for all fungi at 7.7 to 7.8 years. A high prevalence was found for *Cladosporium*, *Aspergillus*, and *Alternaria* compared with *Penicillium*. This age-dependent distribution of sensitization was very specific for fungi, because sensitization to house dust mites (HDMs; IgE to *Dermatophagoides pteronissinus*) showed rapid increasing titers in this group of children, reaching maximum IgE titers at 4 years, without a phase of decline until 14 years (maximum age in the study group). A similar pattern of age-dependent sensitization to different allergens (including fungi) was found in a desert environment, in a group of children aged 3 to 17 years [29]. This suggests that age-dependent sensitization to fungi, especially to *Alternaria*, is not unique to desert climatic conditions, but might be similar in milder and more humid climates.

Fungal Exposure and Association with Clinical Manifestation of Asthma

Interest in the role of sensitization to fungi is increasing since it was found that sensitization to fungi is associated with asthma and also with different manifestations and severity of asthma. In a cross-sectional study in European countries, the frequency of sensitization to fungi was associated with severity of asthma [3]. These observations are in accordance with several observations consistently showing that sensitization to *Alternaria* and (if measured) *Cladosporium* are most strongly associated with different parameters of severity of asthma, which is not found for sensitization to grass, tree, ragweed, pollen, or cat [3]. In a similar study in Australia, indoor exposure to fungi (measured as ergosterol levels) appeared to be a risk factor for sensitization and current asthma [30], but not exposure to HDMs. Associations for fungal sensitization and asthma have been found for (severe) asthma (in children) [26,31–33], life-threatening asthma (adults) [34], death from asthma [35], and visits to emergency rooms [36] (in children) [37]. Furthermore, airway hyperresponsiveness, wheeze, and bronchodilator use in children was associated with increasing exposure to *Alternaria* spores [38]. The association of fungal sensitization with asthma, often found in children, is in accordance with the age-dependent distribution of IgE to fungi, showing maximal sensitization in children [28]. However, although sensitization to fungi shows significantly lower values in older aged groups, sensitization to fungi in adult populations is still associated with life-threatening asthma [34].

The large numbers of spores of *Cladosporium* generally found in the environment can explain its frequency of sensitization. However, the similarity in frequency of sensitization to *Alternaria* and *Cladosporium* in atopic patients and the strong association of severe asthma with sensitization to *Alternaria* are in contrast to the lower number of spores found in the air and the large-size spores (20–60 mm) of *Alternaria*, which predicts limited deposition in lower airways. The weak causal relationship between exposure to fungal spores and clinical manifestation of asthma might be explained by sudden changes of fungal spores in short time intervals (2 hours), called spore plumes [39], and our lack of knowledge of the amount of dead mass of fungal allergens in the air.

Mucosal Airway Defense Against Fungal Spores: Interactive Role of Innate and Cognate Immune Response

Fungal biomass that is deposited on the airway wall is eliminated by the combined action of innate recognition by complement and surfactant proteins that facilitate phagocytosis by alveolar macrophages. Additionally, secretory immunoglobulin A (S-IgA) prevents contact with the epithelial cell layer and allows transport on the epithelial lining fluid (ELF) by coordinated ciliary movement to the oropharyngeal cavity [2]. In healthy airways, this transport will generally remove particles impacted in the larger airways in approximately 4 hours, before germination of fungal spores can occur [9]. Smaller-sized spores can reach the lower airways (*Aspergillus* and *Penicillium*) and can be removed in 12 to 24 hours.

Interaction with Epithelial Cell Surface

Spores that are deposited in large quantities and allergens from dead fungal mass might reach the epithelial cell surface and activate epithelial cells. Epithelial cells are equipped with various receptors, such as Toll-like receptors (TLR), that can recognize surface structures of microorganisms, the so-called pathogen-associated molecular patterns (PAMP) [1,40]. Although the TLR family has been extensively studied for defense against bacteria and viruses, early studies on TLRs indicated a defensive role against fungi [41], which is supported by more recent animal models [42•,43,44•]. Activation of TLR is followed by activation of nuclear factor (NF)-κB and mitogen-activated protein (MAP)-kinase [45]. In addition to activation by TLRs, proteolytic components released by fungi can interact with the epithelial cell surface by disruption of cellular junctional contacts and activation of so-called protease-activated receptors (PAR) that will induce the production of cytokines and prostaglandin (PG)E_2 [46,47]. Fungal extracts have shown differences in their capacity to cause protease-dependent disruption of cellular contacts and release chemokines. Extracts of *Alternaria* were more active in

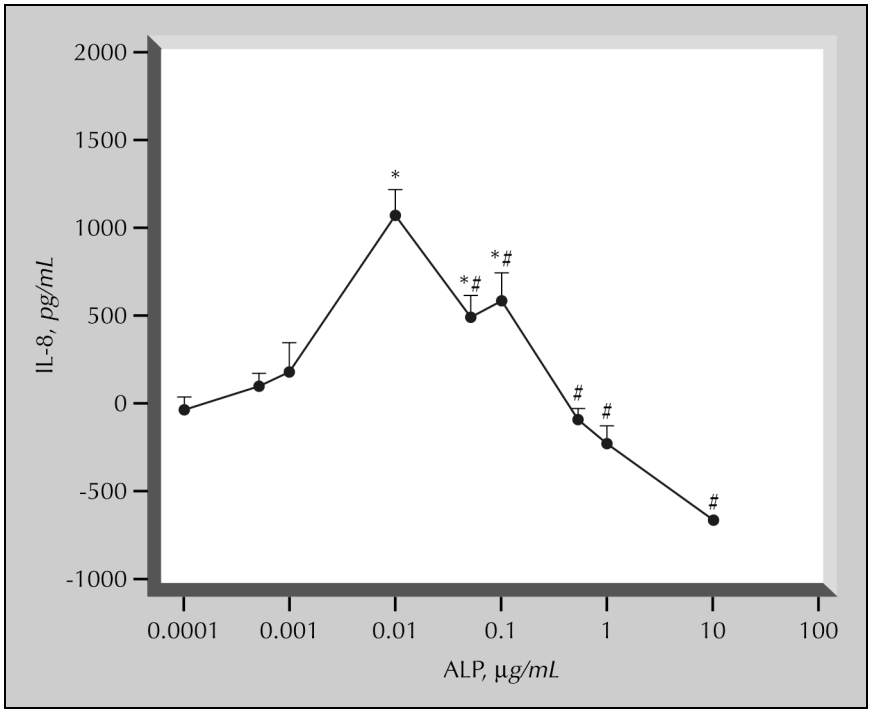

Figure 2. Interleukin (IL)-8 production by A549 epithelial cells incubated with alkaline protease (ALP) from *Aspergillus fumigatus*. A549 cells were incubated with increasing concentrations of ALP and IL-8 measured by enzyme-linked immunosorbent assay [48]. *Zero values* indicate spontaneous production of IL-8 in the absence of fungal protease. *Negative values* indicate inhibition of spontaneous production of protein. *Asterisks* indicate a significant increase of production of cytokine, and # indicates increasing desquamation of A549 cells. (*ALP concentrations provided by* Prof. Dr. M. Monod.)

desquamation and proteinase-dependent activation of epithelial cells, whereas *Cladosporium* did not show desquamation and much less activation [48]. It has been proposed that proteases present in inhaled allergens (including fungi) affecting epithelial cellular contacts might facilitate passage of allergens through the epithelial barrier [49,50]. Activation of TLRs and PARs on epithelial cells, followed by facilitated transport of allergens and production of cytokines and growth factors, might be involved in increased sensitization to (fungal) allergens and inflammatory response and remodeling of the airways [46,50,51]. In a recent animal model, the inhalation of alkaline serine proteinase from *Aspergillus fumigatus* showed a synergistic effect on Asp-f-2–induced inflammatory response [52].

The severe asthmatic attacks as found in allergic bronchopulmonary aspergillosis (ABPA) can be ascribed to the specific capacity of this opportunistic fungus to overcome the innate defense by binding to the epithelial surface and initiating a damaging inflammatory response in the airways [2]. One of the factors that might promote the survival of *Aspergillus* in the airways is the production of proteolytic enzymes that can cleave matrix proteins such as elastin and collagen [53]. A recombinant alkaline serine proteinase of *A. fumigatus* (ALP) added to airway epithelial cells induced cytokine production, showing an activation phase followed by inactivation of the epithelial cells below basal cytokine production (Fig. 2). This characteristic activation and inactivation of airway epithelial cells is similar to the effects of collagen-growth cultures of *A. fumigatus*, containing large quantities of proteolytic enzymes [54]. Such silencing of chemokine production by epithelial cells, which is not found with extracts of *Alternaria* and *Cladosporium*, might be important for hampering successful detection and killing by phagocytic cells. The increased survival of *A. fumigatus* in airway tissue might explain the strong immune responses and eosinophilic inflammatory response found in ABPA [48].

Genes Related to Atopy and/or Airway Reactivity and Susceptibility for Fungus-induced Asthma

During the past decade, the role of atopy as a cause of asthma has been debated. Although early-life exposure to HDMs and cats appears to be related to sensitization, the relation with the development of asthma is still unclear [55]. Inconsistent associations between asthma and atopy have been reported, and other etiologic factors underlying the development of asthma were suggested [56,57]. These epidemiologic data are in keeping with recent studies of application of allergen to the airways, indicating that the atopic status of asthmatic and rhinitis patients does not by itself explain the response to allergen. It has been proposed that other factors, such as increased airway reactivity or pre-existing airway injury, are additional mechanisms underlying asthmatic reactions to allergens [58••,59••]. These observations support current models of asthma theorizing that epithelial cells might show structural abnormalities [60–63]. Damage to the epithelial cell layer by inhaled allergens might induce enhanced production of cytokines and growth factors [64,65], thereby increasing inflammatory responses and remodeling of airway wall structures [66,67], finally resulting in enhanced sensitivity of the airways [50,60,61].

Current research on the genetics of asthma indicates two types of genetic predisposition—genes related to formation of IgE and genes related to development of AHR—which interact to determine the final phenotype of atopic asthma.

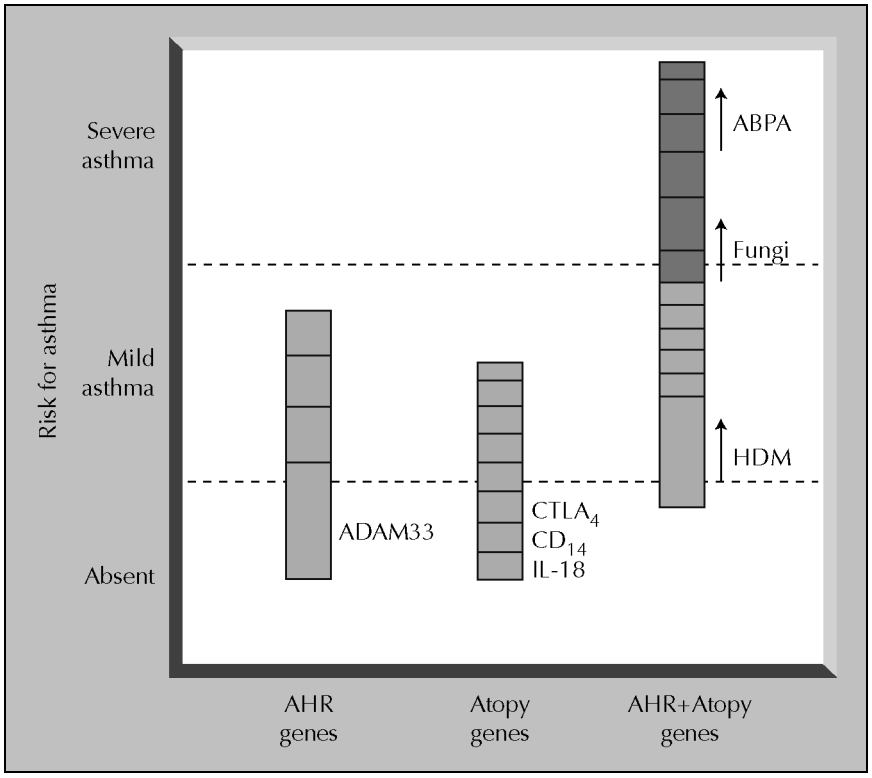

Figure 3. Genetic susceptibility for asthma; influence of fungi. This theoretical model describes the cumulating influence of genes that are associated with airway hyperresponsiveness (AHR), atopy, or the combination of AHR and atopy on the risk for developing asthma. In column 1, *ADAM33* is indicated as one of the several genes that are associated with AHR. The cumulative influence of genes related to atopy on asthma is shown in column 2. Only some of the many (candidate) genes have been indicated. Limited combinations of genes for atopy might result in sensitization without manifestation of asthma, whereas a larger number of genes might increase the chance for atopic asthma. Column 3 shows the combined effect of genes for both AHR and atopy. This combination increases the risk for asthma considerably, by additive and synergistic interactions. As explained in the text, HDM-induced asthma is found in the entire range from weak to severe asthma, whereas fungus-induced asthma is associated with more severe asthma (*dark shaded area*). Allergic bronchopulmonary aspergillosis (ABPA) is found in the group with most severe asthma (*upper part* of column 3). CTLA—cytotoxic T-lymphocyte antigen; HDM—house dust mite; IL—interleukin.

Genome-wide studies have shown the presence of a variety of chromosomal regions that determine the propensity for total IgE and/or specific IgE antibody formation against environmental allergens [68]. In addition to chromosomal regions that show linkage to either IgE and/or AHR, polymorphisms of candidate genes for IgE and/or asthma have been identified (interleukin [IL]-18, cytotoxic T-lymphocyte antigen [CTLA]-4, IL-13, IL-4R) [68–71]. Other genes determine AHR, which is an important condition in asthma [72,73]. Recently, genetic determinants have been identified in asthmatic patients that are specifically associated with airway reactivity and not with atopy [74••,75]. Modified functions of proteins, receptors, cytokines, and enzymes, as outcomes of polymorphism of genes, might interact with each other and determine the final outcome of the immunologic response, airway reactivity, and severity of the asthmatic response. Figure 3 is a "simplified" model that describes how various genes underlying AHR (*cumulative blocks* in column 1) might be associated with increasing risk for (nonatopic) asthma (*y axis*). This group might include patients with, for example, fog- and/or exercise-induced asthma (patients with severe corticosteroid-resistant asthma are not included). The second column shows patients with accumulating influence of genes for atopy (*cumulative blocks* in second column) that might be associated with increasing risk for allergen-induced asthma (*y axis*). Hereditary combination of both genes for AHR and atopy (column 3) might act in an additive and/or synergistic way [76], which might promote the condition for severe asthma. Within this model, the starting-points for allergen-induced asthmatic responses are indicated for HDMs, fungi, and *A. fumigatus*–induced ABPA (*arrows*, Fig. 3). The proteases present in HDMs (especially the cysteine proteinase Der p1) are most potent in breaching the resistance of the epithelial cell layer, facilitating the Th2-type immune response to HDM allergens. Therefore, the association of sensitization to HDMs and asthma will be found in the full range from mild to severe asthma. Fungal asthma is induced by high exposure to allergen, and the proteases involved are less active. Therefore, fungus-induced asthma is found in patients with a high susceptibility of airways for environmental stimuli, which is determined by more cumulative interactions of genes for AHR and/or atopy (*hatched area* in column 3, Fig. 3). This model might explain why fungus-induced asthma is mainly found and associated with more severe asthma. Additionally, ABPA is found in the group with the highest susceptibility, determined by genes that predispose to both severe airway reactivity and IgE formation (*upper part* of column 3, Fig. 3). The group of patients with cystic fibrosis (with a strongly impaired innate defense of the airways) and genetic predisposition for atopy (*eg*, HLA-DR restrictions) seems to be the most susceptible group for ABPA [77].

Conclusions

Fungus-induced sensitization shows an age-dependent distribution, with the highest sensitization in children aged 7 to 8 years, and lower sensitization at increasing age. This pattern of sensitization is in keeping with clinical findings, describing precipitation of severe asthmatic episodes in children associated with sensitization to fungi (*Alternaria*). It is argued that numbers of spores are not similar to exposure to

fungal biomass and that more information on this subject is needed to explain differences between spore counts and corresponding sensitization. A possible procedure for quantifying exposure to fungal allergens is described. A mechanism of interaction between the innate immune defense and fungal antigens is described. The activation of TLRs and PARs by cell-wall components and proteases will facilitate the passage of fungal allergens through the epithelial cell layer, initiating an (Th2-type) immune response and inducing the release of proinflammatory cytokines. In allergic asthmatic patients, the interaction of epithelial cells with environmental components is abnormal and might result in an altered response characterized by a Th2-type response (IgE) and an inflammatory response characterized by eosinophils and Th2-type lymphocytes. Because quantities of allergens released by fungal spores are limited and the activity of the serine proteases is less aggressive (compared with cysteine proteases in HDM), expression of fungus-induced asthma is associated with more severe asthma. It is argued that multiple predispositions for genes determining for both AHR and atopy will be found in those with severe asthma, thereby increasing the chance for fungus-induced asthma.

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