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Birkhäuser Advances in Infectious Diseases

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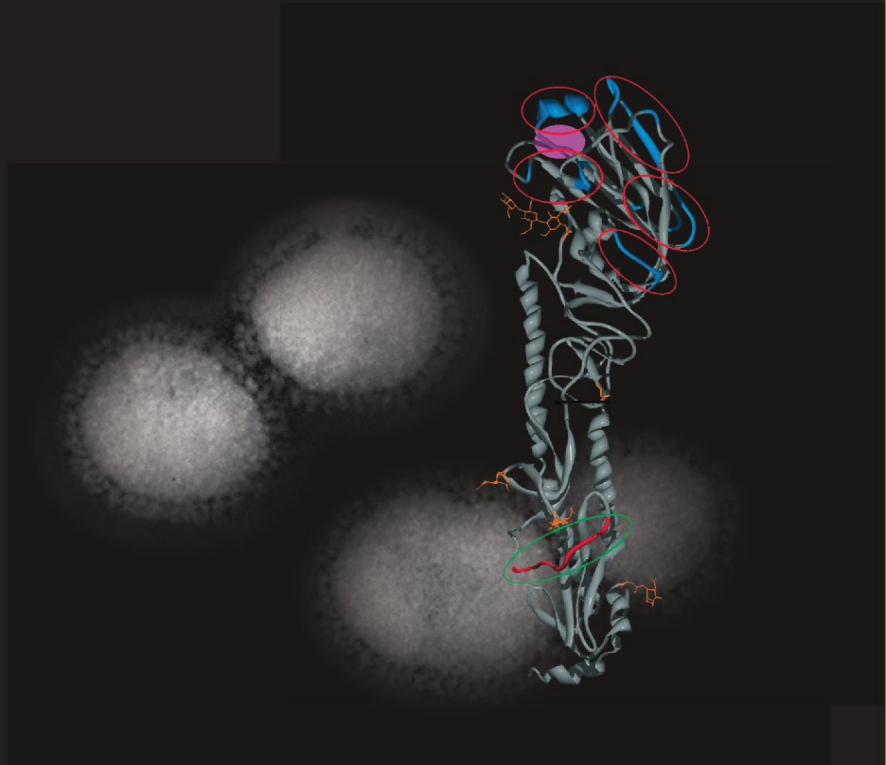
Influenza Vaccines for the Future

Rino Rappuoli
Giuseppe Del Giudice

Editors



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Series Editors

Axel Schmidt, University Witten/Herdecke, Faculty of Medicine, Alfred-Herrhausen-Str. 50, 58448 Witten, Germany

Olaf Weber, Bayer AG, 51368 Leverkusen, Germany

Stefan H.E. Kaufmann, Max-Planck-Institut für Infektionsbiologie, Department of Immunology, Charitéplatz 1, 10117 Berlin, Germany

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Influenza Vaccines for the Future

Rino Rappuoli
Giuseppe Del Giudice

Editors

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Editors

Rino Rappuoli
Giuseppe Del Giudice
Novartis Vaccines
Via Fiorentina 1
53100 Siena
Italy

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List of contributors

Marta Luisa Ciofi degli Atti, National Centre of Epidemiology, Surveillance, and Health promotion, Infectious Disease Unit, Istituto Superiore di Sanità, Roma, Italy

Giuseppe Del Giudice, Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy; e-mail: giuseppe.del_giudice@novartis.com

Peter C. Doherty, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia; Department of Immunology, St Jude Children's Research Hospital, Memphis, TN 38105, USA; e-mail: Peter.Doherty@STJUDE.ORG

Kathryn M. Edwards, Vanderbilt University School of Medicine, Department of Pediatrics, Pediatric Clinical Research Office, Nashville, TN 37232, USA; e-mail: kathryn.edwards@vanderbilt.edu

Anthony Gilbert, Retroscreen Virology Ltd., St Barts and the Royal London Hospital, 2 Royal College Street, London NW1 ONH, UK

Harry Greenberg, Departments of Medicine and Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305 and Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304, USA; e-mail: harry.greenberg@stanford.edu

Carole Guillonneau, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

George Kemble, MedImmune Vaccines, Inc, Mountain View, CA 94043, USA; e-mail: kembleg@medimmune.com

Robert Lambkin-Williams, Retroscreen Virology Ltd., St Barts and the Royal London Hospital, 2 Royal College Street, London NW1 ONH, UK

Maria Lattanzi, Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy; e-mail: maria.lattanzi@novartis.com

Catherine J. Luke, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA; e-mail: cluke@niaid.nih.gov

Mark A Miller, Fogarty International Center, National Institutes of Health, Bethesda Maryland, USA; e-mail: millemar@mail.nih.gov

Justine D. Mintern, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

Emanuele Montomoli, Department of Physiopathology, Experimental Medicine and Public Health, Laboratory of Molecular Epidemiology, University of Siena, Via Aldo Moro 3, 53100 Siena, Italy;
e-mail: montomoli@unisi.it

Samira Mubareka, Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, USA

Derek T. O'Hagan, Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy; e-mail: derek.ohagan@novartis.com

John Oxford, Retroscreen Virology Ltd., St Barts and the Royal London Hospital, 2 Royal College Street, London NW1 ONH, UK
e-mail: j.oxford@retroscreen.com

Peter Palese, Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, USA; e-mail: peter.palese@mssm.edu

Audino Podda, Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy; e-mail: audino.podda@novartis.com

Rino Rappuoli, Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy; e-mail: rino.rappuoli@novartis.com

Caterina Rizzo, National Centre of Epidemiology, Surveillance, and Health promotion, Infectious Disease Unit, Istituto Superiore di Sanità, Roma, and Department of Pharmaco-Biology, University of Bari, Italy; e-mail:
e-mail: caterina.rizzo@iss.it

Lone Simonsen, GWU School of Public Health and Health Services, Washington DC, USA; e-mail: lone@gwu.edu

Kanta Subbarao, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA; e-mail: ksubbarao@niaid.nih.gov

Robert J. Taylor, SAGE Analytica, LLC, Bethesda, Maryland, USA; e-mail: rtaylor@sageanalytica.com

Stephen J. Turner, Department of Microbiology and Immunology, University of Melbourne, Melbourne, Victoria 3010, Australia; Department of Immunology, St Jude Children's Research Hospital, Memphis, TN 38105, USA

Cécile Viboud, Fogarty International Center, National Institutes of Health, Bethesda Maryland, USA; e-mail: viboudc@mail.nih.gov

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Introduction

Rino Rappuoli and Giuseppe Del Giudice

Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

We have been living with influenza as long as history can remember, and we are so used to the fact that this disease is part of life that we usually do not pay much attention to it. Even the science of influenza virus has been dormant for more than half a century. After the excitement of the initial discovery of the virus in 1933 and the development of the first vaccines by growing the virus in the allantoic cavity of embryonated hen's eggs in the 1940s, relatively little has happened in the field: development of the first serological assays, definition of some serological correlates of protection, and little more. There was no real breakthrough until the application of reverse genetics and of novel adjuvant technology to the field. Somehow people thought that the influenza problem had been solved or was not a problem, very little money was available for research. Worldwide only a few laboratories continued to perform research on influenza. Vaccine manufacturers had no incentive to invest in improved influenza vaccines because the low price and the limited market did not justify investments in new technologies. When the 21st century arrived, with every single field was celebrating technological quantum jumps, and the excitements of the human genome permeating the globe, influenza was still happy to use the technologies of 1940s, complacent with the status quo.

Today, for a number of concomitant reasons, the influenza field is about to make the first important changes after more than half a century, and it is likely to take leadership in introducing technological innovations. Since the field is now moving fast and there are many newcomers, there is a risk to re-discover everything, to forget the past experience, and to repeat the mistakes already done in the past. For his reason, this book is designed to provide the background information necessary to understand the state-of-the-art of the influenza field. In the book there are chapters dealing with

the basic biology and molecular biology of the virus, chapters describing the non-recent and recent history of influenza and influenza vaccines, chapters on epidemiology, animal models, correlates of protection, the immune response to influenza infection and vaccines, adjuvants, live-attenuated vaccines, avian viruses, and pandemic vaccines. Overall the messages that come out of the chapters can be summarized as follows.

Influenza is a global underestimated killer

The World Health Organization reports that influenza infects annually 5–15% of the population, causes 3–5 million cases of severe illness, and registers up to 500,000 deaths. Reading the chapter by Simonsen et al., it is clear that, while in developed countries the epidemiology of influenza is difficult and mortality data are confounded by the many secondary diseases that are the ultimate cause of death following an influenza infection, in developing countries there are no data at all. Possibly, a lot of the child mortality recently calculated for pneumococcus (more than 2 million annually) is a consequence of a primary influenza infection. Even in a rich country such as the USA influenza causes 36,000 deaths per year. A simple calculation applying the annual death rate of the USA to the global population would predict an annual global influenza mortality of >846,000. These calculations are obviously inappropriate because the population age is different and the socio-economic conditions vary. However, they can be useful just to suggest that the mortality of influenza and the impact on the global population are underestimated, and that there is an urgent need to learn more about influenza, especially in developing countries and tropical areas. We believe that when appropriate studies are performed, we will find out that the global mortality due to influenza is in excess of 2 million.

Influenza is an important disease for children

The chapters of Edwards and Simonsen et al. both report that the majority of the influenza disease cases occur in infants, children, and the elderly. In the USA, hospitalization rates caused by influenza are 4.5 per 1000 children of less than 6 months of age, and decrease to 1 to 0.5 per 1000 with increasing age. Mortality, up to >150 cases in 2003–2004, is also present in spite of the extremely sophisticated healthcare system. However, while the elderly population, at least in the western world, is covered by vaccination programs, vaccination of infants and children has only been recently recommended in the USA and is not performed elsewhere. The first results of the USA vaccination of schoolchildren suggest that in addition to protecting children, vaccination provides a herd immunity and extends the protection to non-immunized households. The observation that vaccination of children

protects the elderly is very encouraging and suggests that policy makers should urgently introduce infant and children vaccination as a common practice. In spite of the availability of the recently introduced live-attenuated, cold-adapted vaccine (LAIV) and the conventional trivalent inactivated vaccine (TIV), there is the need for improved vaccines for children. In fact, the efficacy of TIV is not optimal, and the LAIV cannot be used in children below 2 years of age. Again, no data on influenza in infants and children are available from developing countries; however, it is easy to imagine that, in the absence of a good healthcare system, influenza can be devastating. We believe that there is an urgent need to generate data and the inclusion of influenza among the vaccine priorities of GAVI for children and pregnant women may be one of the low hanging fruits that would make a great contribution to global health.

Correlates of protection rely on serum antibodies, but live attenuated vaccines use also something else

The licensure of influenza vaccines, described in the chapters by Montomoli and Oxford et al., is based on the induction of serum antibodies. These are measured by either the capacity of sera to inhibit the binding of the virus to the receptors present of the surface of the red blood cells and cause agglutination of the cells (HI), or on the ability of the antibodies to form a complex on red blood cells coated with the influenza antigen, and cause a complement-mediated hemolysis of the cells (single-radial hemolysis or SRH). Both methods use convenient, non-sophisticated read outs, where the color of the red blood cells is a marker that can be scored by the eye. Although not sophisticated, these methods are reliable and well validated by the experience of decades, and we know that an HI titer of 40 correlates with protective efficacy in humans. However, these very old technologies start to show a lot of shortcomings. For instance, HI exhibits a very high degree of variability from one laboratory to another. The situation is even worse for avian viruses such as H5N1 virus strains. Indeed, to measure HI titers against H5N1 viruses, the red blood cells of chicken that we routinely use for conventional vaccines could not be used because the avian virus recognizes mostly avian oligosaccharide receptors containing N-acetyl neuraminic acid linked by α-2,3-galactose, which are not abundant in chicken red blood cells that contain mostly human oligosaccharide receptors linked by an α-2,6-galactose bond. Thus, to determine the HI titers against the H5N1 virus, we had to switch to horse red blood cells, which contain more α-2,3-galactose bonds. However, in doing so we moved away from well standardized, although variable, assays, and today the HI titers reported for H5N1 are even less reproducible and even more variable from laboratory to laboratory in the absence of standardized controls. Neutralization of viral infection is a new, possibly more reliable method that can be biologically

more meaningful than HI and SRH. However, our experience with this new method is limited and there is not yet a titer that we can correlate with efficacy in humans. The limit of the neutralization is often the availability of wild-type viruses that require high containment to perform the assays. In this regard, the pseudotype assays that can use high throughput methods using any viral construct without the need of any containment is a promising assay for the future.

Finally, the most important question that arises from the development and licensure of live attenuated vaccines, well described by Edwards and by Greenberg and Kemble, is that the LAIV vaccine in children is more efficacious than the TIV-inactivated vaccine, in spite of the fact that it induces much lower levels of antibodies. The question raised by this observation, which is of fundamental importance for the understanding of the mechanism of immunity to influenza and also to many other vaccines, is discussed in the chapter by Mintern et al. dealing with the immune response to influenza. The conclusion is that by mechanisms other than serum antibodies (maybe mucosal antibodies or T cells) clearly contribute to the prevention of influenza; however, we have no idea of what they are, how to measure them and how to correlate them with efficacy in humans. Understanding these mechanisms of protection will be of paramount importance to understand the vaccines of the future.

Cell culture: For the first time we have an alternative to eggs for production of influenza vaccines

In 2007, for the first time, the European authority approved a vaccine produced in mammalian cells (MDCK, of canine origin). This step is the first meaningful change since 1945 when the first vaccine produced in embryonated eggs of hens was approved. Other vaccines produced in mammalian cells, using non-human primate (Vero) or human (PerC-6) cells, are in development and are covered mostly in the chapter by Oxford et al. The availability of a cell culture production technology that can simplify the manufacturing process, provides vaccines of increased purity, and produces faster with a more reliable method, is a tremendous step forward in itself. Nevertheless, possibly the real value of this technology is the fact that this provides a platform that allows the development of the vaccines of the future. Today, for instance, most of influenza primary isolates do not easily grow in eggs and therefore cannot be used to make vaccines, so that every year the vaccine composition reflects a compromise between the medical need and what can be grown in eggs. A typical example is the season 2003–2004 when the H3N2 A/Fujan virus strain did not grow in eggs and the vaccine contained a strain that did not cover the highly pathogenic strain circulating that year. Isolating the virus directly in mammalian cells

will allow the selection of the most appropriate vaccine composition every year. In addition, it is well known that passage in eggs selects for mutations in the viral genome that may compromise some of the important epitopes necessary for protection. These changes will no longer happen when vaccine seeds are isolated directly in mammalian cells.

Reverse genetics allows navigating new horizons

The biology of the influenza virus has seen a quantum leap since the end of the century. The chapters by Mubareka and Palese and by Oxford et al. provide a comprehensive analysis of the biology of the virus and the impact of the new discoveries.

Up to very recently, the only way to study the genetics of influenza and generate new strains of viruses has been the co-infection of chicken embryo cells with two viruses and the selection of reassortants each containing the desired combination of the eight RNA fragments that constitute the influenza genome. This technology has been and still is very useful and continues to allow the generation of the seed viruses that are used every year to manufacture the vaccine. In this procedure, the desired vaccine virus is used in a co-infection with the high yield strain A/PR8/34, and reassortants containing the HA and NA segments of the vaccine virus and the other genes from the high yield PR8 virus are selected. The ability to generate new viruses by transfecting cell with plasmids containing the eight fragments of the viral genome first reported in 2000 is one of the most important milestones in the history of influenza, and has already caused a revolution in the way research is performed and vaccines are designed. Now every single gene of influenza can be manipulated at will and viruses that contain the desired RNA fragments can be generated without the co-infection and selection method used so far to generate reassortants. The first important contribution of reverse genetics has been the ability to generate seeds for the H5N1 vaccines. In fact, these reassortants could not be generated by the classical method since the H5N1 virus is pathogenic for avian cells and kills the chicken embryo cells that are usually used to generate the seed viruses for vaccine production. While reverse genetics has already been of fundamental importance by enabling the production of vaccines against H5N1 viruses, the potential of this technology is just at the beginning. Sometime, not in the too distant future, we can imagine that all seed viruses will be synthesized in the laboratory, engineered and optimized at will, and that health authorities will no longer provide seed viruses but files of sequences to be incorporated in vaccines. Another important contribution of reverse genetics has been the ability to generate viruses no longer existing, such as the one that caused the 1918 pandemic, to generate novel live-attenuated viruses containing well-defined genetic defects and to address fundamental questions about the biology of the virus.

Adjuvants will be an essential component of inactivated influenza vaccines

The chapter by Lattanzi reports that the need to improve TIV vaccines was already recognized in 1950 when an inactivated vaccine adjuvanted with mineral oil was given to 18 000 people, who were followed for safety for two decades. The long-term safety turned out to be very satisfactory, and they were able to demonstrate that the adjuvant in the long-term did not have any effect on mortality, malignant diseases or allergy. This was an important finding because at that time people were worried that adjuvants may have increased the frequency of cancer. Conversely, the short-term safety was disappointing, because up to 3% of vaccines developed a delayed local cystic reaction at the site of injection that required surgical intervention. While the vaccine adjuvanted with mineral oil was never licensed because of the local reactions, the findings of the trial, reported in Table 1 of the chapter by Lattanzi, were identical to those reported 50 years later with the new generation of adjuvants. In 1961, Hennessy and Davenport wrote that "Mineral oil adjuvant vaccine is remarkably effective for stimulating high, broad, uniform, and persistent antibody levels against prototype strains of influenza A" and can be helpful to spare the vaccine dose, "a phenomenal economy can be affected in the requirement of antigen" [1].

The need to improve influenza vaccines by adding adjuvants was perceived also in the 1960s and 1970s, and vaccines were licensed for a while using aluminum salts. However, these vaccines were eventually removed because the aluminum salts did not increase immunogenicity but increased reactogenicity. It is interesting in this context to remember that during the last 3 years a big effort has been done to adjuvant H5N1 vaccines with alum, to rediscover that this adjuvant is not useful for TIV vaccines, the same conclusion already achieved 25 years ago!

The need for improved TIV vaccines continued during the 1980s and 1990s when finally a safe, adjuvanted vaccine was licensed for human use in 1997. The chapter by O'Hagan and Podda describes the history and the properties of the new adjuvant named MF59 that represented a fundamental milestone in the history of influenza vaccines and marked the beginning of a new era. The adjuvant is an oil in water emulsion containing a biodegradable oil (squalene), which is present in every eukaryotic organism. In the emulsion the oil is surrounded by droplets of water that are stabilized around the oil by detergents such as Tween and Span. The biodegradable oil (instead of the non-biodegradable mineral oil) and the emulsion, which prevents direct contact of the oil with the biological fluids and cell membranes, solved all the safety problems observed with mineral oil in the 1950s, and allowed the licensure of safe adjuvanted influenza vaccines. During the last 10 years more than 30 million doses of the adjuvanted vaccine have been administered and now the safety on a new adjuvant is well estab-

lished. In general, the adjuvanted vaccine shows increased immunogenicity and broader immunity against non-vaccine strains, similarly to the vaccines adjuvanted with mineral oil, which were tested in the 1950s. The effects of the adjuvanted vaccines are mostly visible in children, the elderly, people with chronic diseases, and when people are naive to an influenza virus as is the case of H5N1. In the case of H5N1, vaccine development was impossible without the presence of oil in water emulsions as adjuvants. In fact, in developing vaccines against avian influenza, non-adjuvanted vaccines were found to be non-immunogenic at meaningful doses, alum-adjuvanted vaccines were found to be useless, with whole virus inactivated vaccines, although immunogenic, they need to take into account that these vaccines were abandoned in the 1960s and 1970s because of the non-acceptable systemic reactogenicity especially in children and non-immune people (see the chapter by Oxford et al.). Against H5N1, the MF59 adjuvanted vaccine was shown to induce a very high immune response at very low antigen content and to induce a broad immune response able to neutralize viruses that were not contained in the vaccine. Other oil in water emulsions containing squalene similar to MF59 have been recently tested in clinical trials with encouraging results. One of them, developed by Glaxo SmithKline and named AS03, is likely to be licensed in the near future. Another one, referred to as AF03 and developed by Sanofi-Pasteur, has been announced recently.

Clearly, improved inactivated influenza vaccines will make use of adjuvants, which we anticipate will become more sophisticated in the next decade by the introduction of Toll-like receptor agonists or other immunomodulatory agents.

Animal models and human challenge are necessary but not perfect

It is not possible to study the virulence, transmissibility, pathogenesis, immune response, and vaccine potency of influenza without the use of appropriate animal models. The chapter by Luke and Subbarao describes the different animal models available for influenza studies. These are mostly mice, ferrets, and guinea pigs. Although each of these models is important, none of them fully reproduces the human infection. Human challenge trials can be performed (see chapter by Oxford et al.), but these are not high throughput and cannot be carried out with highly virulent strains such as the H5N1 and the H1N1, which caused the 1918 pandemic. Improvement of influenza vaccines will require a smart use of each of these models, for instance mice for high-throughput studies, ferrets for protection from infection, and guinea pigs for transmissibility studies. The human challenge can be used to achieve proof of principle in man. In the future we hope that the animal models will improve and we will have mice that can better sustain the influenza infection. This will probably require understanding the spe-

cies-specific innate immunity, which prevents mice from being infected by human viruses, and the development of transgenic mice susceptible to human viruses.

References

1 Hennessy AV, Davenport FM (1961) Relative merits of aqueous and adjuvant influenza vaccines when used in a two-dose schedule. *Public Health Rep* 76: 411–419

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Influenza virus: The biology of a changing virus

Samira Mubareka and Peter Palese

Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, USA

Abstract

Recent discoveries in the field of influenza virology are the focus of this chapter. Influenza viruses are members of the family *Orthomyxoviridae* and include influenza virus types A, B, and C. This introduction provides a detailed overview of influenza virus classification, structure, and life cycle. We also include a brief review of the clinical manifestations of influenza and the molecular determinants for virulence. The genetic diversity of influenza A viruses and their capability to successfully infect an array of hosts, including avian and mammalian species, is highlighted in a discussion about host range and evolution. The importance of viral receptor-binding hemagglutinins and host sialic acid distribution in species-restricted binding of viruses is underscored. Finally, recent advances in our understanding of the seasonality and transmission of influenza viruses are described and their importance for the control of the spread of these viruses discussed.

Introduction

Influenza has had significant historical impact and continues to pose a considerable threat to public health. Our understanding of the biology of influenza virus and its effect on the host has advanced considerably in the last 10 years. Recent events in influenza virus research have contributed to this progress [1]. These include the development of plasmid-based reverse genetics systems [2, 3], the generation of the 1918 pandemic H1N1 influenza virus [4], improved access to biosafety level 3 facilities, and the establishment of international influenza virus sequence databases [5, 6]. Advances have also led to the production of FDA-approved antivirals for influenza and novel vaccine approaches such as the use of live attenuated vaccines. Continued acceleration of influenza virus research has direct implications for the development of improved vaccines and influenza control during pandemic and interpandemic periods.

Overview and classification

Influenza viruses are members of the family *Orthomyxoviridae* and include influenza virus types A, B, and C. Influenza viruses possess seven (influenza C) or eight (influenza A and B) genome segments composed of negative-sense single-stranded RNA. These types differ in various aspects, the most important of which include antigenicity, host range, pathogenicity, transmission, and seasonality. Standard nomenclatures for human influenza viruses include type, geographic location of isolation, isolate number, and year of isolation. For example, an influenza A virus isolated in Panama in 1999 would be referred to as A/Panama/2002/1999. Subtypes of influenza A viruses are described by hemagglutinin (HA) and neuraminidase (NA) designations. To date, 16 HA and 9 NA subtypes have been described.

Influenza A viruses are mostly responsible for seasonal epidemics and global pandemics, and sustain the burden of disease attributable to influenza. Clinical disease is apparent and includes primarily respiratory manifestations, and may be complicated by central nervous system involvement and rarely toxic shock and multi-organ system failure [7, 8]. Circulating strains of influenza A viruses are targets for annual vaccination to mitigate morbidity and mortality imparted by these viruses. In addition to infecting humans, influenza A viruses circulate in other mammals, including swine and horses. Waterfowl harbor several lineages of influenza A viruses, and serve as a reservoir. Transmission amongst wild and domestic fowl and mammalian species is an important characteristic of influenza A, enabling viral reassortment and emergence of novel subtypes in susceptible human populations.

In contrast, influenza B virus has a restricted host range, circulating only in humans, although the virus has been isolated in seals [9]. Influenza B virus demonstrates seasonality and is responsible for human disease, although the clinical manifestations are generally less severe compared to influenza A virus-associated illness. Nonetheless, rare cases of encephalitis and septic shock have been described in children [10, 11]. At present, the two major lineages are represented by influenza B/Victoria/2/1987 and B/Yamagata/16/1988 viruses [12]. Re-emergence of the Victoria lineage after a decade of absence was associated with an outbreak during the 2001–2002 influenza season, affecting healthy immunologically naive children [13]. Influenza B virus is included in inactivated and live attenuated annual influenza vaccines.

Unlike influenza A and B viruses, influenza C viruses lack the NA gene and code for a single surface hemagglutinin-esterase-fusion (HEF) glycoprotein. This virus does not demonstrate marked seasonality and is not included in the annual influenza vaccine, although it has been responsible for occasional outbreaks, predominantly in children [14]. Illness in humans is generally mild and consists of an upper respiratory tract infection. Influenza

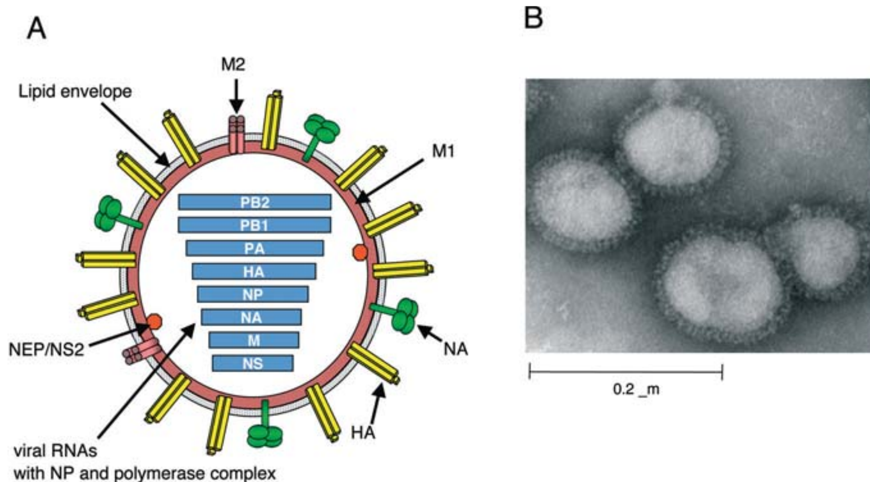

Figure 1. Schematic structure and electron micrograph of influenza virus A.
(A) The viral envelop anchors the hemagglutinin (HA) and neuraminidase (NA) glycoproteins and matrix (M) 2 protein and is derived from the host cell during the process of budding. M1 lies beneath the viral envelope. Nuclear export and nonstructural protein (NEP/NS1) and the core of the virion are contained within. The core consists of eight segments of viral RNA associated with the polymerase complex (PB2, PB1, and PA) and nucleoprotein (NP). Adapted from [1] and kindly provided by M. L. Shaw. (B) Negatively stained electron micrograph of mouse-adapted influenza A WSN/33. Glycoprotein spikes are visible on the surface of the virion. Kindly provided by M. L. Shaw.

C has also been isolated in swine, raising the possibility that this species may serve as a reservoir [15].

Structure and genomic organization

Influenza viruses are enveloped, deriving the lipid bilayer from the host cell membrane during the process of budding. Viral particles are pleomorphic in nature and may be spherical or filamentous, ranging in size from 100 nm to over 300 nm [1]. Spikes consisting of HA and NA project from the surface of virions at a ratio of roughly 4:1 in influenza A viruses (Fig. 1) [1]. The viral envelope is also associated with the matrix (M2) protein, which forms a tetrameric ion channel.

The polymerase proteins PB1, PB2, and PA, the nucleoprotein (NP), and the virion RNA comprise the ribonucleoprotein (RNP) complex. This complex is present in the core of virions, which also includes the nuclear

Table 1. Influenza A genes and primary functions of their encoded proteins

Genome segment[1]	Length in nucleotides	Encoded proteins	No. of amino acids	Function
1	2341	PB2	759	Polymerase subunit, mRNA cap recognition
2	2341	PB1	757	Polymerase subunit, endonuclease activity, RNA elongation
		PB1-F2[2]	87	Pro-apoptotic activity
3	2233	PA	716	Polymerase subunit, protease activity, assembly of polymerase complex
4	1778	HA	550	Surface glycoprotein, receptor binding, fusion activity, major viral antigen
5	1565	NP	498	RNA binding activity, required for replication, regulates RNA nuclear import
6	1413	NA	454	Surface glycoprotein with neuraminidase activity, virus release
7	1027	M1	252	Matrix protein, interacts with viral RNPs and glycoproteins, regulates RNA nuclear export, viral budding
		M2[3]	97	Integral membrane protein, ion channel activity, uncoating, virus assembly
8	890	NS1	230	Interferon antagonist activity, regulates host gene expression
		NEP/NS2[3]	121	Nuclear export of RNA

[1]Influenza A/Puerto Rico/8/1934
[2]Encoded by an alternate open reading frame
[3]Translated from an alternatively spliced transcript

export and nonstructural proteins (NEP/NS1). Influenza virus genes, gene products, and primary functions are summarized in Table 1.

Influenza virus life cycle

Attachment, entry, and nuclear import

In humans, influenza viruses are transmitted by the respiratory route. Host cellular receptors consist of oligosaccharides residing on the surface of respiratory epithelial cells. Specificity of binding is imparted by the linkage of the penultimate galactose (Gal) to N-acetylsialic acid (SA). α2,6 linkage (SAα2,6Gal) is distributed in the human respiratory tract and is associated with binding to human influenza virus HA. In contrast, waterfowl and domestic poultry harbor sialic acids with α2,3 linkage (SAα2,3Gal), which is distributed in the gastrointestinal tract, reflecting the fecal-oral mode of

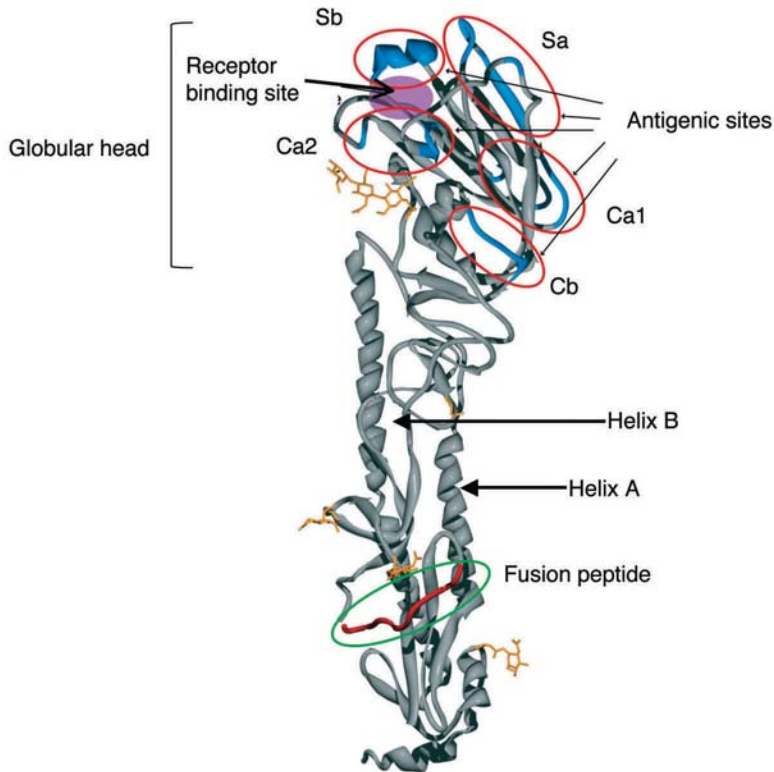

Figure 2. Ribbon structure of the 1918 influenza virus hemagglutinin
The sialic acid receptor binding site and the five antigenic sites are located on the globular head. This structure also possesses a cleavage site where HA is cleaved into HA1 and HA2 for fusion of viral and endosomal membranes and subsequent uncoating. Adapted from [1] and kindly provided by J. Stevens and I. Wilson.

transmission of avian influenza strains in these species [16]. Specificity of viral HA binding is imparted by the receptor binding pocket on the surface of the HA molecule (Fig. 2). The HA is a rod-shaped trimer anchored in the virion's envelope and contains three primary ligand-binding sites on a globular head [17], [18]. Specificity of binding has been linked to certain amino acid residues in the HA receptor-binding domain. In H3 subtypes, amino acid 226 is one such residue, where the presence of leucine allows binding of SAα2,6Gal, whereas the presence of glutamine at this position permits binding of SAα2,3Gal. Amino acid changes in the HA of other subtypes, such as H1 viruses (including the H1N1 virus responsible for the 1918 pandemic), have been associated with adaptations in receptor binding specificity, translating into a switch in host specificity with disastrous consequences [19, 20].

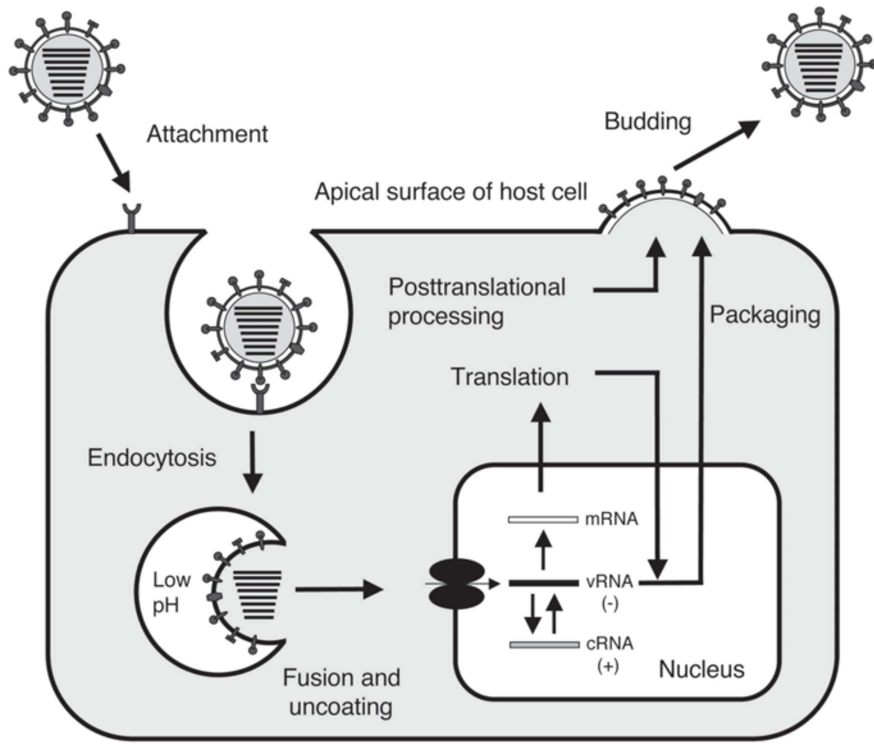

Figure 3. Influenza virus replication cycle
The virus is endocytosed after initial binding of the HA to host cell sialic acid receptors. Acidification of the cleaved HA facilitates approximation of viral and endosomal membranes and release of ribonucleoprotein (RNP). Transcription follows importation of RNPs into the nucleus. Assembly occurs at the apical surface of the host cell where budding and release occur. Adapted from [1] and kindly provided by M. L. Shaw. See text for detail.

Several possible pathways for the entry of influenza viruses into host cells have been postulated and recently reviewed [21]. Endocytosis is a multi-step process consisting of surface receptor-mediated binding, internalization, and intracellular trafficking. Clathrin-mediated and clathrin-independent internalization *via* caveolae, as well as caveolae-independent endocytosis have been demonstrated [22, 23]. An initial acidification step in early endosomes is followed by trafficking to low-pH late endosomes, a process mediated by members of the Rab host protein family. Fusion of influenza virus to the endosome is triggered by low pH conditions and mediated by the fusion peptide of HA2 after cleavage of HA, creating a pore in the endosome through fusion of viral and endosomal membranes (Fig. 3) [1].

Subsequent steps in the uncoating process involve the influenza virus tetrameric M2 protein, which is involved in the release of RNP into the host

cell cytoplasm through ion channel activity [24, 25]. Viral RNA (vRNA) synthesis occurs in the nucleus, and viral RNPs must therefore be imported. This process is primarily mediated by viral NP, which coats viral RNA and possesses nuclear localization signals (NLS), including an unconventional NLS which binds host karyopherin-alpha and is essential for energy-dependent RNP nuclear import [26, 27].

Transcription, replication, and nuclear export

Viral RNA serves as a template for the production of messenger RNA (mRNA) and subsequent transcription, as well as for the generation of complementary RNA (cRNA), which is positive sense and functions as a template for the generation of more vRNA (viral replication). RNA segments are coated by NP through nonspecific interactions between the arginine-rich positively charged NP and the negatively charged RNA phosphate backbone [1]. The viral polymerase complex consists of tightly associated PB1, PB2, and PA and associates with NP-coated RNA without disrupting this interaction [28]. PB1 is an endonuclease involved in both replication and transcription, and binds the promoter region of RNA segments [29]. It functions as an RNA-dependent RNA polymerase and catalyzes RNA chain elongation. Interaction with PA is required for this function and viral replication [30]. PB2 binds both NP and PB1 *via* separate binding sites [31]. Initiation of transcription is reliant on PB2, which binds the cap on host pre-mRNA, and this cap serves as a primer for transcription [32, 33]. In addition, interactions between PB2 and host proteins may also be species specific, and may play a role in restricting host range [34]. PA is a component of the polymerase heterotrimer, is co-transported into the nucleus with PB1, and is thus important in the formation of this complex [35, 36].

Synthesis of mRNA begins with a host cell 5' capped primer, generated by host cell RNA polymerase II and obtained from host pre-mRNA [33]. Transcription is thus initiated and synthesis on the template occurs in a 3' to 5' direction. A polyadenylation signal consisting of five to seven uridines at the 5' end of vRNA prematurely terminates transcription after inducing stuttering of the viral polymerase [37–39]. The generation of NP and NS1 tends to occur earlier after infection compared to the generation of surface glycoprotein and M1 mRNAs [1]. Mechanisms for the regulation of gene expression remain evasive, although NP has been implicated in the control of gene expression [40].

Viral replication requires the synthesis of vRNA, which is primer-independent and occurs through a cRNA intermediate. Nascent cRNA is therefore not capped or polyadenylated upon termination. The notion that cRNA synthesis is initiated after a switch from mRNA synthesis has recently been challenged [41].

RNP complexes subsequently associate with M1 at its C-terminal domain, and aggregation of this complex leads to inhibition of transcription [42]. M1 also interacts with NEP at its C-terminal domain [27, 43]. NEP, in turn, associates with host nuclear export receptor Crm1 *via* the NEP N-terminal domain [43], thus orchestrating the export of viral RNP from the nucleus.

Viral assembly, budding, and release

Post-translational modification of the HA consists of glycosylation in the Golgi apparatus [44]. Along with viral RNP, protein components of the virion are coordinately trafficked to the apical surface of the host cell for assembly into progeny virus.

Two models for the packaging of viral RNA segments exist, and include the “random incorporation” [45, 46] and the ‚selective incorporation’ models [47, 48]. The latter implies that each RNA segment possesses a packaging signal, resulting in virions with exactly eight segments. Putative packaging signals in coding regions of polymerase genes, spike glycoprotein genes, and the NS gene have been identified [47, 49–52].

Viral assembly is coordinated by the M1 protein, which associates with the cytoplasmic tails of the viral glycoproteins [53, 54], as well as RNP and NEP, as described above. Lipid rafts navigate viral membrane glycoproteins to the apical surface of the host cell [55, 56]. In addition, there is evidence that targeting of NP and polymerase proteins to the apical surface also involves lipid rafts [57].

Genomic packaging and viral assembly occurs at the apical membrane, and is associated with accumulation of M1 and the formation of lipid rafts. The M1 protein has also been implicated in viral morphology [58, 59]. Because the HA binds cell surface sialic acid receptors, virions must be released. The NA functions as a sialidase and cleaves sialic acids from the host cell and viral glycoproteins to minimize viral aggregation at the cell surface [60]. Balance between the HA and NA is thus required for optimal receptor binding and destruction [53, 61]. In addition to its receptor-destroying activity, NA is a viral spike glycoprotein and important surface antigen [62].

Evolution

Amongst the influenza virus types, influenza A demonstrates the most genetic diversity and is capable of successfully infecting an array of hosts, including avian and mammalian species. Influenza A viruses exhibit an evolutionary pattern that is complex, and consists of antigenic drift and shift. Drift occurs on an annual basis and has been attributed to low fidelity

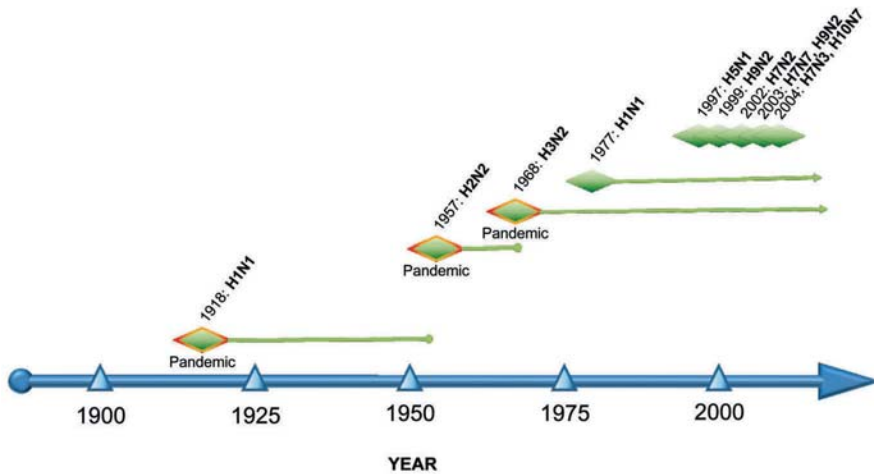

Figure 4. Influenza A virus subtypes in humans
Three pandemics occurred during the 20th century, including the "Spanish" influenza pandemic of 1918, the "Asian" pandemic in 1958, and the "Hong Kong" pandemic in 1968. H1N1 viruses re-emerged in 1977 and continue to circulate in the human population, along with the H3N2 subtype. In addition, H5N1 viruses have been reported to infect humans throughout Asia and Africa. Several other avian viruses have also recently caused sporadic infection in humans. Adapted from [68].

of the RNA polymerase and subsequent selection from immune pressure exerted by the host [63]. This results in antigenic diversity of the HA and NA glycoproteins, and is one of the major challenges to vaccine production, requiring annual changes to vaccine components. The HA1 domain contains several epitopes, and is the most dynamic as a consequence, demonstrating clusters of antigenic variance over time [64]. Antigenic shift results after a viral reassortment event where exchange of one or more of the viral segments with that of another strain may result in a novel serotype, potentially diversifying the host range of the virus. It is in this setting that pandemic strains have emerged in immunologically naive populations in the past, including the H2N2 (with new HA, NA, and PB1 segment) subtype in 1957 and the H3N2 influenza virus (with new HA and PB1 segments), which caused a pandemic in 1968 (Fig. 4). To tackle the challenge of understanding the evolution of influenza virus, large-scale collaborative efforts such as the Influenza Genome Sequencing Project have been undertaken. The presence of several co-circulating clades in the human population has been described, accounting for reassortment. This can result in limited vaccine effectiveness, as seen with A/Fujian/411/2002-like virus during the 2003–2004 season [6]. Genetic evolution appears to be a relatively gradual process; however, antigenic changes in the HA1 domain tend to cluster [64]. Ongoing changes of

the H3 hemagglutinin in the human population result from selective pressure exerted by the host immune system. In contrast, the H3 lineage in birds has remained relatively stable [65]. The rate of change of the H3 subtype is greater when compared to H1 viruses and influenza B, with estimated nucleotide changes per site per year of 0.0037 for H3, 0.0018 for H1, and 0.0013 for influenza B [66]. As greater numbers of influenza virus genome sequences become available and we gain insight into antigenic patterns of change, this knowledge may be applied to annual vaccine development. Prediction of future influenza sequences could lead to the more timely development of effective vaccines [67], although modeling methods have yet to be validated.

Recently, several avian influenza viruses, including H5N1, H7N2, H7N3, H7N7, H9N2, and H10N7 subtypes have infected humans [68], although limited evidence for person-to-person spread exists [69]. Lack of transmission amongst humans remains a barrier to pandemic spread of these viruses. The H5N1 subtype isolated from avian species has undergone genetic reassortment, and several genotypes exist. Genotypes Z and V are largely responsible for outbreaks of highly pathogenic influenza viruses (HPAI) in domestic birds in Southeast Asia beginning in 2003 [69]. H5N1 viruses may also be divided into clades 1 and 2 based upon genomic analysis of the HA genes, and clade 2 is further divided into subclades [70]. Less than 1% divergence from avian isolates has been reported in viruses isolated from humans in Asia [5].

Host range

Influenza A virus is a zoonotic pathogen capable of infecting birds (waterfowl and chickens), swine, horses, felines, and other species. Host range restriction of different types of influenza viruses is observed. Species-restricted binding of viruses is mediated by different types of receptor-binding hemagglutinins [71–75]. The distribution of different types of SA linkages has recently been elucidated in humans, although the type of cell infected (ciliated vs non-ciliated) is currently under debate [76, 77]. SA with α2,6Gal linkage predominates on epithelial cells of the upper airway, including nasal mucosa, sinuses, bronchi, and bronchioles [78]. In human tracheobronchial epithelial (HTBE) cells, oligosaccharides with SA with α2,6Gal linkage predominated on nonciliated epithelial cells [77], although these oligosaccharides have been described on ciliated and goblet cells in the human airway [78]. Lower airways also contain SA with mostly α2,3Gal linkage, in addition to SA with α2,6Gal linkage [79, 80].

Host restriction is not absolute, and human infections with avian influenza viruses (including H5N1, H9N2, and H7N7 viruses) have been

extensively described [81–86]. H5N1 binds type II pneumocytes and macrophages of the lower respiratory tract in humans [78, 80, 87]. H5N1 infection of ciliated cells in HTBE cell culture with limited cell to cell spread [88] and of human nasopharyngeal, adenoid, and tonsillar *ex vivo* cell cultures have been shown [89]. Binding of H5N1 viruses to saccharides terminating in α2,6Gal SA linkage has been achieved by mutating HA amino acids residues at positions 182 and 192, suggesting potential for adaptation to the human host [90].

Differences in influenza virus receptors amongst avian species have been described, and is reflected in binding of different types of avian influenza viruses. Although chicken and duck influenza viruses preferentially bind α2,3Gal-linked SA, viruses from chickens had greater affinity for SA where the third sugar moiety was a β(1-4)GlcNAc-containing synthetic sialylglycopolymer. Duck viruses preferred β(1-3)GalNAc sugar moieties in the third position [91]. Distribution of influenza virus receptors reflects the sites of replication. In chickens and waterfowl, SA with α2,3Gal linkage is found in the upper respiratory tract and intestines. Some species demonstrate the ability to support replication of both human and avian influenza viruses. The respiratory tract and intestines of quail contain both α2,3Gal and α2,6Gal-linked terminal sialic acids [92]. In swine, oligosaccharides with both types of linkages may be found, and suggest this species serves as a mixing vessel where human, avian, and swine influenza viruses can reassort [93, 94]. However, no *in vivo* evidence has been provided that pandemic strains have emerged from this source.

Clinical manifestations, pathogenesis, and virulence

Clinical manifestations

Uncomplicated influenza in humans is an upper respiratory tract infection characterized by cough, headache, malaise, and fever (influenza-like illness). These symptoms are nonspecific and are not predictive of influenza virus infection, particularly in individuals <60 years old [95]. Pulmonary and extra-pulmonary complications may arise. The latter consist of central nervous system involvement (encephalitis, acute necrotizing encephalopathy, Reye's syndrome, and myelitis) [7], myositis/rhabdomyositis [96], myocarditis [96, 97], increased cardiovascular events [98], disseminated intravascular coagulation [96], and toxic and septic shock (bacterial and non-bacterial) [8, 96, 99]. Pulmonary complications include primary viral pneumonia, secondary bacterial pneumonia, and exacerbation of chronic lung disease [96, 100]. Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), multi-organ failure, profound lymphopenia, and hemophagocytosis have been associated with H5N1 infection and carry high mortality rates [8, 81, 101–103].

Pathogenesis

Few human histopathological studies of uncomplicated influenza exist. Pathological findings from post-mortem examination of 47 fatal pediatric influenza A cases included major airway congestion (90%), inflammation (73%) and necrosis (50%) [100]. Lower airway pathology included hyaline membranes (67%), interstitial cellular infiltrates (67%) and diffuse alveolar damage (DAD). Secondary pneumonia, intra-alveolar hemorrhage, and viral pneumonitis were noted in a quarter of cases [100]. Fulminant DAD with acute alveolar hemorrhage and necrosis followed by paucicellular fibrosis and hyaline membrane formation is observed in H5N1-infected human lungs [104]. Extra-pulmonary pathology includes reactive hemophagocytosis in the hilar lymph nodes, bone marrow, liver and spleen [105]; white matter demyelination [104] and cerebral necrosis [87]; and acute tubular necrosis of the kidneys [101]. Despite the presence of diarrhea and H5N1 virus replication in the gastrointestinal tract of humans, no pathological lesions have been described in the bowel [87, 102]. Immune dysregulation has been implicated in the pathogenesis of ARDS and reactive hemophagocytosis. Elevated levels of neutrophil, monocyte and macrophage chemoattractants (IL-8, IP-10, MIG, and MCP-1) and pro-inflammatory cytokines (IL-10, IL-6 and IFN-γ) are observed in H5N1-infected humans [81]. In addition, increased levels of IL-2 (in a human case) [101] and RANTES (in primary human alveolar and bronchial epithelial cells) [106] have also been reported. Contribution of pro-inflammatory mediators to lung pathology has also been demonstrated using a Toll-like receptor 3-knockout mouse infected with mouse-adapted WSN influenza A virus. These mice demonstrated enhanced survival, despite higher virus replication and lower levels of RANTES, IL-6, and IL-12p40/p70 compared to wild-type mice [107].

Virological determinants of virulence

The HA, PA, PB1, PB2, PB1-F2, NA and NS1 gene products have been implicated in virulence. Virulence determinants have been explored using the reverse genetic system for influenza viruses and mammalian (ferret and mouse) models for influenza virus pathogenicity.

The polymerase gene complex, consisting of PA, PB1, and PB2 genes are involved in replication and transcriptional activity. A single gene reassortant containing the PB2 from A/Hong Kong/483/97 (H5N1, which is fatal in mice) in a background of A/Hong Kong/486/97 (H5N1, causing mild respiratory infection in mice) demonstrated a lethal phenotype in this animal model [108]. In addition, reassortants containing polymerase complex genes

from A/chicken/Vietnam/C58/04 (H5N1), a non-lethal virus, in the background of A/Vietnam/1203/04, (H5N1) influenza virus isolated from a fatal human case were attenuated in an animal model [109]. When a single point mutation K627E in the PB2 gene was generated in A/Vietnam/1203/04 [109] and in A/Hong Kong/483/97 [108], virulence was reduced in mice, although in other studies this substitution did not reduce virulence substantially [110]. The molecular mechanism(s) responsible for virulence have yet to be elucidated.

PB1-F2 is the gene product arising from a second reading frame of the PB1 gene and has been implicated in immune cell apoptosis through the VDAC1 and ANT3 mitochondrial pathways [111]. Knockout of PB1-F2 did not alter viral replication, but enhanced clearance of the virus and reduced lethality in mice was demonstrated, suggesting that PB1-F2 may play a role in viral pathogenesis [112]. Enhanced pathogenicity was observed in mice infected with recombinant influenza virus containing the PB1-F2 gene from a highly pathogenic H5N1 virus isolated from a fatal human case in Hong Kong in 1997 [112].

Evasion of the host immune response is a key virulence determinant, permitting viruses to establish sustainable infection. The innate immune system is the first line of host defense and the influenza virus possesses the ability to interfere with this response. Type I IFNs (IFN-α/β) are central to establishing an antiviral state in host cells. IFN antagonism has been primarily attributed to the NS1 protein of influenza virus, which sequesters double-stranded RNA, thus preventing the activation of IFN transcription factors (for review, see [113]).

The effect of avian influenza virus NS1 on IFN production has also been explored. A/goose/Guangdong/1/96, which differs by one amino acid from A/goose/Guangdong/2/96 at position 149, is lethal in chickens and antagonizes IFN-α/β [114]. In addition, the C terminus of the NS1 protein contains a PDZ ligand domain, capable of binding PDZ protein interaction domains of host proteins, thus potentially disrupting host cellular pathways. Viruses causing pathogenic infection in humans from 1997 and 2003 contained avian motifs at the NS1 PDZ ligand-binding site. These and the motif found in the 1918 influenza virus NS1 had stronger binding affinities to PDZ domains of human cellular proteins compared to low pathogenicity influenza viruses [115].

Neurovirulence has been associated with glycosylation of the NA glycoprotein [116]. The HA glycoprotein has also been implicated in virulence. Although cleavability of the HA protein has been primarily implicated in pathogenicity in chickens, lethality has also been demonstrated in mice. Replacement of the multibasic cleavage site in a high pathogenicity H5N1 virus from Hong Kong (HK483) with an amino acid sequence typical of low pathogenicity viruses resulted in attenuation [108].

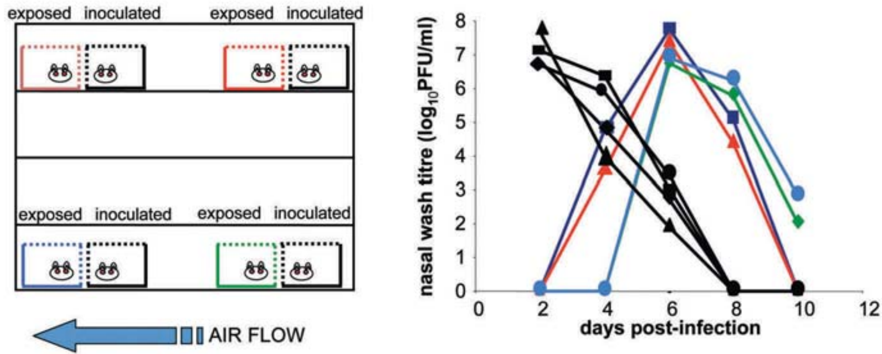

Figure 5. Close range transmission of human influenza A amongst guinea pigs
Inoculated animals placed in proximity to uninoculated animals (without direct contact) spread Pan99 to all exposed animals. Adapted from [110].

Seasonality and transmission

Influenza A and B viruses exhibit marked seasonality and this pattern dictates the annual vaccination schedule. Several theories with respect to the mechanism(s) dictating this seasonal pattern have been proposed (for review, see [117]). Year-round human influenza virus activity in equatorial regions may be a reservoir for annual outbreaks in the northern and southern hemispheres. As research progresses in this area, factors determining seasonality may be exploited for the control of the spread of influenza virus [118].

Transmission of influenza virus amongst humans is poorly understood and the mode(s) of spread are currently under debate [119, 120]. It is widely accepted that influenza virus is transmitted by the respiratory route in humans, although the contribution of small particle aerosols relative to large respiratory droplets is unknown. In addition, the role of fomites is questionable. Until recently, ferrets have served as the only animal model for the study of influenza virus transmission. A novel mammalian model using the guinea pig has recently been developed to overcome the limitations imposed by the ferret model. Guinea pigs are highly susceptible to infection with an unadapted human H3N3 (A/Panama/2002/1999, or Pan99) influenza virus, with a 50% infectious dose of 5 PFU, and this virus grows to high titers in the upper respiratory tract and to moderate titers in the lungs. Transmission of Pan99 by direct contact and aerosol in this system is 100% (Fig. 5) [121]. Control of influenza virus spread during interpandemic and pandemic periods through physical means will be paramount to abrogating person-to-person transmission and is particularly crucial where viruses are resistant to currently available antivirals.

Perspectives

Effective and timely vaccine development depends on in-depth understanding of influenza virus biology. Although recent advances have been made, ongoing research will be required to fulfill this goal. Identification and characterization of the molecular signatures required for transmission will be of utmost importance to preventing further influenza virus pandemics. Globalization of H5N1 infection in humans requires parallel efforts on behalf of virologists in conjunction with epidemiologists and other members of the public health community to translate the growing body of knowledge into means by which influenza spread can be controlled.

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References

1 Shaw ML, Palese P (2007) Orthomyxoviridae: The viruses and their replication. In: DM Knipe, PM Howley (eds): *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelpha, 1647–1689

2 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A (1999) Rescue of influenza A virus from recombinant DNA. *J Virol* 73: 9679–9682

3 Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E et al (1999) Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci USA* 96: 9345–9350

4 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A (2005) Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310: 77–80

5 World Health Organization Global Influenza Program Surveillance Network (2005) Evolution of H5N1 avian influenza viruses in Asia. *Emerg Infect Dis* 11: 1515–1521

6 Ghedin E, Sengamalay NA, Shumway M, Zaborsky J, Feldblyum T, Subbu V, Spiro DJ, Sitz J, Koo H, Bolotov P et al (2005) Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* 437: 1162–1166

7 Studahl M (2003) Influenza virus and CNS manifestations. *J Clin Virol* 28: 225–232
8 Sion ML, Hatzitolios AI, Toulis EN, Mikoudi KD, Ziakas GN (2001) Toxic shock syndrome complicating influenza A infection: A two-case report with one case of bacteremia and endocarditis. *Intensive Care Med* 27: 443
9 Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA (2000) Influenza B virus in seals. *Science* 288: 1051–1053
10 Newland JG, Romero JR, Varman M, Drake C, Holst A, Safranek T, Subbarao K (2003) Encephalitis associated with influenza B virus infection in 2 children and a review of the literature. *Clin Infect Dis* 36: e87–95
11 Jaimovich DG, Kumar A, Shabino CL, Formoli R (1992) Influenza B virus infection associated with non-bacterial septic shock-like illness. *J Infect* 25: 311–315
12 Chen JM, Guo YJ, Wu KY, Guo JF, Wang M, Dong J, Zhang Y, Li Z, Shu YL (2007) Exploration of the emergence of the Victoria lineage of influenza B virus. *Arch Virol* 152: 415–422
13 Hite LK, Glezen WP, Demmler GJ, Munoz FM (2007) Medically attended pediatric influenza during the resurgence of the Victoria lineage of influenza B virus. *Int J Infect Dis* 11: 40–47
14 Matsuzaki Y, Abiko C, Mizuta K, Sugawara K, Takashita E, Muraki Y, Suzuki H, Mikawa M, Shimada S, Sato K et al (2007) A nationwide epidemic of influenza C virus infection in Japan in 2004. *J Clin Microbiol* 45: 783–788
15 Yuanji G, Desselberger U (1984) Genome analysis of influenza C viruses isolated in 1981/82 from pigs in China. *J Gen Virol* 65: 1857–1872
16 Wright PF, Neumann G, Kawaoka Y (2007) Orthomyxoviruses. In: DM Knipe, PM Howley (eds): *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, 1714–1715
17 Eisen MB, Sabesan S, Skehel JJ, Wiley DC (1997) Binding of the influenza A virus to cell-surface receptors: Structures of five hemagglutinin-sialyloligosaccharide complexes determined by X-ray crystallography. *Virology* 232: 19–31
18 Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. *Annu Rev Biochem* 69: 531–569
19 Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, Tumpey TM, Basler CF, Taubenberger JK, Palese P (2005) A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. *J Virol* 79: 11533–11536
20 Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA (2004) Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* 303: 1866–1870
21 Sieczkarski SB, Whittaker GR (2005) Viral entry. *Curr Top Microbiol Immunol* 285: 1–23
22 Matlin KS, Reggio H, Helenius A, Simons K (1981) Infectious entry pathway of influenza virus in a canine kidney cell line. *J Cell Biol* 91: 601–613
23 Nunes-Correia I, Eulalio A, Nir S, Pedroso de Lima MC (2004) Caveolae as an additional route for influenza virus endocytosis in MDCK cells. *Cell Mol Biol Lett* 9: 47–60
24 Takeda M, Pekosz A, Shuck K, Pinto LH, Lamb RA (2002) Influenza A virus

M2 ion channel activity is essential for efficient replication in tissue culture. *J Virol* 76: 1391–1399
25 Pinto LH, Holsinger LJ, Lamb RA (1992) Influenza virus M2 protein has ion channel activity. *Cell* 69: 517–528
26 Wang P, Palese P, O'Neill RE (1997) The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza A virus nucleoprotein NP is a nonconventional nuclear localization signal. *J Virol* 71: 1850–1856
27 Cros JF, Garcia-Sastre A, Palese P (2005) An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein. *Traffic* 6: 205–213
28 Area E, Martin-Benito J, Gastaminza P, Torreira E, Valpuesta JM, Carrascosa JL, Ortin J (2004) 3D structure of the influenza virus polymerase complex: Localization of subunit domains. *Proc Natl Acad Sci USA* 101: 308–313
29 Jung TE, Brownlee GG (2006) A new promoter-binding site in the PB1 subunit of the influenza A virus polymerase. *J Gen Virol* 87: 679–688
30 Perez DR, Donis RO (2001) Functional analysis of PA binding by influenza A virus PB1: Effects on polymerase activity and viral infectivity. *J Virol* 75: 8127–8136
31 Poole E, Elton D, Medcalf L, Digard P (2004) Functional domains of the influenza A virus PB2 protein: Identification of NP- and PB1-binding sites. *Virology* 321: 120–133
32 Fechter P, Mingay L, Sharps J, Chambers A, Fodor E, Brownlee GG (2003) Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. *J Biol Chem* 278: 20381–20388
33 Krug RM, Bouloy M, Plotch SJ (1980) RNA primers and the role of host nuclear RNA polymerase II in influenza viral RNA transcription. *Philos Trans R Soc Lond B Biol Sci* 288: 359–370
34 Labadie K, Dos Santos Afonso E, Rameix-Welti MA, van der Werf S, Naffakh N (2007) Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. *Virology* 362: 271–282
35 Kawaguchi A, Naito T, Nagata K (2005) Involvement of influenza virus PA subunit in assembly of functional RNA polymerase complexes. *J Virol* 79: 732–744
36 Fodor E, Smith M (2004) The PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. *J Virol* 78: 9144–9153
37 Li X, Palese P (1994) Characterization of the polyadenylation signal of influenza virus RNA. *J Virol* 68: 1245–1249
38 Luo GX, Luytjes W, Enami M, Palese P (1991) The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. *J Virol* 65: 2861–2867
39 Zheng H, Lee HA, Palese P, Garcia-Sastre A (1999) Influenza A virus RNA polymerase has the ability to stutter at the polyadenylation site of a viral RNA template during RNA replication. *J Virol* 73: 5240–5243
40 Ye Q, Krug RM, Tao YJ (2006) The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. *Nature* 444: 1078–1082

41 Vreede FT, Brownlee GG (2007) Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA *in vitro*. *J Virol* 81: 2196–2204
42 Baudin F, Petit I, Weissenhorn W, Ruigrok RW (2001) *In vitro* dissection of the membrane and RNP binding activities of influenza virus M1 protein. *Virology* 281: 102–108
43 Akarsu H, Burmeister WP, Petosa C, Petit I, Muller CW, Ruigrok RW, Baudin F (2003) Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). *EMBO J* 22: 4646–4655
44 Gallagher PJ, Henneberry JM, Sambrook JF, Gething MJ (1992) Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. *J Virol* 66: 7136–7145
45 Enami M, Sharma G, Benham C, Palese P (1991) An influenza virus containing nine different RNA segments. *Virology* 185: 291–298
46 Bancroft CT, Parslow TG (2002) Evidence for segment-nonspecific packaging of the influenza A virus genome. *J Virol* 76: 7133–7139
47 Watanabe T, Watanabe S, Noda T, Fujii Y, Kawaoka Y (2003) Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes. *J Virol* 77: 10575–10583
48 de Wit E, Spronken MI, Rimmelzwaan GF, Osterhaus AD, Fouchier RA (2006) Evidence for specific packaging of the influenza A virus genome from conditionally defective virus particles lacking a polymerase gene. *Vaccine* 24: 6647–6650
49 Liang Y, Hong Y, Parslow TG (2005) cis-Acting packaging signals in the influenza virus PB1, PB2, and PA genomic RNA segments. *J Virol* 79: 10348–10355
50 Fujii K, Fujii Y, Noda T, Muramoto Y, Watanabe T, Takada A, Goto H, Horimoto T, Kawaoka Y (2005) Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. *J Virol* 79: 3766–3774
51 Fujii Y, Goto H, Watanabe T, Yoshida T, Kawaoka Y (2003) Selective incorporation of influenza virus RNA segments into virions. *Proc Natl Acad Sci USA* 100: 2002–2007
52 Gog JR, Afonso ED, Dalton RM, Leclercq I, Tiley L, Elton D, von Kirchbach JC, Naffakh N, Escriou N, Digard P (2007) Codon conservation in the influenza A virus genome defines RNA packaging signals. *Nucleic Acids Res* 35:1897–1907
53 Schmitt AP, Lamb RA (2005) Influenza virus assembly and budding at the viral budozone. *Adv Virus Res* 64: 383–416
54 Chen BJ, Takeda M, Lamb RA (2005) Influenza virus hemagglutinin (H3 subtype) requires palmitoylation of its cytoplasmic tail for assembly: M1 proteins of two subtypes differ in their ability to support assembly. *J Virol* 79: 13673–13684
55 Zhang J, Pekosz A, Lamb RA (2000) Influenza virus assembly and lipid raft microdomains: A role for the cytoplasmic tails of the spike glycoproteins. *J Virol* 74: 4634–4644
56 Barman S, Adhikary L, Chakrabarti AK, Bernas C, Kawaoka Y, Nayak DP (2004) Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza A virus neuraminidase in raft association and virus budding. *J Virol* 78: 5258–5269

57 Carrasco M, Amorim MJ, Digard P (2004) Lipid raft-dependent targeting of the influenza A virus nucleoprotein to the apical plasma membrane. *Traffic* 5: 979–992
58 Bourmakina SV, Garcia-Sastre A (2003) Reverse genetics studies on the filamentous morphology of influenza A virus. *J Gen Virol* 84: 517–527
59 Elleman CJ, Barclay WS (2004) The M1 matrix protein controls the filamentous phenotype of influenza A virus. *Virology* 321: 144–153
60 Palese P, Tobita K, Ueda M, Compans RW (1974) Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* 61: 397–410
61 Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, Kobasa D, Kawaoka Y (2000) Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. *J Virol* 74: 6015–6020
62 Colman PM (1994) Influenza virus neuraminidase: Structure, antibodies, and inhibitors. *Protein Sci* 3: 1687–1696
63 Fitch WM, Leiter JM, Li XQ, Palese P (1991) Positive Darwinian evolution in human influenza A viruses. *Proc Natl Acad Sci USA* 88: 4270–4274
64 Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA (2004) Mapping the antigenic and genetic evolution of influenza virus. *Science* 305: 371–376
65 Bean WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, Webster RG (1992) Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. *J Virol* 66: 1129–1138
66 Ferguson NM, Galvani AP, Bush RM (2003) Ecological and immunological determinants of influenza evolution. *Nature* 422: 428–433
67 Plotkin JB, Dushoff J, Levin SA (2002) Hemagglutinin sequence clusters and the antigenic evolution of influenza A virus. *Proc Natl Acad Sci USA* 99: 6263–6268
68 National Institute of Allergy and Infectious Diseases, NIH (2007) http://www3.niaid.nih.gov/news/focuson/flu/illustrations/timeline/
69 Horimoto T, Kawaoka Y (2005) Influenza: Lessons from past pandemics, warnings from current incidents. *Nat Rev Microbiol* 3: 591–600
70 Webster RG, Govorkova EA (2006) H5N1 influenza – Continuing evolution and spread. *N Engl J Med* 355: 2174–2177
71 Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, Kawaoka Y (1998) The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. *J Virol* 72: 7626–7631
72 Rogers GN, Paulson JC (1983) Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 127: 361–373
73 Suzuki Y, Ito T, Suzuki T, Holland RE Jr, Chambers TM, Kiso M, Ishida H. Kawaoka Y (2000) Sialic acid species as a determinant of the host range of influenza A viruses. *J Virol* 74: 11825–11831
74 Rogers GN, Pritchett TJ, Lane JL, Paulson JC (1983) Differential sensitivity of human, avian, and equine influenza A viruses to a glycoprotein inhibitor of infection: Selection of receptor specific variants. *Virology* 131: 394–408
75 Rogers GN, D'Souza BL (1989) Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* 173: 317–322

76 Thompson CI, Barclay WS, Zambon MC, Pickles RJ (2006) Infection of human airway epithelium by human and avian strains of influenza A virus. *J Virol* 80: 8060–8068

77 Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci USA* 101: 4620–4624

78 Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y (2006) Avian flu: Influenza virus receptors in the human airway. *Nature* 440: 435–436

79 Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, Holtzman MJ, Brody SL (2006) Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol* 80: 7469–7480

80 van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T (2006) H5N1 virus attachment to lower respiratory tract. *Science* 312: 399

81 de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC et al (2006) Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 12: 1203–1207

82 Kandun IN, Wibisono H, Sedyaningsih ER, Yusharmen, Hadisoedarsuno W, Purba W, Santoso H, Septiawati C, Tresnaningsih E, Heriyanto B et al (2006) Three Indonesian clusters of H5N1 virus infection in 2005. *N Engl J Med* 355: 2186–2194

83 Oner AF, Bay A, Arslan S, Akdeniz H, Sahin HA, Cesur Y, Epcacan S, Yilmaz N, Deger I, Kizilyildiz B et al (2006) Avian influenza A (H5N1) infection in eastern Turkey in 2006. *N Engl J Med* 355: 2179–2185

84 Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JS, Guan Y (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol* 43: 5760–5767

85 Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, Meijer A, van Steenbergen J, Fouchier R, Osterhaus A, Bosman A (2004) Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* 363: 587–593

86 Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum G J et al (2004) Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci USA* 101: 1356–1361

87 Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, Nicholls JM, Chokephaibulkit K, Vanprapar N, Auewarakul P (2005) Influenza A H5N1 replication sites in humans. *Emerg Infect Dis* 11: 1036–1041

88 Thompson CI, Barclay WS, Zambon MC, Pickles RJ (2006) Infection of human airway epithelium by human and avian strains of influenza A virus. *J Virol* 80: 8060–8068

89 Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL, Wong MP, Chui WH, Poon LL, Tsao SW et al (2007) Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. *Nat Med* 13: 147–149

90 Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto

Y, Ito M, Kiso M, Horimoto T et al (2006) Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature* 444: 378–382

91 Gambaryan AS, Tuzikov AB, Bovin NV, Yamnikova SS, Lvov DK, Webster RG, Matrosovich MN (2003) Differences between influenza virus receptors on target cells of duck and chicken and receptor specificity of the 1997 H5N1 chicken and human influenza viruses from Hong Kong. *Avian Dis* 47: 1154–1160

92 Wan H, Perez DR (2006) Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. *Virology*. 346: 278–286

93 Shu LL, Lin YP, Wright SM, Shortridge KF, Webster RG (1994) Evidence for interspecies transmission and reassortment of influenza A viruses in pigs in southern China. *Virology* 202: 825–833

94 Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG (1993) Genetic reassortment between avian and human influenza A viruses in Italian pigs. *Virology* 193: 503–506

95 Call SA, Vollenweider MA, Hornung CA, Simel DL, McKinney WP (2005) Does this patient have influenza? *JAMA* 293: 987–997

96 Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos A M, Posey DL, Klimov A, Lindstrom SE et al (2005) Influenza-associated deaths among children in the United States, 2003–2004. *N Engl J Med* 353: 2559–2567

97 Nolte KB, Alakija P, Oty G, Shaw MW, Subbarao K, Guarner J, Shieh WJ, Dawson JE, Morken T, Cox NJ, Zaki SR (2000) Influenza A virus infection complicated by fatal myocarditis. *Am J Forensic Med Pathol* 21: 375–379

98 Davis MM, Taubert K, Benin AL, Brown DW, Mensah GA, Baddour LM, Dunbar S, Krumholz HM, American Heart Association, American College of Cardiology et al (2006) Influenza vaccination as secondary prevention for cardiovascular disease: A science advisory from the American Heart Association/American College of Cardiology. *J Am Coll Cardiol* 48: 1498–1502

99 Jaimovich DG, Kumar A, Shabino CL, Formoli R (1992) Influenza B virus infection associated with non-bacterial septic shock-like illness. *J Infect* 25: 311–315

100 Guarner J, Paddock CD, Shieh WJ, Packard MM, Patel M, Montague JL, Uyeki TM, Bhat N, Balish A, Lindstrom S et al (2006) Histopathologic and immunohistochemical features of fatal influenza virus infection in children during the 2003–2004 season. *Clin Infect Dis* 43: 132–140

101 Chan PK (2002) Outbreak of avian influenza A (H5N1) virus infection in Hong Kong in 1997. *Clin Infect Dis* 34: S58–64

102 Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, Lochindarat S, Nguyen TK, Nguyen TH, Tran TH et al (2005) Avian influenza A (H5N1) infection in humans. *N Engl J Med* 353: 1374–1385

103 Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W, Chunsuthiwat S, Sawanpanyalert P, Kijphati R, Lochindarat S, Srisan P, Suwan P, Osotthanakorn Y et al (2005) Human disease from influenza A (H5N1), Thailand, 2004. *Emerg Infect Dis* 11: 201–209

104 Ng WF, To KF, Lam WW, Ng TK, Lee KC (2006) The comparative pathology of severe acute respiratory syndrome and avian influenza A subtype H5N1 – A review. *Hum Pathol* 37: 381–390

105 To KF, Chan PK, Chan KF, Lee WK, Lam WY, Wong KF, Tang NL, Tsang DN, Sung RY, Buckley TA et al (2001) Pathology of fatal human infection associated with avian influenza A H5N1 virus. *J Med Virol* 63: 242–246
106 Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS (2005) Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir Res* 6: 135
107 Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M (2006) Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* 2: e53
108 Hatta M, Gao P, Halfmann P, Kawaoka Y (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293: 1840–1842
109 Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ et al (2006) The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *J Exp Med* 203: 689–697
110 Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P et al (2005) Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J Virol* 79: 11788–11800
111 Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P (2005) Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. *PLoS Pathog* 1: e4
112 Zamarin D, Ortigoza MB, Palese P (2006) Influenza A virus PB1–F2 protein contributes to viral pathogenesis in mice. *J Virol* 80: 7976–7983
113 Garcia-Sastre A, Biron CA (2006) Type 1 interferons and the virus-host relationship: A lesson in detente. *Science* 312: 879–882
114 Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H (2006) The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. *J Virol* 80: 11115–11123
115 Obenauer JC, Denson J, Mehta PK, Su X, Mukatira S, Finkelstein DB, Xu X, Wang J, Ma J, Fan Y et al (2006) Large-scale sequence analysis of avian influenza isolates. *Science* 311: 1576–1580
116 Li S, Schulman J, Itamura S, Palese P (1993) Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. *J Virol* 67: 6667–6673
117 Lofgren E, Fefferman N, Naumov YN, Gorski J, Naumova EN (2007) Influenza seasonality: Underlying causes and modeling theories. *J Virol* 81: 5429–5436
118 Stone L, Olinky R, Huppert A (2007) Seasonal dynamics of recurrent epidemics. *Nature* 446: 533–536
119 Tellier R (2006) Review of aerosol transmission of influenza A virus. *Emerg Infect Dis* 12: 1657–1662
120 Brankston G, Gitterman L, Hirji Z, Lemieux C, Gardam M (2007) Transmission of influenza A in human beings. *Lancet Infect Dis* 7: 257–265
121 Lowen AC, Mubareka S, Tumpey TM, Garcia-Sastre A, Palese P (2006) The guinea pig as a transmission model for human influenza viruses. *Proc Natl Acad Sci USA* 103: 9988–9992

Influenza Vaccines for the Future
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Influenza vaccines have a short but illustrious history

John Oxford, Robert Lambkin-Williams and Anthony Gilbert

Retroscreen Virology Ltd., London Bioscience Innovation Centre, 2 Royal College Street, London NW1 ONH, UK

Abstract

Isolation of the causative virus of influenza in 1933, followed by the discovery of embryonated hen eggs as a substrate, quickly led to the formulation of vaccines. Virus-containing allantoic fluid was inactivated with formalin. The phenomenon of antigenic drift of the virus HA was soon recognized and, as WHO began to coordinate the world influenza surveillance, it became easier for manufacturers to select an up-to-date virus. Influenza vaccines remain unique in that the virus strain composition is reviewed yearly but modern attempts are being made to free manufacturers from this yolk by investigating internal virus proteins including M2e and NP as "universal" vaccines covering all virus sub types. Recent technical innovations have been the use of Vero and MDCK cells as the virus cell substrate, the testing of two new adjuvants and the exploration of new presentations to the nose or epidermal layers as DNA or antigen mixtures. The international investment into public health measures for a global human outbreak of avian H5N1 influenza is leading to enhanced production of conventional vaccine and to a new research searchlight on T cell epitope vaccines, viral live attenuated carriers of influenza proteins and even more innovative substrates to cultivate virus, including plant cells.

Introduction

When the influenza A virus first emerged from a presumed avian reservoir at the end of the ice age 10 000 or so years ago, there was a distinct difficulty in finding new human victims. For example, at that time, only a few hundred settlers were in the London region near the Royal London Hospital, now a community of four million people. At that time a traveler would have to walk a 100 miles to find another small settlement, perhaps at Stonehenge near Salisbury.

Nowadays we have a truly global community of six billion people, linked so that two million people are moving each day by plane, while perhaps 10 million are journeying in their homelands. Influenza, like all viruses, is opportunistic. In 1918, it had the unprecedented opportunity to spread at the end of the first global war. Ten million soldiers began the move home-

wards and every steamship was packed as they fanned out from France to England, Europe, the US, Canada, Australia, India and SE Asia [1–3]. How perfect for a virus spread by aerosol droplets, close contact and contamination of towels, cups and every day utensils. A virgin population, which had never before encountered the avian virus (H1N1), was on the stage of this theatre of infection. Perhaps a billion people were infected in the next 18 months, and 50–60 million died, making this by far the biggest outbreak of infectious diseases ever recorded, with an impact many times greater than the so-called bubonic plague outbreaks in Medieval Europe. However, more than 2 billion people survived. The overall mortality was around 1%, although in a few semi-closed societies of hunter-gatherers in the Arctic, the mortality from the disease and subsequent starvation as young hunters died and husky dogs attacked and ate the survivors exceeded 90% [4–7]. While most people in the world were infected, we are forced to view the innate protective power of our immune system with awe [8, 9]. We are equipped with 100 000 genes, 7 million years of evolution and 80 000 years of specialization since our emergence from Africa. In contrast, influenza is a miniscule eight-gene vehicle. A recent study [10] of the reproductive number (R0) of the 1918 virus suggests that, unexpectedly, it may have been quite low, not exceeding three persons infected with a single case. This would place pandemic influenza not far above the lowly group of viruses such as small pox and SARS and not reaching the heights that measles has attained. But this unexpected theoretical analysis, if it is not flawed, gives us more practical opportunities to break a chain of infection of a pandemic with antivirals, hygiene and vaccines [11–13].

The new world of the 21st century, although harboring in some countries a few old-fashioned attitudes, akin to “influenza and pneumonia is the old person’s friend” nevertheless has the capability for the first time to defend itself against Mother Nature and her threat of influenza. For the first time in history, intense surveillance by the World Health Organization, early identification of a new pandemic influenza virus by molecular diagnostics, application of vaccination and antiviral chemoprophylaxis and possible quarantine and masks, could actually prevent a pandemic arising. For the expressed intention of WHO and the world community of infectious disease researchers is to deflect the first wave of the first pandemic of the 21st century. In this endeavor, our huge resources of natural innate immunity, assisted by new vaccines, will help us. But the formulation of the vaccines and their stockpiling alongside anti-neuraminidase (NI) antivirals will need significant investment of time and money and this has started with a 3 billion euro investment from the USA and EU.

Most recently [14], Baroness Findlay of Glandaff put the epidemiology of influenza H5N1 situation succinctly in the House of Lords Report of Pandemic Influenza “We believe the risk of a pandemic of human-to-human transmissible virus is to be taken very seriously. We believe that it may not happen in the very short time. To explain why we came to this stance; we

believe that the problem, if it does emerge is more likely to emerge in Asia. Asia is where fire fighting must be done today”. The Baroness had just heard the background science, that China alone holds 700 million domestic ducks, a possible Trojan Horse of virus persistence, which approximates to 70% of the worlds domestic duck population. Expert evidence from FAO had summarized that China, Indonesia and Vietnam represented the core of the problem, but only 160 million dollars were available at that point in 2005/2006 to help, and bio-security is not imposed strictly, while veterinary services are haphazard.

We are not the first generation of virologists to recognize the influenza pandemic threat, but we are the first to have the knowledge of the avian reservoir and the tools to deal with the problem in a scientific manner. The world capacity for influenza vaccine today of 300 million doses did not arrive by accident: it came to us from the hard work and dedication of four generations of dedicated scientists and doctors. The intention here is to give just tribute to these pioneers and their new discoveries. Using the vaccine methods developed over six decades we can for the first time confront influenza as it emerges, surround it and actually prevent a pandemic. We no longer need to be passive observers at a theatre of infection. Churchill coined the phrase “Give us the tools and we will finish the job”. Well, we now have them and we will. Such is the essence and spirit of this chapter.

A snapshot of the first six decades of influenza virology

The serendipitous discovery of infection of ferrets, which produce clinical signs, and the cross infection of a student from a ferret was the first technology foundation stone [9]. Ferrets are used today as a key model to investigate new vaccines.

The two most important technologies, which form the granite like foundation of influenza vaccine research, are the hemagglutination inhibition test (Fig. 1) and the cultivation of virus in embryonated hen’s eggs (Fig. 2), first reported in 1941 and 1946 respectively [15, 16].

If one adds two other vital scientific observations, that of Hobson et al. [17] who correlated a HI titer of 40 with protective efficacy, in volunteers in 1972 and then the discovery of a single-radial diffusion for standardization of the hemagglutinin (HA) content of vaccines by Schild in 1973, it is quite apparent that the technologies are all now well tried and tested [18]. The elucidation of the structure of the fragmented influenza genome [19] has quickly led to techniques, genetic reassortment and correlation of functions with certain genes (Fig. 3). From a practical viewpoint, some old much passaged viruses such as A/PR/8/34 (H1N1) grew to extraordinary infectious titers in the egg allantoic cavity, exceeding a new wild-type virus by 100-fold or more. Why not create a reassortant in the laboratory with six replicative genes of A/PR/8/34 to give high replication while having the two new HA

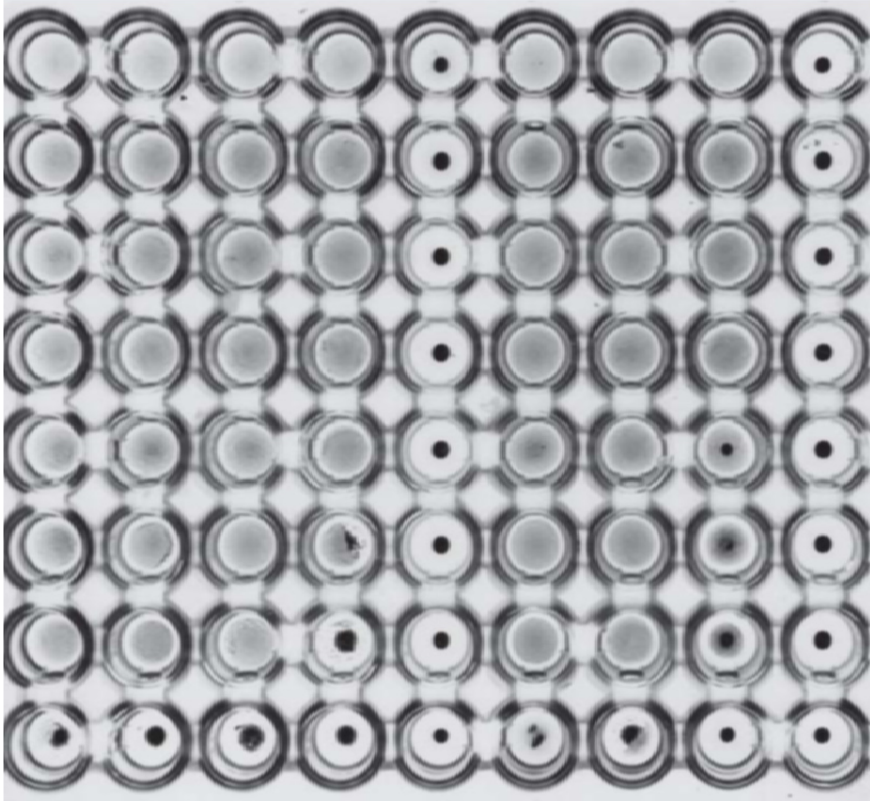

Figure 1. The classic hemagglutination inhibition test
The test depends upon interaction of eight HA units of virus that would normally agglutinate 0.5% turkey red blood cells. Pre-incubation of this standard virus with dilutions of serum antibody abrogate the agglutinating property of the virus (vertical rows 5 and 9). No antibody is detectable in rows 1, 2, 3, 4, 6, 7, 8.

and neuraminidase (NA) genes of the new epidemic virus? This technique proved to be a masterstroke and in the last quarter of a century three laboratories, CSL in Melbourne, NIBSC in London and Ed Kilbourne's laboratory in New York, have rushed each year to produce the new candidate vaccine viruses prefixed IVR-, NIB- and X-, respectively. The almost made-to-order technique of gene reassortment with influenza was also central to producing host range mutants with attenuation genes for live vaccines.

Undoubtedly the simultaneous discovery of the reverse genetics [20, 21] by the three laboratories in New York, Wisconsin and Oxford was a masterstroke in technical advance, which has enabled mutations to be placed, at will, into the genomes of the negative-strand viruses. The conjunction of older and newer techniques with the licensing of the mammalian cell lines from monkey kidney (Vero) [22], dog kidney (MDCK) [23] or human tis-

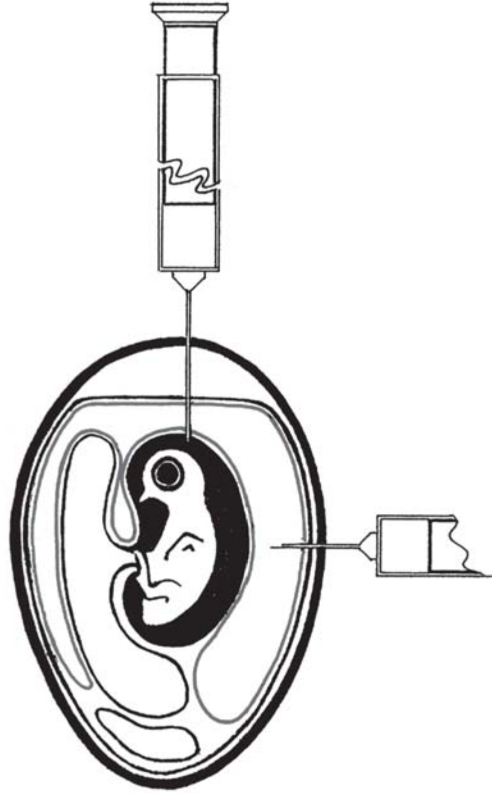

Figure 2. Inoculation of embryonated hens eggs to grow influenza virus for vaccine
Virus is inoculated through the shell of a 10-day-old embryonated hen's egg and more rarely in the research laboratory into the amniotic cavity (top). After 2 days incubation at 37 °C, the clear fluids are removed and titrated for HA by hemagglutination.

sue (PER-6) has led directly to the newly emerging influenza vaccines of the 21st century upon which societies will place such weight in the face of threats of influenza epidemics and pandemics.

The historical steps in killed vaccine development

The first experiments on the attempted immunization of animals were made in the USA by Francis Magill [24] and in England by Andrewes and Smith in 1937 [25]. Mouse lung suspensions or filtrates were used after inactivation with formaldehyde, and it was found relatively easy to protect mice against intranasal infection with influenza. Immunization experiments in man were accelerated when allantonic fluid preparations of virus formed the starting

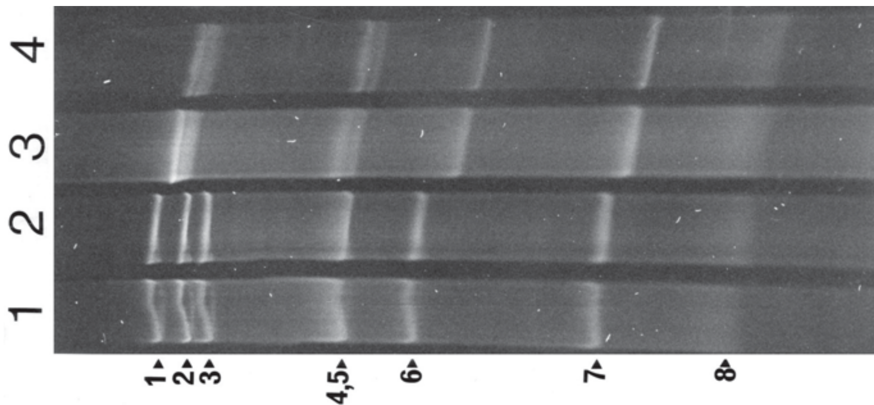

Figure 3. The influenza genome is in eight fragments.
The genome could be labeled with ^{32}P extracted and separated on polyacrylamide gels.

material soon after the technique of allantoic inoculation of fertile hen's eggs was discovered [16]. The first field trial demonstrating short-term protection by inactivated vaccine took place in the United States during a sharp epidemic of influenza in 1943 (Commission Influenza, 1944) [26].

Progress with the development of purer, more potent vaccines has proceeded steadily since those early days and technical advances with ultracentrifugation, by methods producing richer cultures and chemical inactivation avoiding too great a modification of the surface HA and NA antigens have all helped. To avoid the relatively high rate of local and general systemic reactions caused by inactivated whole-virus vaccines, chemical treatment to disrupt the particle and to separate the wanted antigens (HA and NA) from other constituents of the virus has led to a variety of different split or subunit vaccines (Figs 4–6). Ether extraction [27, 28], deoxycholate treatment [29] and treatment with other detergents have been introduced. Some methods have provided subunit vaccines causing fewer clinical side reactions than the older whole-virus particle vaccines, but drawbacks have appeared, including that of reduced antigenicity. Adjuvants of oily emulsions promised potent vaccines with excellent antibody responses, and few reactions were first encountered. But a rare abscess at the site of inoculation caused much distress and this early approach had to be abandoned. In spite of attempts to develop safer materials none have yet been developed commercially until very recently. Thus, after 60 years work, the hope of an ideal inactivated vaccine, free from the induction of clinical reactions and yet potent immunogenically has just been fulfilled with pandemic H5N1 vaccines.

In 1946, a major antigenic deviation of influenza A virus occurred with the appearance of A/CAM/46 (H1N1) virus in Australia. In the USA and Europe outbreaks of influenza occurred early in 1947, which were due to

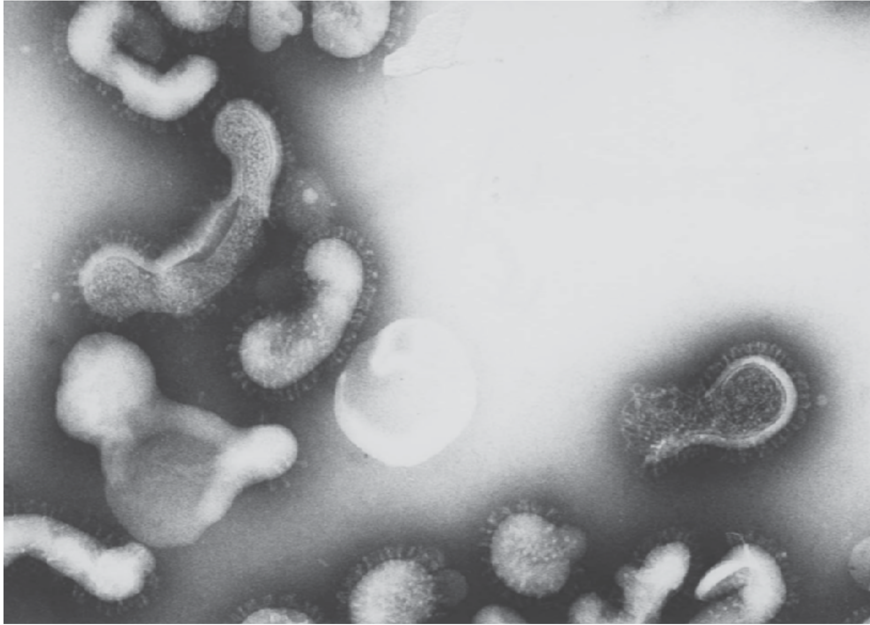

Figure 4. Whole-virus vaccine
Influenza viruses are pleomorphic with a fringe of HA and NA spikes.

the same virus; some communities previously receiving vaccine containing PR8 and Weiss viruses (H0N1 in the old classification and now reclassified H1N1) were attacked. This time the vaccine did not protect against the new virus typified by the prototype A/FM/1/47 (H1N1) [30, 31] and this led to realization of the enormous importance of the updated antigenic make-up of inactivated vaccine.

Yet other difficulties have become appreciated, one of which is the inappropriate antibody response occurring sometimes after inoculation, when the vaccine induces cross-reacting antibody to heterologous viruses or the first virus in the subtype which the vaccinee first experienced, rather than that appropriate to the specific antigen – HA – of the vaccine virus. This response is probably allied to the phenomenon of “original antigenic sin”.

Vaccine purification historical and present

The starting materials for almost all types of inactivated vaccine are allantoic fluids from fertile hen’s eggs previously inoculated with a seed culture, the yield of which is enhanced using a recombinant virus, one parent of which is a high yielding laboratory strain (A/PR8/34) and the other acts as the donor

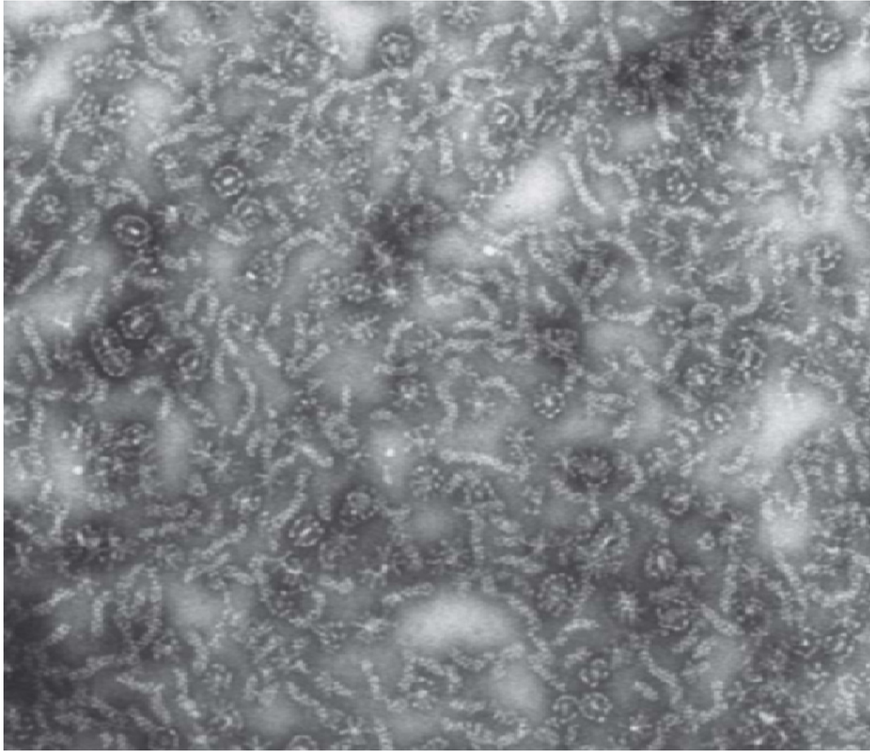

Figure 5. Split influenza virus vaccine
The whole virus is disrupted with detergent, which dissolves the lipid membrane releasing HA, NA and internal NP, seen as “lamb tails”.

of the requisite surface HA and NA antigens from a wild-type virus [32]. Purification from unwanted egg material is accomplished by ultracentrifugation on a zonal ultracentrifuge [33]. Whole-virus particles thus separated are inactivated by formalin or β propiolactone, the HA content being as high as possible commensurate with the necessity to avoid febrile reactions after inoculation. Children were sensitive to the older whole-virus vaccines; as many as 30% under 2 years developed fever after 0.25 ml vaccine and up to 8% of 6-year-old children were similarly affected after 0.5 ml [34]. The precise constituent producing the fever was not clearly identified, but the viral proteins were believed to be concerned [35, 36].

Separation of the HA and NA by means of detergents such as Tween 80 or Triton N101 produced split-virus or subunit vaccine and general experience suggested that these materials are less pyrogenic, but less immunogenic, than whole-virus vaccine [37]. This was particularly well demonstrated by studies during the swine influenza campaign in the USA in 1976, when many

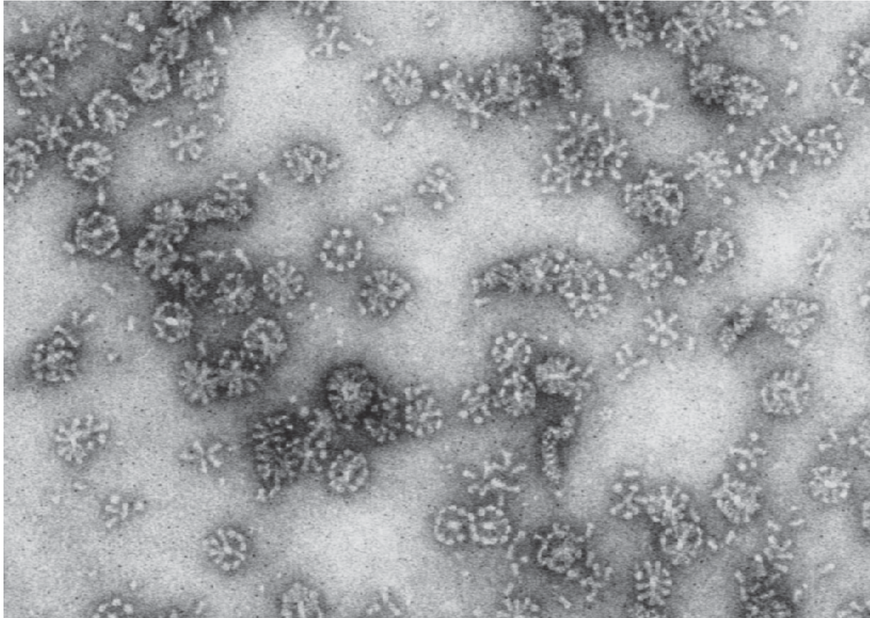

Figure 6. Subunit influenza virus vaccine
The split virus is fractioned in a sucrose gradient and the HA and NA subunits are separated from NP, and M, and standardized by SRD and used for vaccine.

observers reported results, which ultimately led to the recommended use in children of split-type rather than whole-virus vaccines. Such recommendations continue at the present time. In adults, too, the older whole-virus vaccines gave a higher proportion of febrile reactions than split virus [38].

Early progress: The standardization of potency, composition and dosage of inactivated vaccines

Former methods for assays of the potency of inactivated vaccine depended upon measuring the HA activities of the vaccines with erythrocyte suspensions using the Salk pattern technique of Miller and Stanley [15]. In a major technological breakthrough, Schild et al. [18] proposed a method of assay based on single-radial immunodiffusion (SRD) (Fig. 7). The HA antigen content of vaccines was estimated using SRD tests in agarose gels containing specific HI antibodies. The SRD method was modified and refined by Wood et al. [39]. The SRD technique was valid for both whole-virus and split-virus vaccines and was quickly adopted for international use and is still the gold standard. In this test, vaccine virus preparations and refer-

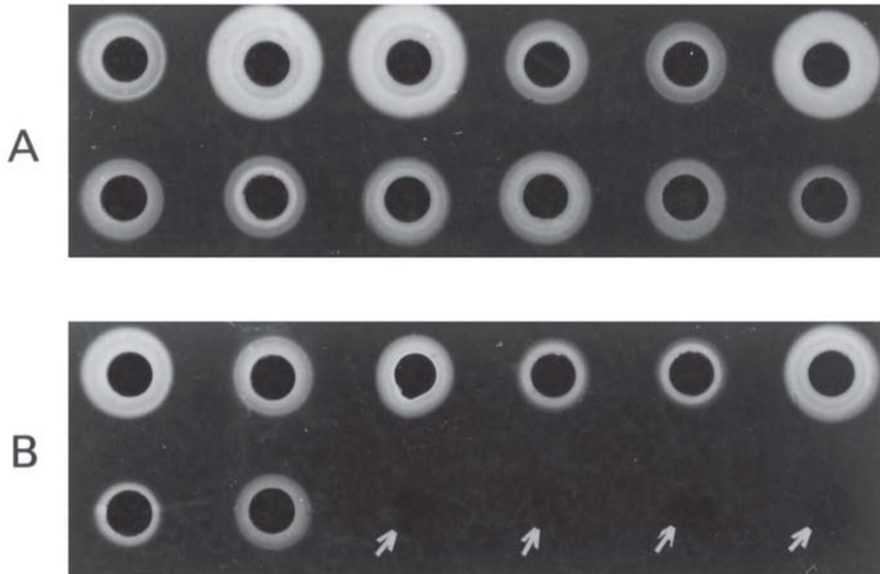

Figure 7. Single-radial diffusion (SRD) test to standardize HA
Vaccine antigen is pipetted into 3-mm wells in an agar plate containing specific anti-HA, -NA and -NP antibodies. After a few hours incubation a zone of precipitation is quantified and the area is proportional to the quantity of HA in the vaccine.

ence antigen calibrated in terms of micrograms of HA are disrupted with detergent and dilutions of the treated antigens are introduced into wells in SRD immunoplates. The size of the precipitation ring obtained for the vaccine is compared with that obtained with a reference antigen of calibrated HA content titrated on the same plate. The vaccine potency is measured in terms of micrograms of HA per vaccine dose. Inactivated influenza vaccines frequently contain two or more virus strains and the HA content of each component (15 μg) is assayed independently.

HA dosage of vaccines and relationship to HI antibody response

It has been known for many years that the serological response to inactivated vaccine depends on the previous experience of the recipient to infection by viruses of the same subtype of influenza A virus as that present in the vaccine. Whereas a single subcutaneous injection of (H1N1) vaccine gave as good a response as two doses prior to 1957, the advent of the new pandemic A/Asian (H2N2) virus produced a different effect. Thus, Holland et al. [40] demonstrated that two doses at an interval of 2 or more weeks produced a better response to one dose and in this regard the vaccine induced immune

response was much inferior to that noted before the change in virus subtype. Such an experience was again noted during the first year of circulation of A/Hong Kong (H3N2) virus and also when the A/New Jersey/76 (Hsw1N1) vaccine was used in children and young adults. Also, in the circumstances of 1977–1978, when most persons under 25 years of age had no previous antibody to the re-circulating H1N1 virus, a two-dose regimen for children and young adults produced a more satisfactory response than a single injection [41]. The contrast between the effects of a single dose of vaccine in persons infected with H1N1 viruses at least 20 years earlier was very striking. These data have immediate relevance today in terms of H5N1 vaccine.

Several factors are of importance in the determination of the quantity and the precise composition of the antibody response to the surface antigens of the virus present in inactivated vaccine. First and foremost, the quantities of HI and NI antibodies induced by vaccine are broadly related to the quantity of antigen present in a single dose. Secondly, the precise composition of the antibodies formed in response to influenza A virus is important. Thus, reinforcement of previously acquired antibodies by the orientation of the B lymphocyte response to the first infection by the particular subtype of virus experienced in childhood or later may take precedence over the strain-specific antibody response to the vaccine virus. Thirdly, the precise response is influenced by the route by which the vaccine is presented to the body's immune system.

First then, several earlier studies reported a graded relationship between the quantity of antigen inoculated and the antibody response that results. This was so in the study of Mostow et al. [42], who gave increasing doses of vaccine in a single injection containing 300–4600 chick cell agglutination units (CCA) containing A/Japan/57 (H2N2) virus groups of volunteers. The serum HI response was tested with four different H2N2 viruses isolated 1962–1967 and also the homologous virus. With more than a 10-fold increase in HA from the least to the highest dose, the geometric mean titer (GMT) of antibody increased only 5-fold. Similar results were obtained by Potter et al. [43], who inoculated student volunteers with vaccines ranging in dosage from 5 to 400 International Units and containing A/Port Chalmers/73 (H3N2) virus. The vaccine was a surface-antigen detergent-treated material [44] adsorbed to aluminum hydroxide gel. GMT HI serum titers increased against homologous virus from 8- to 174-fold with the increase in dose of vaccine HA. Three other H3N2 strains and A/Singapore/57 (H2N2) virus were also tested and all three H3N2 viruses showed graded HI antibody responses proportional in magnitude to increase in antigen dose, as did the homologous virus.

The Pandemic Working Group of the MRC Committee on Influenza Vaccine [45] gave graded doses of whole-virus vaccine containing the A/New Jersery/76 (Hsw1N1) strain to groups of volunteers in 1976. Those less than 44 years of age, who did not possess significant serum HI antibody to the virus before immunization, showed a post-vaccination antibody titer ranging from 64 to 148 GMT with a nearly 8-fold increase in dose from 8 to

61 μg HA. Above this age, in those 45 to 64 with pre-existing Hsw1 antibody, there was an increase in antibody titer from 7 to 36 times (GMT) with a change in HA from 4 to 61 μg. Thus, the effect of increasing the potency of this vaccine on the antibody response was much greater in those sera, which indicated that they had been exposed to the antigen, presumably by infection with a related virus, than in those with no such exposure. Both whole and detergent-split-virus vaccines showed a relatively poor HI response in volunteers less than 25 years of age whose initial serum had no significant amount of pre-vaccination or post-infection HI antibody. In this group of subjects two doses of vaccine gave a better antibody response than did one, but the resultant post-vaccination GMT was half that obtained with a single dose in the vaccinees over 25 years of age.

These examples underline the practical importance of a considerable degree of antigenic drift within a subtype comprising HI antibody response. Also, the recall of antibodies induced by previous infection illustrates the general rule that an up-to-date monovalent vaccine reinforces antibodies against former members of the subtype, while also inducing specific antibodies to the vaccine virus. This was clearly shown by direct comparison of monovalent and polyvalent vaccines such as the MRC Committee on Influenza Vaccine's trials [46–49].

The quantitative dose response already described for HI is also found with NI antibody, but is less consistent. Thus, Potter et al. [50] noted that there was a 2- to 6-fold increase in NI antibody as vaccine potency was increased from 5 to 400 IU HA. Yet the trial of A/New Jersey/76 (Hsw1N1) vaccine conducted by the Pandemic Working Group of the MRC Influenza Vaccine Committee [45] found only a slight increase in NI antibody after an increased dose from 100 to 200 IU using 100 IU HA in the vaccine. Nicholson et al. [41] gave a whole-virus vaccine of the A/USSR/77 (H1N1) virus, which ranged in potency up to 6-fold, and found, in those under 25, a 3-fold increase in NI antibody. However, in those over 25 years of age an increase in dose of vaccine had a less constant effect on NI antibody formation. One possible reason for the variation in the effect of different vaccines on the NI antibody is the lack of consistency in the NA content [51]; however, another possibility may be that immunological priming to the HA in the vaccine can in some way suppress the immunogenicity of the NA antigen, which may be physically associated with the HA.

The second important variable in the immune response to inactivated vaccine arises from the relative amounts of cross-reactive and strain-specific antibodies that are generated. The differentiation of these requires special techniques such as SRD and the adsorption studies. Webster et al. [52] compared, in adults, the response to an A/Port Chalmers/73 (H3N2) subunit vaccine to homologous and heterologous H3N2 viruses. Most of the antibody was cross-reactive with A/Hong Kong/68 virus but when higher doses of the vaccines were employed, strain-specific A/Port Chalmers/73 antibody was produced in addition to that against heterologous virus. Oxford et al.

[53, 54] compared whole- and split-virus vaccines containing A/Victoria/75 or A/Scotland/74 viruses and using single-radial-hemolysis and adsorption techniques showed that in an immunized adult, cross-reactive antibody was induced much more frequently than specific antibody against homologous virus. They showed the same phenomenon in adults during infection with A/Port Chalmers/73 virus, who frequently also developed antibody rises to A/Hong Kong antigens from 1968. Oxford et al. [54] used similar techniques to analyze sera from children aged 3–6 years immunized with a surface-antigen vaccine containing A/Victoria/75 (H3N2) antigens. Most children produced a strain-specific serum antibody to the vaccine antigens, whereas adults similarly vaccinated tended to produce antibody cross-reacting with all variants of the H3N2 subtype tested. Post-epidemic sera from those of various ages recently infected by A/Texas/77 –like strain showed cross-reactive antibody in adults but in contrast mostly strain-specific responses in children. Strain-specific antibody is considered to be more protective.

The route of vaccination

The influence of the route of immunization with inactivated vaccine has been studied in the past by many observers. The chief alternative to the subcutaneous–intramuscular route is intradermal injection using a reduced amount of vaccine. The advantages of this route are economy and the avoidance of febrile reaction. The principal disadvantage is the fact that the antibody response is less consistent. It was shown by Appleby et al. [55] that the GMT after intradermal vaccine was less than half that obtained with subcutaneous vaccine, and this seemed logical in that only one-tenth of the vaccine dose was given intradermally. McCarroll and Kilbourne [56] found little difference in the antibody responses to intradermal and subcutaneous vaccines in equivalent doses. Tauraso et al. [57] reinvestigated the question using a two-dose regime before the arrival of the A/Hong Kong/68 (H3N2) epidemic. In the equivalent amount of 0.1 ml vaccine, antibodies formed in higher titer after intradermal than subcutaneous vaccine. However, the titers after 0.5 ml vaccine subcutaneously were little different than after intradermal injection of 0.1 ml. It is considered advisable, however, in practice to limit intradermal vaccination to when the vaccine is in short supply or when, in children or the aged, reactions after subcutaneous vaccine might pose problems.

The nasal route of inoculation either by instillation of drops or by spray was first studied in detail by Waldman et al. [58]. Compared with the subcutaneous vaccine in a dose of 0.5 ml, antibodies capable of neutralizing the virus A/Taiwan/64 (H3N2) increased to a greater extent in sputum and nasal secretions after repeated nasal inoculation with a total volume of 3.6 ml vaccine. In contrast, the intranasal vaccine produced a much lower rise in serum antibody, the GMT being only one-sixth that after subcutaneous vaccine. Waldman et al. [59], using an aerosol spray found that a better

serum antibody response occurred with a small-sized particle spray than a larger one, but the nasal antibody response was better after the latter or with nasal drops. Absorption studies showed that a majority of the secretory antibody (IgA) response in nasal secretion was cross-reactive with heterologous viruses (A/Hong Kong/68 H3N2). Phillips et al. [60] compared subcutaneous or intradermal vaccine in nurses with vaccine dropped intranasally. The subcutaneous route produced the best serum antibody rises, and intradermal vaccine was superior to the intranasal route in terms of antibody response. The nasal antibody titers after immunization by either subcutaneous or respiratory routes paralleled those in serum.

The fact that nasal antibodies increase after subcutaneous vaccine [61, 62] is important because the lack of a good response in serum antibody in those given the same vaccine intranasally is a limitation hardly offset by local nasal secretory changes. Challenge of immunized groups of persons by live attenuated virus also supports the view that nasal antibodies play a supplementary role to serum HI antibody [63].

Early quantification of side reactions to vaccines: Whole virus *versus* split and subunit

The field trials of inactivated vaccines in 1976 and 1977 added to knowledge concerning the reactogenicity of different preparations. The split-virus type of vaccine then used unquestionably caused fewer systemic febrile responses in both children and adults. The fact that reactions with whole-virus vaccines used at the time were unpleasantly severe for those without serum antibodies to the vaccine virus before inoculation had not been fully appreciated. In the case of children aged 6–18 in the American trials of A/New Jersey/76 (Hsw1N1) virus, the most potent vaccines caused fever in up to 63% of vaccinees. In the UK, the Pandemic Working Group of the MRC Committee on Influenza Vaccine found that a dose of 61 μg HA (1000 IU) of whole-virus vaccine with the same Hsw1N1 strain produced, in adults, local reactions in 50% and systemic effects in over 60% of volunteers. Even the lower doses of 18–27 μg HA caused local reactions in 50% and systemic effects in 40%. The A/USSR/77 (H1N1) virus vaccine trial in 1978 in Britain showed that adsorbed or aqueous split-virus vaccine produced fewer reactions than did whole virus [51]. After a second dose of the same vaccine, fewer volunteers experienced reactions than seen after the first dose. Later studies of the endotoxin content of various pools of inactivated type A or B vaccines using the limulus lysate test gave no hint of a parallel between the occurrence of general reactions and the endotoxin content [64].

Neurological illness is a recognized sequel to immunization with a variety of vaccines but had not previously been observed with any frequency after influenza virus vaccines. Wells [65] noted the rare instance of Guillain-Barré Syndrome (GBS), which appeared in excess among the persons

recently vaccinated with A/Swine vaccine compared with the numbers in unvaccinated individuals. Of 1098 persons with GBS reported from 1 October 1976 to the 31 January 1977, 532 had received vaccine prior to the onset of neurological symptoms. The overall risk of GBS was calculated as ten cases per million vaccinated. The rate of occurrence during the 10-week swine vaccine period was five to six times greater than in unvaccinated persons. However, the excess in number was greater in the 2nd and 3rd weeks after inoculation than either the 1st or subsequent weeks. As reported by Langmuir [66], GBS was not associated with a particular variety of vaccine or age group. However, that numbers were slightly greater in those aged 25–44 than in middle aged or elderly persons appears to rule out the possibility that the syndrome was, in some way, related to the absence of antibodies to the swine virus before immunization, for most of those over 45 would have been exposed to antigens of this virus many years before. After the swine influenza campaign was terminated, surveillance was continued, and during the period 1978–1979, when 12.5 million doses of ordinary inactivated vaccine were estimated to have been used, the related risk of GBS was 1.4 times the incidence in unvaccinated persons. This risk was regarded as not significant [67]. No clue to the cause of the marginally increased risk of GBS in immunized persons in1976 has yet been obtained, but could be virus strain related.

Advent of the 1968 pandemic virus and use of inactivated vaccines

At the time when A/Hong Kong /68 (H3N2) virus was spreading in Asia, plans were made by the MRC Committee on Influenza Vaccine to protect children in residential schools and other groups in a controlled manner. Inactivated polyvalent vaccine containing two H2N2 viruses (A/England/64 and A/England/66) and a B strain were compared with an H3N2 A/Hong Kong whole or deoxycholate-treated virus vaccine in initial serological trials. Antibody formation even in those without detectable serum HI antibody gave GMTs over 100 in those receiving A/Hong Kong vaccine intramuscularly. However, controlled trials in two boarding schools showed no convincing evidence of protection. In uncontrolled trials in other schools either the polyvalent or the A/Hong Kong vaccine were given or no vaccine at all. There were 12 schools where epidemics of influenza occurred in January and February 1969 but no evidence of protection was found in those receiving A/HK vaccines. The only clue obtained concerning the vaccine failure was first that only one dose of vaccine had been given, and this is known to be inadequate to give a satisfactory antibody response in previously seronegative persons, and secondly, there was an interval between vaccine administration and infection of 2–4 months. These two factors may have combined to explain the absence of protection because of the inadequacy of the antibody response at the time of challenge. It would be

fair to add that others [68, 69] did obtain protection from A/Hong Kong/68 whole-virus vaccine during the first outbreak of influenza due to this virus in the USA. The use of modern adjuvanted H5N1 vaccine in two doses is anticipated to give protective effects.

First studies with live influenza vaccines

The use of living but attenuated virus as an immunizing agent developed slowly from the initial studies of Mawson and Swan [70] in Australia and the USSR. The major difficulty of the lack of a laboratory test to indicate that cultured virus had lost its pathogenicity, while retaining infectivity for man, meant that deliberate intranasal inoculation of volunteers furnished the only way to select a suitable strain for infection without causing clinical reaction. In spite of the widespread adoption of live vaccines selected by this method and given as an intranasal spray in the USSR, little interest was exhibited in most other countries. From 1956 onwards, trials took place in volunteers in England and Wales to provide evidence of safety and immunogenicity of cultured viruses, and the drawback of a reduced infectivity of well-attenuated viruses handicapped progress. The necessity to observe a match between the antigens of epidemic viruses and those present in the vaccine was a further drawback until the technique of reassortment of characters between two strains, one of which was of proven attenuation, was utilized to yield seed viruses with the desirable clinical and antigenic properties. Other disadvantages of live viruses appeared during the intensive researches of the 1980s particularly in the USA and in England [71, 72]. It cannot yet be claimed that the ideal live attenuated virus vaccine has been formulated, but reverse genetics and increased knowledge of virulence genes have now lead to a resurgence of interest.

In the 1980s, genetic studies were intensively pursued in attempts first to define the particular gene or combination of genes, donated by the attenuated virus that confers the property of attenuation upon the reassortant strain. It was found that the biological properties of excreted virus may be altered compared with those of the original virus in the vaccine and the manner of this alteration was also studied genetically. Such work is essential in achieving the goal of an effective and safe vaccine virus for human use. Experimental inoculations were carried out initially in small-scale tests in volunteers under semi-isolation to permit close observation (see below).

Host-range virus mutants as live vaccines

Multiple cultivation and passage of viruses either in animal hosts such as ferrets and mice, or in developing chick embryos or tissue cultures had been practiced even before the use of temperature-sensitive (*ts*) or cold-adapted

(*ca*) mutants was suggested. Early workers in Britain used the PR8/34 virus as a host range mutant, which, although noninfective for man, has retained animal pathogenicity even after many passages in eggs. As a donor parent with good powers of multiplication in the laboratory, PR8 was mated with various strains of wild-type influenza A viruses to obtain recombinants with up-to-date surface HA and NA antigens. This method was preferable to simple laboratory cultivation because some viruses failed to alter in pathogenicity after as many as 30 serial passes in cultures [73], although other virus strains appeared to become attenuated with only a few passages in eggs.

PR8 virus was chosen also by workers in Belgium who prepared reassortants from a number of viruses, some of which were licensed for human use [74]. To select recombinants with as high proportion of RNA components as possible derived from the host range mutant PR8, Florent et al. [75] used RNA-RNA hybridization to identify gene origins. Later the gene constellation of four of the candidate vaccine viruses were determined and Florent [76] found that some clones of Beare and Hall's [77] recombinants of PR8 and A/Englannd/69 (H3N2) containing five genes from PR8 were satisfactorily attenuated. However, one clone though containing six PR8 genes was nevertheless clinically virulent to volunteers. A further genetic study of PR8 host range recombinants using viruses tested clinically by Beare and Reed [78] was made by Oxford et al. [79]. It was again found that recombinants from PR8 and A/England/69 viruses could contain only the surface HA and NA genes from wild-type virus and yet retain virulence for man.

Additional attempts to stabilize the attenuation of candidate viruses were made both by Beare at the Medical Research Council's laboratories at Salisbury and the RIT workers by rendering the virus resistant to an inhibitor present in normal horse serum. This property was present in the RIT series of recombinants. It seems strange that stabilization has not been pursued since, nor has cultivation of host range mutant viruses, such as PR8, at abnormally low temperatures, such as 25 °C. This method was found by Sabin [80] to be preferable to normal temperatures when attenuating polio viruses, and it was exploited by both workers in the USA and USSR.

Marker tests, which can be equated with attenuation of virulence for man, were sought with relatively variable results. One such test used weanling rats that were inoculated intranasally first with virus and later with cultures of *Haemophilus influenzae*. Virulent virus induces bacteremia and meningitis and using this method Jennings et al. [81] successfully separated a number of reassortant viruses and obtained some correlation with clinical virulence. Yet the host range mutant parent PR8/34 and RIT 4050, which are both attenuated in man, were classed as virulent by the rat.

A new approach at that time used an avian (duck) virus, which was found to have only low pathogenicity for squirrel monkeys inoculated intranasally and was proposed as a donor of attenuation. A reassortant with a virulent humanA/Udorn/72 (H3N2) virus behaved as did the avian parent in the squirrel monkey, although immunizing the latter against the virulent

parent. Clinical trials have suggested that this virus is attenuated for man and is immunogenic, but has not been investigated since [82].

Temperature-sensitive virus mutants as live vaccines

Most work on the development of viruses with restricted multiplication at temperatures above the normal range for cultivation has been affected by Chanock, Murphy and associates at the National Institutes of Health, Bethesda [83]. The technique employed chemically produced mutation in virus RNA by cultivation in the presence of the mutagenic agent 5-fluorouracil. After cultivation and plaquing at 33°, 37° and 38°C, mutant viruses with the requisite temperature sensitivity were obtained. Intranasal inoculation of hamsters confirmed temperature restriction, in that much lower titers of virus were found in the hotter lungs than in the cooler upper respiratory tract.

Spread from inoculated volunteers to adults in contact was not observed and no evidence of a change in virulence was found in viruses recovered from adult recipients of vaccine [84]. But in seronegative children, the A/Hong Kong/68-*ts*-l [E] virus both produced mild febrile reactions and a virus that had lost its properties was recovered from some who were infected.

A second series of *ts*-1a2 was then developed by combining two defective ts viruses, each of which belonged to a different complementation group in respect of the genetic defect. The progeny exhibited greater temperature restriction than the *ts*-1[E] line of viruses. It was termed A/Udorn/72 *ts*-1A2, and it was recombined with three further viruses; wild-type A/Victoria/3/75, A/Alaska/77 (H3N2) and also A/Hong Kong/77 (H1N1). These *ts*-1A2 viruses were highly immunogenic and exhibited temperature restriction of multiplication in cell cultures and reduced replication in the hamster lung. The A/Victoria/3/75-*ts*-1A2 recombinant retained its ts properties after inoculation into doubly seronegative children. Unfortunately, when the A/Alaska/77-*ts*-1A2 virus was similarly tested in a single child after tests in adults had shown genetic stability, the nasal secretions of the vaccinee yielded a ts-positive virus that produced plaques at 39ºC even though the child had shown no symptoms or fever. The recombinant 1A2 virus with A/HongKong/77 (H1N1) parent exhibited a capacity to infect 70% of doubly seronegative adults and was attenuated compared with the wild-type parent. Nevertheless, it appeared possible that a virus such as the A/Alaska-*ts*-1A2 might, if transferred to contacts from an inoculated child, result in clinical illness and clinical studies with this particular virus were not pursued.

Cold-adapted virus mutants as live vaccines

Beginning with a strain of H2N2 virus recovered in Ann Arbor, Michigan in 1960 by cultivation of throat washings in tissue cultures at 36ºC, Maassab

[85, 86] evolved a virus, A/Ann Arbor/6/60 (H2N2), which has acted as a donor of attenuation to other viruses by genetic reassortment. Earlier passages were made in chick kidney tissue cultures followed by intranasal passages in mice and then a gradual adaptation to lower temperatures, in tissue cultures and in developing hens' eggs inoculated allantoically, led to a virus with good powers of multiplication at 25 °C. The ca variant was found to retain the infectivity of the original strain for both the mouse and ferret, although it produced no deaths in mice and no fever or turbinate lesions in ferrets, whereas the original virus was pathogenic for both species. The virus proved to be temperature sensitive with a shut-off temperature of 37 °C [87]. Recombinants with wild-type viruses of both H2N2 and H3N2 subtypes were prepared, studied in the laboratory and in volunteers and analyzed genetically. The original A/Ann Arbor/6/60 (H2N2) virus was not, however, tested in fully susceptible persons presumably because of the difficulty in that period of finding seronegative adults. A few persons with low titers of serum neutralizing antibodies (1:4 to 1:6) were inoculated and as judged by antibody responses, became infected without undergoing clinical illnesses. More rigorous clinical studies have been pursued with recombinants, in particular those with H3N2 antigens, and details of the results have been bought together and earlier data summarized by Kendal [72]. The donor ca parent has been more recently reassorted with H5N1 genes.

It is clear that infectivity and immunogenicity were fully retained for seronegative adults of whom 111 received H3N2 recombinants. Among those receiving three of four recombinants, clinical reactions were minimal or negligible but with the fourth, derived from the A/Scotland/74 parent, in 4 of 12 volunteers receiving $10^{8.5}$ and in 1 receiving $10^{7.5}$ $TCID_{50}$, there were clinical illnesses. Viruses re-isolated from the vaccinees retained *ts* properties and so did those given recombinants of A/Victoria/75 (H3N2) and A/Alaska/77 (H3N2). However, some loss of *ca* restriction was found in virus re-isolated from volunteers given the A/Scotland/74 recombinant.

Cold-adapted recombinants with A/USSR/77 (H1N1)-like virus have also been studied in adult volunteers and found to be less immunogenic as judged by HI antibody responses. A better response was obtained by Wright et al. [88] in children in Nashville given$10^{6.5}$ $TCID_{50}$ of strain CR 35 (H1N1) and none of 11 children developed adverse clinical reactions even though 8 became infected. All re-isolated viruses retained the ts phenotype. The failure to elicit serum antibody response in adults given this same virus recombinant is puzzling. Using the ELISA enzyme-linked assay, Murphy and others [89] found that by this more sensitive method antibody rises could be demonstrated and the results tallied better with the ability to re-isolate viruses from the inoculated volunteers than did the serum HI responses.

The Leningrad group of workers led by Smorodinstev [90] were the first to obtain a virus indirectly attenuated by cultivation at 25 ºC. The group used strains selected by inoculating volunteers with several viruses derived from cultures repeatedly incubated at 25–26 °C to speed up attenuation. Some

5–7 months were required for the preparation and production of new strains even using genetic recombination to incorporate new surface HA and NA antigens. Although Alexieva et al. [91] found that cold cultivation was not successful in producing reliably attenuated viruses for use in children, the technique was adopted for general use. Genetic studies of the Leningrad viruses are described briefly by Kendal et al. [72] and these parent ca viruses are currently the center of new interest for attenuated H5N1 vaccines.

Usually, preliminary studies were made in the USSR in 18–21-year-old seronegative adults who receive virus twice at intervals of 10–14 days administrated by nasal spray. Viruses were attenuated by passage for varying periods at 25 °C and both donor viruses and recombinants proved temperature sensitive. In 1961–1964, when H2N2 viruses were circulating, 5165 children aged from 1 to 6 years received the ca A/Leningrad/57 (H2N2) virus. Some febrile reactions occurred but only in less than 1% of the children. Further studies of recombinants with H3N2 or H1N1 antigens and the same Leningrad H2N2 parent after 47 serial passages under cold conditions of cultivation (25 °C) were conducted in children, half of whom had no detectable serum antibody to the vaccine strain. No reactions occurred and over 90% of the children responded with antibody production. It is clear from the earlier papers by Alexieva et al. [91, 92] that intranasal administration of children aged 7–15 years were too reactogenic and that this is the reason why the peroral route has been chosen for routine administration in the USSR.

A Japanese virus recovered in 1957, (A/Okuda/57(H2N2), was found to be attenuated for children and served as a donor of attenuation both in Japan and in England. Japanese workers, Zhilova et al. [92] developed a recombinant virus (KO-1) from ultraviolet-irradiated A/Okuda/57 and wild-type A/Kumamoto/22/76 (H3N2). Serial passaging in eggs, in the presence of normal horse serum was followed by plaque purification and later clinical tests in a few children. The M (membrane) gene was found to have been donated by the Okuda parent. From reassortants with other human viruses, a candidate WRL 105 virus was selected and underwent clinical trials without harmful clinical effects [93] but has been little investigated since that time.

Mammalian cell culture vaccines

Cultivation of influenza viruses in mammalian cells rather than eggs initially encouraged two manufactures to invest in cell culture fermenters for vaccine production [22, 23]. Many more groups are now researching these technologies. Capacity can be increased to cope with a surge in demand for a pandemic virus vaccine. Moreover, the final vaccine has the theoretical advantage of the absence of egg proteins. The cell culture vaccine virus is also easier to purify. Where clinical isolates of influenza viruses are culti-

vated in mammalian cells and eggs in parallel, different antigenic variants may be selected [94]. The biological variants have amino acid substitutions in the receptor binding site in proximity to an antigenic site on the HA, and an amino acid change in this region can alter antigenicity. Of the two virus subpopulations that can be selected, the virus which is grown on MDCK (or Vero) cells rather than in eggs appears more closely related to the wild-type clinical virus. There is some indication that cell-grown virus vaccines offer greater protection in animal models than the corresponding egg-grown vaccine. These are all powerful arguments in favor of the new generation of influenza vaccines being cultivated currently in Vero [22] or MDCK [23] or Per 6 cells.

Unlike historical vaccines: could newly developed 21st century vaccines induce protection across the different virus subtypes?

There are 16 known subtypes of the HA of influenza A virus. Only three subtypes have caused pandemics in humans, H1, H2 and H3, while H5, H7 and H9, predominantly circulating in birds have crossed the species barrier into humans and caused human outbreaks. We do not know whether these latter three subtypes could mutate into human-to-human transmitters and thereby acquire pandemic potential. At present H5N1 is causing considerable concern in SE Asia. An important question therefore is whether a vaccine could be engineered to give so-called heterotypic or cross-subtype immunity to protect against all these potentially pandemic viruses. It is well known that the internal proteins of influenza A virus such as M1, M2 and NP are shared by all influenza A viruses. These internally situated proteins are certainly immunogenic (particular NP) but could the immunity induced, either T cell or antibody, be broadly reacting?

To back up the central core of this approach, it has been known for 40 years that mice infected with an influenza A (H1N1) virus would later resist a lethal challenge from an influenza A (H3N2) virus. Given the lack of genetic and antigenic relatedness between the H1 and H3 proteins, or indeed the corresponding N1 and N2 proteins, this strong cross-immunity was attributed to an internal protein such as NP or M. However, it has been difficult to construct a solid database and there has been a lingering doubt about this so-called cross-protective immunity. Most virologists deduced, virtually by elimination, that a cross-reactive portion of the HA (HA2) could have provided the cross protection. Furthermore, this cross protection is particularly seen in the mouse model, leading some to conclude that the mouse recognized cross protection epitopes that perhaps humans did not.

Fundamental studies to correlate the genetics and immunology of NP and M established the cytotoxic T cell response to portions of these proteins. However, the work clearly showed that M2 could be a cross-reactive immunogen, although a relatively weak one [95]. The M2 protein is an integral

membrane protein of influenza A viruses that is expressed at the plasma membrane of virus-infected cells and is also present in small amounts on virions. The important extracellular domain, potentially targeted by antibodies and T cells, is conserved by virtually all influenza A viruses. Even the 1918 pandemic virus differs only in one amino acid. The first indication that the M2 was immunologically active was the observation that an anti-M2 monoclonal antibody reduced the spread of virus cell culture. Not unexpectedly, the antibody reacted with the extracellular domain of M2. Even more excitingly, the antibody reduced the replication of virus in mouse lungs. Immunization studies with M2 constructs, however, have given more mixed results. Immunization of mice with DNA plasmid of M1 and M2 gene gave protection mainly *via* T helper cell activity. An alternative approach utilized a hepatitis B core and M2 fusion protein. The cross protection resided in antibodies, although M2-specific antibodies did not neutralize the virus *in vitro*. Presumably, protection was mediated by an indirect mechanism such as complement-mediated cytotoxicity or antibody-dependant cytotoxicity. However, the protection induced in the mouse model was considerably less than that induced by a conventional sub unit HA/NA vaccine.

It could be argued that weak heterotypic immunity may be present already in the community and that this is helping to prevent the emergence of chicken influenza A (H5N1) in SE Asia [96]. Certainly with evidence of tens of millions of domestic birds infected since late 2003 in 13 countries in SE Asia, with only a handful of human infections and only human-to-human transmission in family groups, there is a possibility that the unique co-circulation since 1977 of two influenza A viruses (H1N1 and H3N2) may have enhanced heterotypic immunity in most communities, which in turn abrogates the emergence of chicken influenza A (H5N1) into humans. It would be foolhardy, though, to take this argument to a fuller conclusion and relax preparations for a new pandemic influenza A virus.

The historical use of volunteers to study influenza and vaccines

At present, with the unprecedented research investment into influenza vaccines, there are new discoveries of adjuvants and vaccine formulations to be tested as well as fundamentals of virus transmission, infectiousness and pathogenicity. The ultimate test is in influenza-infected volunteers. This specialized work was initiated over 60 years ago.

During the great pandemic of 1918, when the precise nature of the causative microbe of the Spanish influenza had not been established, a group of American scientists asked for young volunteers from the army and navy. The quest was to probe the nature of the microbe that was already causing devastation in their own country and where, by 1919, 500,000 young people were to die. However, this was not the first study into the precise nature of the microbe. The infection had first been documented a year earlier as a her-

ald wave in the great city-sized military base and encampment of Etaples [6, 12]. Here the British Army constructed the largest establishment [97] in its history, where 100,000 newly recruited soldiers each day intermingled with thousands of wounded soldiers, pigs and, in the nearby villages and markets, with ducks, domestic chickens and geese. These are now recognized as the necessary biological features of an epicenter for the creation of a pandemic virus. We surmise, in retrospect, that an avian virus from a silently infected goose or duck could have crossed species either to a pig or to a soldier, already infected with a human strain of influenza. This is the mixing bowl hypothesis. Indeed, common epidemic influenza was known to be circulating in the winter of 1916–1917 in Etaples. Another factor in Etaples could have been the hundreds of tons of gas of 25 varieties contaminating the landscape of the nearby Somme battlefield, as well as many of the wounded soldiers brought by the night trains into the 12 hospitals on site and causing respiratory distress. A group of pathologists there and at Abbeville, led by G Gibson, raised the question of the nature of the microbe. Could it be a Gram-negative bacterium such as *H. influenzae*, already described by Pfeiffer as the cause of the previous influenza pandemic of 1889? Or could it be a virus? Viruses were rather unknown entities at that time but had been identified by their filter-passing nature. So Gibson's experiment was quite simply to take sputum from a soldier victim and filter it through a Berkefield candle filter, which would hold back any known bacterium but allow the passage of the much smaller ultrafilterable virus. But what then? Gibson had not even considered that a human volunteer would receive the filtrate. In fact, he gave it to a series of macaques and, inadvertently, to himself. He died and the macaques became ill. His premature discovery of new virus influenza has lain undiscovered and hitherto unquoted in the archives of the First World War [98].

Meanwhile, in the USA, a more vigorous decision had been taken, and army and navy volunteers were infected intranasally with filtered material from Spanish influenza victims. Some volunteers were placed 0.5 m from dying servicemen, who coughed in their faces. The incredible result of this heroic endeavor is that not a single volunteer became ill, whereas all around the USA their companions were dying. It is more than possible that the volunteers had already been subclinically infected in the early summer outbreak of 1918, which was less virulent than the autumn virus and would be expected to give cross immunity.

The MRC common cold and influenza quarantine unit in Salisbury (UK)

As soon as the Second World War was over, the Medical Research Council in the UK established the Common Cold Unit in Salisbury at the Harvard Hospital. The hospital was a donation from the USA to cope with expected

bomb casualties from London. In the event, this fully equipped multi-building facility was used as an acute surgical hospital for servicemen. With Christopher Andrewes as its first chief scientist, the unit recruited volunteers to unravel the virological mysteries of respiratory disease. For the next 40 years, a small team of virologists and clinicians infected volunteers and discovered the first human coronavirus, the common cold virus, and were the first to describe the clinical effects of interferons. Essentially similar units were set up in the USA and Russia.

Estimates of vaccine protection obtained in the past by deliberate challenge in quarantine units

The considerable difficulties encountered in mounting field trials led to experiments in which immunized volunteers were subjected to deliberate inoculation with live virus either in the form of attenuated strain or modified wild-type strain. This protocol was suggested by Henle et al. [99], who immunized a group of children with inactivated influenza A (H1N1) virus vaccine and then inoculated them with egg-cultured virus of the same subtype but recently isolated, by inhalation of an aerosol. High rates of infection (75%) were produced in 28 unimmunized children of whom 10 became ill. Those receiving vaccine either escaped subsequent infection or developed serological changes; only 1 child of the 42 children thus challenged became ill. Although this study illustrated the outstanding success of the immunized protocol, there are probably few observers today who would be prepared to submit their children to a similar risk of deliberately induced illness. Such a risk is, of course, experienced during epidemics and Bell et al. [100] undertook a similar experiment in adult volunteers some of whom were immunized with a single dose of inactivated A/Japan/305/57 (H2N2) virus vaccine soon after the A/Asian epidemic began. The volunteers were isolated before being given intranasally pooled nasopharyngeal washings from patients with influenza and this caused clinical illness in 87% of volunteers previously given a placebo. As 50% of the vaccinated volunteers developed fever after challenge in this experiment, the single injection of inactivated vaccine proved relatively ineffective, presumably because of its inadequate immunogenicity.

The information obtained by deliberate challenge of immunized volunteers has been explored in the past using modified attenuated virus strains. Beare et al. [73] did this in their comparison of inactivated or live influenza B vaccines in which a challenge from the live virus B strain was used to assess the comparative efficacy of the two vaccines. Re-inoculation with live virus was resisted better by those receiving the same material a month previously than by those injected with inactivated vaccine.

Couch [101] has reported a number of trials in volunteers after inactivated vaccine using a low dose of an essentially unmodified H3N2 virus

that had received one or two passages in human embryonic kidney culture. It was first established by Greenberg et al. [102] that previous infection by homotypic H3N2 virus gave protection against deliberate exposure for up to 4 years after the original infection. Comparison of inactivated vaccine (A/HongKong/68 (H3N2) given intranasally or subcutaneously showed that following challenge with live virus only those who had developed a serum antibody response after vaccine by either route resisted infection.

In a further trial of an anti-NA inactivated vaccine made from an Heq1N2 virus, it was shown that a reduced frequency of illness and a reduced titer of virus in nasal wash specimens resulted following live H3N2 virus challenge compared with the findings in control subjects. The number of those who contracted infection was also reduced somewhat by the inactivated NA vaccine, thus supporting the suggestion of Schulman et al. [103] that NA antibody, although incapable of neutralizing viral infectivity, could limit the extent of viral replication. Beutner et al. [104] also immunized children with an NA-specific vaccine and noted that antibody to NA had a role protecting against illness rather than against infection. Slepushkin et al. [105] and Monto and Kendal [106] came to similar conclusions with regard to NA vaccine and the clinical evidence of protection from illness.

A series of experiments on volunteers, designed to obtain evidence of protection from vaccines containing viruses that were homotypic or heterologous to the challenge virus, is important in relation to the determination of the best composition of inactivated vaccine. Potter et al. [43] gave one of four inactivated monovalent H3N2 virus vaccines to groups of students, measured their pre- and post-immunization antibodies by HI and NI tests and later challenged all the groups with a live intranasal H3N2 virus (WRL 105). This virus was antigenically nearest to the A/Port Chalmers/73 virus and vaccine from this latter strain and also that containing A/Scotland/74 virus gave better protection against infection than earlier H3N2 virus vaccines; the result thus correlated with the induced HI antibody titers.

Larson and others [107] also challenged the immunity produced by inactivated vaccine made from A/Port Chalmers/73 (H3N2) virus with that from a strain developed by the Pasteur Institute [108]. This virus (30c) with an antigen closely similar to A/England/72 (H3N2) was selected in the laboratory by a method analogous to natural selection by antigenic drift, and thus represents the first human attempt to anticipate antigen variation in nature. Challenge of those immunized with one or the other vaccines showed that protection by the heterologous 30c virus was about one-quarter as effective as that produced by the homologous A/Port Chalmers/73 virus.

Experiences related by Couch also confirm [101] that antibody effective against the homologous HA of the challenging virus is more protective than that formed by heterologous antigen. Protection was also compared after inactivated vaccine by intranasal or subcutaneous routes, which showed that the important mediator of immunity was the serum IgG content of anti-HA rather than the respiratory secretion content of specific IgA.

Figure 8. A volunteer room at the Common Cold and Influenza Unit, Harvard Hospital, Salisbury in the 1980s. Volunteers would stay for 2 weeks in this country-placed unit to be infected and carefully studied for clinical symptoms.

A new quarantine unit in London

We have established a new quarantine unit, based in London (www.retroscreen.com), but very much centered upon the experience and ethos of the Common Cold Unit of the past [109]. In a series of experiments over the past 2 years, we have infected over 250 young volunteers with influenza A (H3N2), influenza B and influenza (H1N1) virus and more recently respiratory syncytial virus, and now have fully characterized virus pools [110]. In the USA, a quarantine unit had already been established in Virginia and also at Baylor, and pioneered work into the new NA inhibitors of influenza using an influenza A virus isolated in 1991 [111]. So far our own unit has focused on evaluating new influenza vaccines [112]. We use groups of 20 young volunteers and quarantine them in a student hostel or hotel along with clinicians and scientists (Fig. 8). The MRC Common Cold Unit was rooted strongly in the post-war era with deck chairs, free run rabbits, coun-

try walks, afternoon cream teas and two-course English meals. Our new unit reflects a more diverse community, so chicken tikka is as common on the menu as roast lamb and baked potatoes, but the wish of many of the volunteers is the same: to contribute to knowledge.

Conclusion

Influenza A virus has a proven record as a "bioterrorist" virus but driven not in Churchill's words by the "evil forces of perverted science" but by the vast unfathomable laws of nature and emergence, re-emergence, and resurgence of natural disease. Information from the human genome project, whereby a significant proportion of the 30 000 active genes are already known to be involved in innate and acquired immunity, provides reassurance that the immune system will continue to provide some protection against new viruses.

Gauguin in his last great painting "Who are we, where have we come from, where are we going?" asks crucial questions about the future of humankind. But it was the medieval painter Breugel who asked the major question, yet to be answered in the 21st century. His medieval painting "The Triumph of Death" shows a horseman on a white charger scything at random and gathering souls during an outbreak of *Pasteurella pestis* in medieval times. The question haunting the painting is "why do some persons survive while others die". Even in 1918 in most communities 99% of persons infected with the virus survived. But why did some die and exactly how were they killed by such a minute and fragile form of life that we know as the orthomyxovirus influenza? Was the immune reaction and ensuing cytokine storm overwhelming, or was virus replication in the endothelial cells of the air sacs more important?

An extraordinary clear message is emerging, which tells us to build our public health infrastructure and continue and expand our epidemiological vigilance and surveillance against all these infectious viruses and bacteria. For pandemic influenza, every country needs a detailed and practical plan and a supply of antiviral drugs and new vaccines at hand. We would then be "at the end of the beginning" as regards protection of all citizens. Influenza was the 20th century's weapon of mass destruction. Nature is the greatest bioterrorist of our world and emerging viruses could do for us all, as easily and as quickly, or even more so, than the Great Influenza of 1918, except for the fact that we now have the ammunition to fight back: knowledge of virus transmission and effective antivirals and vaccines.

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References

1 Phillips H, Killingray D (2002) *The Spanish influenza pandemic of 1918–1919: New perspectives*. Routledge Social History of Medicine Series
2 Churchill WS (1993) *The Great War*, Vols 1 and 2. George Newnes Ltd, London
3 Crosby AW (1918) *America's Forgotten Pandemic*. Cambridge University Press, New York
4 Medical Research Committee (1919) Special Report Series No 36. *Studies of influenza in Hospitals of the British Armies in France, 1918*. HM Stationery Office, London, 112
5 *Reports on the Pandemic of Influenza 1918–1919* (1920) Reports on Public Health and Medical subjects, No 4. Stationery Office, London
6 Oxford JS (2000) Influenza A Pandemics of the 20th century with special reference to 1918: Virology, pathology and epidemiology. *Rev Med Virol* 10: 119–133
7 Macpherson WG, Herringham WP, Elliott TR, Balfour A (1927) *Medical Services Diseases of the War*. Vol. 2. *Medical Aspects of Aviation and Gas Warfare and Gas Poisoning*. HMSO, London
8 Collier L, Oxford JS (2007) *Human Virology: A Text for Students of Medicine.* Oxford University Press, Oxford
9 Stuart-Harris CH, Schild GC, Oxford JS (1983) *Influenza: The Viruses and the Disease.* Edward Arnold, London
10 Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S, Burke DS (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437: 209–214
11 Barry JM (2004) *The Great Influenza, the epic story of the deadliest plague in history.* Viking, New York
12 Oxford JS (2005) Preparing for the first influenza pandemic of the 21st century. *Lancet Infect Dis* 5: 129–132
13 Oxford JS, Lambkin-Williams R, Sefton A, Daniels R, Elliot A, Brown R Gill D (2005) A hypothesis: The conjunction of soldiers, gas, pigs, ducks, geese and horses in Northern France during the Great War provided the conditions for the emergence of the "Spanish" influenza pandemic of 1918–1919. *Vaccine* 23: 940–945
14 House of Lords Report on Pandemic Influenza (2005)
15 Miller GL, Stanley WM (1944) Quantative aspects of the red blood cell agglutination test for influenza virus. *J Exp Med* 79: 185
16 Burnet FM (1941) Growth of influenza virus in the allantoic cavity of the chick embryo. *Aus J Exp Biol Med Sci* 19: 291
17 Hobson D, Curry RL, Beare AS, Word-Gardner A (1972) The role of serum HI antibody in protein against challenge infection with influenza A and B viruses. *J Hyg* 70: 767–777
18 Schild GC, Wood TM, Newman RW (1975) A single radial immunodiffusion technique for the assay of haemagglutinin antigen. *WHO Bull* 52: 223–231
19 Palese P, Schulman JL (1976) Mapping of the influenza virus genome:

Identification of the haemagglutinin and neuraminidase genes. *Proc Natl Acad Sci USA* 73: 2142–2146

20 Hoffman E, Neumann G, Kawaoka Y, Hoborn G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci USA* 97: 6108–6113

21 Schickli JH, Flandorfer A, Nakaya T, Martinez-Sobrido L, Garcia-Sastre A, Palese P (2001) Plasmid-only rescue of influenza A virus vaccine candidates. *Philos Trans R Soc London B* 356: 1965–1973

22 Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F (1998) Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. *Vaccine* 16: 960–968

23 Palache AM, Brands R, van Scharrenburg G (1997) Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells or fertilised chicken eggs. *J Infect Dis* (Suppl 1): S20–S23

24 Francis T Jr, Nagill TP (1935) Immunological studies with the virus of influenza. *J Exp Med* 62: 505

25 Andrewes CH, Smith W (1937) Influenza: Further experiments on the active immunisation of mice. *Br J Exp Pathol* 18: 43

26 Commission on Influenza, Board of Influenza and other epidemic diseases in the Army (1944) A clinical evaluation of vaccination against influenza. *J Am Med Assoc* 124: 982

27 Davenport FM, Hennessy AV, Brandon FM, Webster RG, Barrett CD Jr, Lease GO (1964) Comparisons of serological and febrile responses in humans to vaccination with influenza viruses or their haemagglutinins. *J Lab Clin Med* 63: 5–13

28 Brandon FB, Cox F, Lease GO, Timm EA, Quinn E, McLean IW Jr (1967) Respiratory virus vaccines. III. Some biological properties of sephadex-purified ether-extracted influenza virus antigens. *J Immunol* 98: 800–805

29 Duxbury AE, Hampson AW, Sievers JGM (1968) Antibody response in humans to deoxycholate-treated influenza virus vaccines. *J Immunol* 101: 62–67

30 Francis T Jr, Salk JE, Quilligan JJ Jr (1947) Experience with vaccination against influenza in the Spring of 1947. *Am J Public Health* 37: 1013–1016

31 Loosli CG, Schoenberger J, Barnett G (1948) Results of vaccination against influenza during the epidemic of 1947. *J Lab Clin Med* 33: 789

32 Kilbourne ED (1969) Future influenza vaccines and use of genetic recombinants. *Bull World Health Organ* 41: 643–645

33 Reimer CB, Baker RS, van Frank RM, Newlin TE, Cline GB, Anderson NG (1967) Purification of large quantities of influenza virus by density-gradient centrifugation. *J Virol* 1:1207–1216

34 Glezen WP, Loda FA, Denny FW (1969) A field evaluation of inactivated, zonal-centrifuged influenza vaccines in children in Chapel Hill, North Carolina, 1968–1969. *Bull World Health Organ* 41: 566–569

35 Symposium of influenza A (H1N1) (1977) *J Infect Dis* Suppl 136

36 Salk JE (1948) Reactions to concentrated influenza vaccines. *J Immunol* 58: 369

37 Potter CW, Jennings R, Clark A (1977) The antibody response and immunity to

challenge infection induced by whole inactivated and Tween-Ether split influenza vaccines. *Dev Biol Stand* 39: 323–328

38 Ennis FA, Mayner RE, Barry DW, Manischewitz JE, Dunlap RC, Verbonitz MW, Bozeman RM, Schild GC (1977) Correlation of laboratory studies with clinical responses to A/New Jersey influenza vaccines. *J Infect Dis* (Suppl) 136: S397–406

39 Wood JM, Schild GC, Newman RW Seagroatt V (1977) Application of an improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen content of whole virus and subunit vaccines. *Dev Biol Stand* 39: 193–200

40 Holland WW, Isaacs A, Clarke SKR, Heath RB (1958) A serological trial of Asian influenza vaccine after the Autumn epidemic. *Lancet* I: 820–822

41 Nicholson KG, Tyrrell DAJ, Harrison P, Potter CW, Jennings R. Clark A (1979) Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine; serological responses and clinical reactions. *J Biol Stand* 7: 123–136

42 Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS, Hierholzer JC (1970) Studies on inactivated influenza vaccines. II. Effect of increasing dosage on antibody with resistance to influenza in man. *Am J Med* 92: 248–256

43 Potter CW, Jennings R, Nicholson K, Tyrrell DAJ, Dickinson KG (1977) Immunity to attenuated influenza virus WRL 105 infection induced by heterologous, inactivated influenza A virus vaccines. *J Hyg (Camb)* 79: 321–332

44 Brady MI, Furminger IGS (1976) A surface antigen influenza vaccine 1. Purification of haemagglutinin and neuraminidase proteins. 2. Pyrogenicity and antigenicity. *J Hyg (Camb)* 77: 161–172

45 Pandemic Working Group of Medical Research Council's Committee on Influenza and other respiratory virus vaccines (1977) Antibody responses and reactogenicity of graded doses of inactivated influenza A/New Jersey/76 whole-virus vaccine in humans. *J Infect Dis* 136: S475

46 Medical Research Council Committee on Influenza Vaccine (1953) Clinical trials of influenza vaccine. *Br Med J* 2: 1173–1173

47 Medical Research Council Committee on Influenza Vaccine (1957) Clinical trials of influenza vaccine *Br Med J* 2: 1–7

48 Medical Research Council Committee on Influenza Vaccine (1958) Trials of an Asian influenza vaccine. *Br Med J* 1: 415–418

49 Medical Research Council Committee on Influenza Vaccine (1964) Clinical trials of oil-adjuvant influenza vaccine, 1960–3. *Br Med J* 2: 267–271

50 Potter CW, Jennings R, Phair JP, Clarke A, Stuart-Harris CH (1977) Dose-response relationship after immunisation of volunteers with a new surface-antigen-adsorbed influenza virus vaccine. *J Infect Dis* 135: 423–431

51 Kendal AP, Bozeman FM, Ennis FA (1980) Further studies of the neuraminidase content of inactivated influenza vaccines and the neuraminidase antibody responses after vaccination of immunologically primed and unprimed populations. *Infect Immun* 29: 966–971

52 Webster RG, Kasel JA, Couch RB, Laver WG (1976) Influenza virus subunit vaccines. II. Immunogenicity and original antigenic sin in humans. *J Infect Dis* 134: 48–58

53 Oxford JS, Schild GC, Potter C, Jennings R (1979) The specificity of the anti-haemagluttinin antibody response induced in man by inactivated vaccines and by natural infection. *J Hyg (Camb)* 82: 51–56
54 Oxford JS, Haaheim LR, Slepushkin A, Werner J, Kuwert E, Schild GC (1981) Strain specificity of serum antibody to the haemagglutinin of influenza A (H3N2) viruses in children following immunisation or natural infection. *J Hyg (Camb)* 86: 17–26
55 Appleby JC, Himmelweit F, Stuart-Harris CH (1951) Influenza virus A vaccines. Comparison of intradermal and subcutaneous routes. *Lancet* 1: 1384–1387
56 Mc Carroll JR, Kilbourne ED (1958) Immunisation with Asian strain influenza vaccine – Equivalence of the subcutaneous and intradermal routes. *N Engl J Med* 259: 618–621
57 Tauraso NM, Gleckman R, Pedreira FA, Sabbaj J, Yahwak R, Madoff MA (1969) Effect of dosage and route of inoculation upon antigenicity of inactivated influenza virus vaccine (Hong Kong strain) in man. *Bull World Health Organ* 41: 507–516
58 Waldman RH, Case JA, Fulk RV, Togo Y, Hornick RB, Heiner GG, Dawkin Jun AT, Mann JJ (1968) Influenza antibody in human respiratory secretions after subcutaneous or respiratory immunisation with inactivated virus. *Nature* 218: 594–595
59 Waldman RH, Wigley FM, Small PA Jr (1970) Specificity of respiratory secretion antibody against influenza virus. *J Immunol* 105: 1477–1483
60 Phillips CA, Forsythe BR, Christmas WA, Gump DW, Whorton EB, Rogers I, Rudin A (1970) Purified influenza vaccine; clinical and serological response to varying doses and different routes of immunisation. *J Infect Dis* 122: 26–32
61 Potter CW, Stuart-Harris CH, McClaren C (1972) Antibody in respiratory secretions following immunisation with influenza virus vaccines. In, FT Perkins, RHS Regamey (eds): *International Symposium Series Immunological standardisation* 20. Karger, Basel, 198
62 Ruben FL, Potter CW, Stuart-Harris CH (1975) Humoral and secretory antibody responses to immunisation with low and high dosage split influenza virus vaccines. *Arch Virol* 47: 157–166
63 Downie JC, Stuart-Harris CH (1970) The production of neutralising activity in serum and nasal secretions following immunisation with influenza B virus. *J Hyg (Camb)* 68: 233–244
64 Ennis FA, Dowdle WR, Barry DW, Hochstein HD, Wright PF, Karzon DT, Marine WM, Meyer HM Jr (1977) Endotoxin content and clinical reactivity to influenza vaccines. *J Biol Stand* 5: 165–167
65 Wells CEC (1971) A neurological note on vaccinations against influenza. *Br Med J* 3: 755–756
66 Langmuir AD (1979) Guillain-Barré syndrome: The swine influenza virus vaccine incident in the United States of America, 1976–77. *JR Soc Med* 72: 660–669
67 Hurwitz ES, Schonberger LB, Nelson DB, Holman RC (1981) Guillain-Barré syndrome and the 1978–1979 influenza vaccine. *N Engl J Med* 304: 1557–1561
68 Mogabgab WJ, Liederman E (1970) Immunogenicity of 1967 polyvent and 1968 Hong Kong influenza vaccines. *J Am Med Assoc* 211: 1672–1676

69 Knight V, Couch RB, Douglas RG, Tauraso NM (1971) Serological responses and results of natural infectious challenge of recipients of zonal ultracentrifuged influenza.A2/AICHI/2/68 vaccine. *Bull World Health Organ* 45: 767–771
70 Mawson J, Swan C (1943) Intranasal vaccination of humans with living attenuated influenza virus strains. *Med J Aust* 1: 394
71 Stuart-Harris CH (1980) Present status of live influenza virus vaccine. *J Infect Dis* 142: 784
72 Kendal AP, Maasab H.F, Alexandrova GI, Ghendon YZ (1981) Development of cold-adapted recombinant live attenuated influenza A vaccines in the USA and USSR. *Antiviral Res* 1: 339
73 Beare AS, Bynoe ML, Tyrrell DAJ (1968) Investigation into attenuation of influenza viruses by serial passage. *Br Med J* 4: 482–484
74 Huygelen C, Petermans J, Vascoboinic E, Berge E, Colinet G (1973) Live attenuated influenza virus vaccine *in vitro* and *in vivo* properties. In: FT Perkins, RHS Regamey (eds): *International Symposium on Influenza Vaccines for Man and Horses. Series Immunobiological Standards*, vol 20. Karger, Basel, 152
75 Florent G, Lobmann M, Beare AS, Zygraich N (1977) RNA's of influenza virus recombinants derived from parents of known virulence for man. *Arch Virol* 54: 19–28
76 Florent G (1980) Gene constellation of live influenza A vaccines. *Arch Virol* 64: 171–173
77 Beare AS, Hall TS (1971) Recombinant influenza A viruses as live vaccine for man. *Lancet* II: 1271–1271
78 Beare AS, Reed S (1977) The study of antiviral compounds in volunteers. In: JS Oxford (ed): *Chemoprophylaxis and Viral Infections of the Respiratory Tract*, vol 2. CRC Press, Cleveland, 27
79 Oxford JS, McGeoch DJ, Schild GC, Beare AS (1978) Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. *Nature* 273:778–779
80 Poliomyelitis Congresses (1948–61) Papers and discussions at 1st, 2nd, 3rd, 4th and 5th International Poliomyelitis Congresses 1948, 1951, 1954, 1957 and 1961. Lippincott, Philadelphia
81 Jennings R, Potter CW, Teh CZ, Mahmud MI (1980) The replication of Type A influenza viruses in the infant rat: A marker for virus attenuation. *J Gen Virol* 49: 343–354
82 Murphy BR, Clements ML, Maasab HF, Buckler-White AJ, Tian S-F, London WT, Chanock RM (1984) The basis of attenuation of virulence of influenza virus for man. In: CH Stuart-Harris, CW Potter (eds): *Molecular Virology and Epidemiology of Influenza*. Academic Press, London, 211
83 Chanock RM, Murphy BR (1979) Genetic approaches to control of influenza. *Perspect Biol Med* 22: S37
84 Richman DD, Murphy BR, Chanock RM, Gwaltney JM Jr, Douglas RG, Betts RF, Blacklow NR, Rose FB, Parrino TA, Levine MM, Caplan ES (1976) Temperature-sensitive mutants of influenza A virus XII. Safety, antigenicity, transmissibility and efficacy of influenza A/Udorn/72-*ts*-1[E] recombinant viruses in human adults. *J Infect Dis* 134: 585–594

85 Maassab HF (1967) Adaptation and growth characteristics of influenza virus at 25 °C. *Nature* 213: 612–614
86 Maassab HF (1969) Biological and immunologic characteristics of cold-adapted influenza virus. *J Immunol* 102: 728–732
87 Spring SB, Maassab HF, Kendal AP, Murphy BR, Chanock RM (1977) Cold adapted variants of influenza A. II. Comparison of the genetic and biological properties of *ts* mutants and recombinants of the cold-adapted A/Ann Arbor/6/60 strain. *Arch Virol* 55: 233–246
88 Wright PF, Okabe N, McKee KT Jr, Maasab HF, Karzon DT (1982) Cold-adapted recombinant influenza A virus vaccines in young seronegative children. *J Infect Dis* 146: 71–79
89 Murphy BR, Tierney EL, Barbour BA, Yolken RH, Alling DW, Holley HP Jr, Mayner RE, Chanock RM (1980) Use of the enzyme-linked immunosorbent assay to detect serum antibody responses of volunteers who received attenuated influenza A virus vaccine. *Infect Immun* 29: 342–347
90 Alexandrova GI, Smorodintsev AA (1965) Obtaining of an additionally attenuated vaccinating cryophilic influenza strain. *Roum Rev Inframicrobiol* 2: 179
91 Alexieva RB, Petrova SM, Janceva BN (1971) Studies on some biological properties of vaccinal influenza strains cultivated at low temperatures. In: B Gusic (ed): *Proceedings of the Symposium on Live Influenza Vaccine*. Yugoslav Academy of science and Arts, Zagreb, 43
92 Zhilova GP, Alexandrova GI, Zykov MP, Smorodintsev AA (1977) Some problems with modern influenza prophylaxis with live vaccine. *J Infect Dis* 135: 681–686
93 Morris CA, Freestone DS, Stealey VM, Oliver PR (1975) Recombinant WRL 105 strain live attenuated influenza vaccine. Immunogenicity, reactivity and transmissibility. *Lancet* II: 196–199
94 Schild GC, Oxford JS, de Jong JC (1983) Evidence for host-cell selection of influenza virus antigenic variants. *Nature* 303: 706–709
95 Neiryncks S, Deroot T, Saelens X, Vanland Schoot P, Tou WM, Friers W (1999) A universal influenza A vaccine based on the extra cellular domain of the M2 protein. *Nat Med* 5: 1157–1163
96 Rimmelzwaan GF, Baars M, van Beek R, van Amerongen G, Lövgren-Bengtsson K, Claas EC, Osterhaus AD (1997) Induction of protective immunity against influenza virus in a macaque model: Comparison of conventional and ISCOM vaccines. *J Gen Virol* 78: 757–765
97 Britain V (1989) *Testament of Youth: An Autobiographical Study of the Years 1900–1925*. Penguin, New York
98 Gibson HG, Bowman FB, Connor JI (1919) The etiology of influenza: A filterable virus as the cause (with some notes on the culture of the virus by the method of Noguchi). In: *Studies of Influenza in Hospitals of the British armies in France, 1918*. HMSO, London, 36, 19–36
99 Henle W, Henle G, Stokes J Jr (1943) Demonstration of the efficacy of vaccination against influenza type A by experimental infection of human beings. *J Immunol* 46: 163
100 Bell JA, Ward TG, Kapikian AZ, Shelokov A, Reichelderfer TE, Huebner RJ

(1957) Artificially induced Asian influenza in vaccinated and unvaccinated volunteers. *J Am Med Assoc* 165: 1366–1373

101 Couch RB (1975) Assessment of immunity to influenza virus using artificial challenge of normal volunteers with influenza virus. *Dev Biol Stand* 28: 295–306

102 Greenberg SB, Couch RB, Kasel JA (1973) Duration of immunity to type A influenza. *Clin Res* 21: 600

103 Schulman JL, Khakpour M, Kilbourne ED (1968) Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. *J Virol* 2: 778–786

104 Beutner KR, Chow T, Rubi U, Strussenberg J, Clement J, Ogra PL (1979) Evaluation of a neuraminidase-specific influenza A virus vaccine in children. Antibody responses and effects on two successive outbreaks of natural infection. *J Infect Dis* 140: 844–850

105 Slepushkin AN, Schild GC, Beare AS, Chinn S, Tyrrell DAJ (1971) Neuraminidase and resistance to vaccination with live influenza A2 Hong Kong vaccine. *J Hyg (Camb)* 69: 571–578

106 Monto AS, Kendal AP (1973) Effect of neuraminidase antibody on Hong Kong influenza. *Lancet* I: 623–625

107 Larson HE, Tyrrell DAJ, Bowker CH, Potter CW, Schild GC (1978) Immunity to challenge in volunteers vaccinated with an inactivated current or earlier strain of influenza A (H3N2). *J Hyg (Camb)* 80: 243–248

108 Fazekas de St. Groth S, Hannoun C (1973) Sélection par pression immunologique de mutants dominants du virus de la grippe A (Hong Kong). *C R Acad Sci de Paris D* 276: 1917

109 Tyrrell D, Fielder M (2002) *Cold Wars; The Fight Against the Common Cold*. Oxford University Press, Oxford

110 Fries L, Lambkin-Williams R, Gelder C, White G, Burt D, Lowell G, Oxford J (2004) FluInsure^{TM}, an inactivated trivalent influenza vaccine for intranasal administration, is protective in human challenge with A/Panama/2007/99 (H3N2) virus. In: Y Kawaoka (ed): *Options for the Control of Influenza, V*. International Congress Series. Elsevier, London, 1263, 661–665

111 Treanor JJ, Hayden FG (1998) Volunteer challenge studies. In: KG Nicholson, RG Webster, AJ Hay (eds): *Textbook of Influenza*. Blackwell Science, Oxford

112 Jones S, Evans K, McElwaine-John H, Sharpe M, Oxford J, Lambkin-Williams R, Mant T, Nolan A, Zambon M (2008) DNA vaccination protects against an influenza challenge in a phase 1b double blind randomised placebo controlled clinical trial (submitted)

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The epidemiology of influenza and its control

Lone Simonsen[1], Cécile Viboud[2], Robert J. Taylor[3] and Mark A. Miller[2]

[1]George Washington University School of Public Health and Health Services, Washington, DC, USA; [2]Fogarty International Center, National Institutes of Health, Bethesda, Maryland, USA; [3]SAGE Analytica, LLC, Bethesda, Maryland, USA

Abstract

In this chapter we highlight how recent advances in influenza epidemiology can inform existing strategies for disease control. As a field, influenza epidemiology has benefited greatly from analysis of large data sets regarding hospitalization, mortality, and outpatient visits. These data have allowed comparison of the impact of influenza in various climates and the evaluation of the direct and indirect benefits of vaccination, the latter through the vaccination of "transmitter populations" such as school children, to achieve herd immunity. Moreover, the resolution of influenza epidemiology has undergone a leap to the molecular level due to the integration of new antigenic and viral genomic data with classical epidemiological indicators. Finally, the new data have led to an infusion of quantitative studies from the fields of evolutionary and molecular biology, population genetics and mathematics.

The progress can be seen in many forms. The emerging field of molecular influenza epidemiology is providing deeper insight into global patterns of viral emergence, the important role of reassortment in generating genetic novelty, and global diffusion of virus variants – including the mysterious but crucial role of the Tropics, especially Southeast Asia, as a source of new variants. Deeper stratification of contemporary and historic epidemiological data is providing a more detailed picture of the effect of age and other host characteristics on outcomes, as well as better estimates of the transmissibility of pandemic and seasonal influenza viruses. Re-examination of observational studies of vaccine effectiveness in seniors is leading to reconsideration of seasonal and pandemic vaccine priorities, while mathematical modelers have developed tools to explore optimal strategies for mitigating a future pandemic. The field of influenza epidemiology has rapidly progressed in the past decade and become truly multidisciplinary. Progress could be sustained in the next decade by even closer ties with virology, evolutionary biology, immunology, and genetics.

Introduction

Influenza viruses evolve continuously, challenging mammalian and avian hosts with new variants and causing complex epidemic patterns with regard

to age, place and time. Human influenza viruses cause disease through a variety of direct and indirect pathological effects. The direct effects include destruction of infected cells, damage to respiratory epithelium, and immunological responses that cause general malaise and pneumonia. Indirect consequences of infection include secondary bacterial infection pathogens as a result of tissue damage and exacerbation of underlying co-morbid conditions such as cardiovascular disease, renal disease, diabetes or chronic pulmonary disease [1, 2]. Morbidity and mortality associated with influenza is frequently cited as that which falls within broad disease categories, such as pneumonia and influenza (P&I), respiratory illness, or all-cause (AC) mortality. In the latter case, influenza infection is usually not laboratory confirmed but its health impact can be determined through statistical inference, based on seasonal coincidence of virus circulation and disease outcomes [3–5].

Given the difficulty of directly measuring influenza morbidity and mortality, time-series models have been developed to elucidate patterns of disease within various age groups and populations [5–13]. Such models allow for quantification of disease burden by season and severity of circulating strains [9]. Historical archived data have also elucidated the links between influenza transmission across geographic regions and population movements [14], and allowed comparison of the impact and transmissibility of past pandemics and epidemics in multiple countries [15–24]. Similar models applied to prospective syndromic surveillance data have allowed the study of the epidemiological signature of recurring and reemerging strains of influenza on populations [25]. Mathematical modeling and statistical analyses of influenza activity in tropical countries have rekindled interest in the old mystery of the seasonal drivers of influenza, and offered new insights into the circulation patterns of this virus at the global and regional scales [26–28] (Fig. 1).

The field of influenza epidemiology has recently undergone a quantum leap in resolution due to the increased availability of antigenic and viral genomic data and the integration of these data with classical epidemiological indicators [29–33]. The emerging field of molecular influenza epidemiology (also known as "phylodynamics" [29]) has already provided a much clearer picture of the complex dynamics of global influenza virus circulation and reassortment patterns. The growing number of available influenza genome sequences from specimens collected around the world has started to create a more coherent picture of the global epidemiology of influenza, in particular the interplay between virus evolution, population immunity and impact.

Throughout this chapter, our intention is to highlight how influenza epidemiology can help refine existing strategies for influenza control, especially vaccination. We begin by examining the spatial and temporal spread of seasonal influenza, and we discuss how old and new analytical tools are reshaping quantitative thinking in influenza epidemiology and control. We

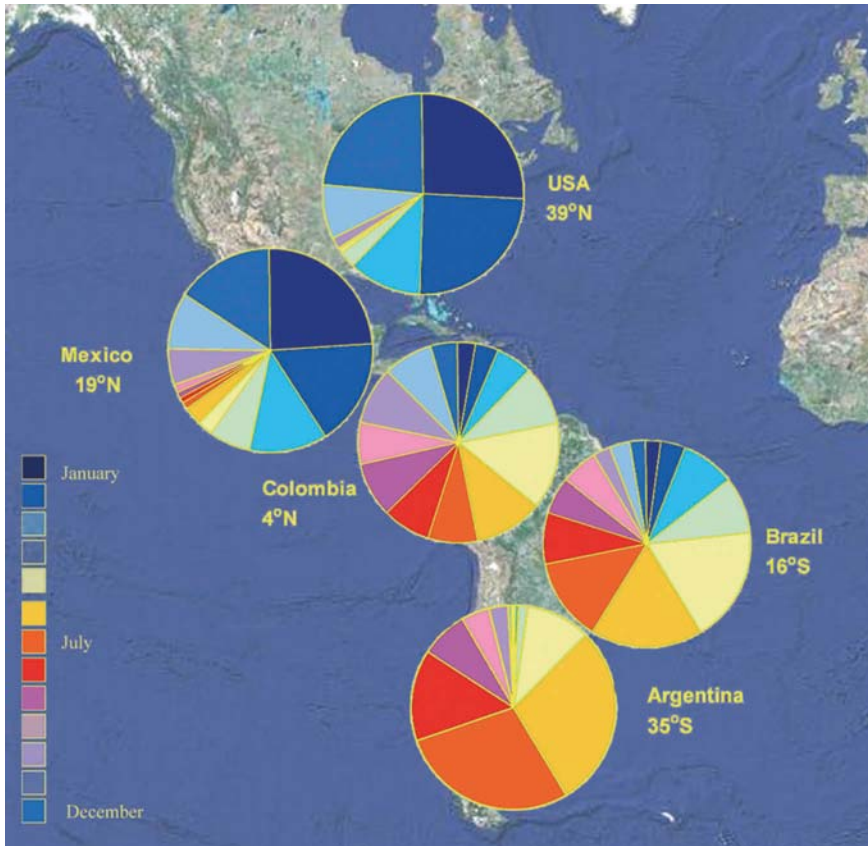

Figure 1. Comparison of influenza virus seasonal patterns in temperate and tropical countries in the Americas.
Pie charts represent the percent distribution of influenza virus isolation by month as compiled from WHO data between 1997 and 2005 (color bar). Note the transition in seasonal patterns from North to South. The latitude of the capital city is indicated for each country in the legend. Adapted from Viboud et al. [27].

then move on to several aspects of historical patterns of disease observed during the three pandemics of the 20th century, and touch upon the epidemiology of the recent avian A/H5N1 influenza threat. We conclude with an in-depth review of what is known about vaccine benefits in seniors – the group that bears the greatest influenza-related mortality burden – and a discussion of the implications of influenza epidemiology for pandemic planning. Readers looking for a more comprehensive treatment of the vast field of influenza epidemiology should consider supplementing this chapter with some of the classical reviews published over the last decades [2, 34–38].

Seasonal influenza: New insights

The health impact of annual influenza epidemics varies greatly in terms of hospitalizations and deaths. In the United States, clinical illness affects 5–20% of the population and asymptomatically infects a larger number [37]. Infants, who are exposed to influenza epidemics as a novel antigenic challenge after maternal antibodies decline, may have attack rates as high as 30–50% in their first year of life, depending on the frequency of contacts with older siblings [39]. For reasons not fully understood, influenza viruses cause seasonal epidemics in the Northern and Southern Hemisphere during their respective winters. In the tropics the timing of activity is less defined, with sometimes year-round circulation or bi-seasonal peaks during the year (Fig. 1) [27, 28, 40–43].

Methods used to estimate the mortality burden of influenza

Estimates of the number of influenza-related deaths are typically inferred through statistical analysis. The syndromic diagnosis "influenza-like illness" is rarely laboratory confirmed and is often caused by non-influenza respiratory viruses. Moreover, influenza is often a precipitating factor that brings about death from secondary bacterial pneumonia or an underlying chronic disorder. In these cases, the underlying disorders are typically identified as the cause of death. Because of these ascertainment problems, determining the magnitude of influenza-related deaths requires indirect approaches in which mathematical or statistical models are applied to broad death categories. This approach was first used in 1847 by William Farr to characterize an influenza epidemic in London and was further developed and extensively used throughout the 20th century. The refinements include Serfling-like cyclical regression models [6, 12, 18, 21, 44–47] and Arima models [7, 8, 48, 49], which are applied to monthly or weekly time series of P&I or AC mortality. Overall, investigators from at least 17 countries have used variants of these Serfling-type models to estimate the mortality burden of influenza. Similar issues and statistical approaches apply to the estimation of the influenza burden on hospitalization [10, 11, 50]. The various statistical approaches all attribute "excess deaths" in winter months to influenza. Such seasonal approaches are not suited to studying disease burden of influenza in countries with tropical climates because they require an annual seasonal pattern of viral activity interrupted by influenza-free periods.

More recently, the US Centers for Disease Control and Prevention (CDC) has used an approach to measure hospitalization and mortality burden based on a new generation of seasonal regression models integrating laboratory surveillance data on influenza and respiratory syncytial virus (RSV) [5, 11]. In such models, winter seasonal increases in deaths or hospitalizations are directly proportional to the magnitude of respiratory virus

activity. In the US between 1980 and 2001, Thompson et al. [5, 11] estimated that seasonal influenza epidemics were associated with 17 deaths per 100 000 on average (range 6–28 per 100,000) depending on the severity of the circulating strains. Reassuringly, different model approaches yield similar average estimates of the influenza mortality burden in the US [13, 51, 52], while estimates from Europe and Canada are similar to those from the US [45, 53, 54]. The integration of hospitalization or death indicators and viral surveillance data is particularly useful for the study of influenza in tropical areas (we return to this later).

Age and time variability in influenza-related mortality in temperate climates

Influenza-related deaths contribute ~5% (range 0–10%) of all winter mortality in seniors over 65 in the US, with similar proportion in Italy and Canada [12, 54, 55]. Seasons dominated by the influenza A/H3N2 subtype are typically associated with 2–3-fold higher mortality than seasons dominated by influenza A/H1N1 and influenza B viruses. But the pattern is not always clear; there have been influenza A/H3N2 dominated seasons with little excess mortality (e.g., 2005–2006 Northern hemisphere season). The age-specific risk of influenza-related (excess) mortality rates rises sharply past age 65 (Fig. 2). People aged ≥80 years are at approximately 11-fold higher risk than people aged 65–69 years. Moreover, in recent decades about 90% of all influenza-related deaths occurred among seniors ≥65 years, 75% occurred among seniors aged ≥70 years, and 55% occurred among seniors over 80 years [12]. As the population in the US and other developed countries has aged substantially over the last decades, the crude number of influenza-related deaths has been rising. Because the risk of influenza-related death increases exponentially with age in the later decades of life, it is essential to standardize for age when comparing mortality impact in different countries and over time [12, 55].

Burden and circulation patterns of influenza in the tropics

Because most seasonal influenza models ("Serfling approaches") depend on winter seasonality in the data, they are not generally useful for tropical countries. However, such models can be used for unusually severe epidemics and pandemics, where the excess disease burden is many fold greater than in average years [44]. Integration of viral surveillance data with death or hospitalization indicators is the most useful approach in tropical settings, although long-term historical surveillance data is usually lacking [27]. A series of studies in Hong-Kong and Singapore recently found that annual influenza-related hospitalization and mortality rates in wealthy

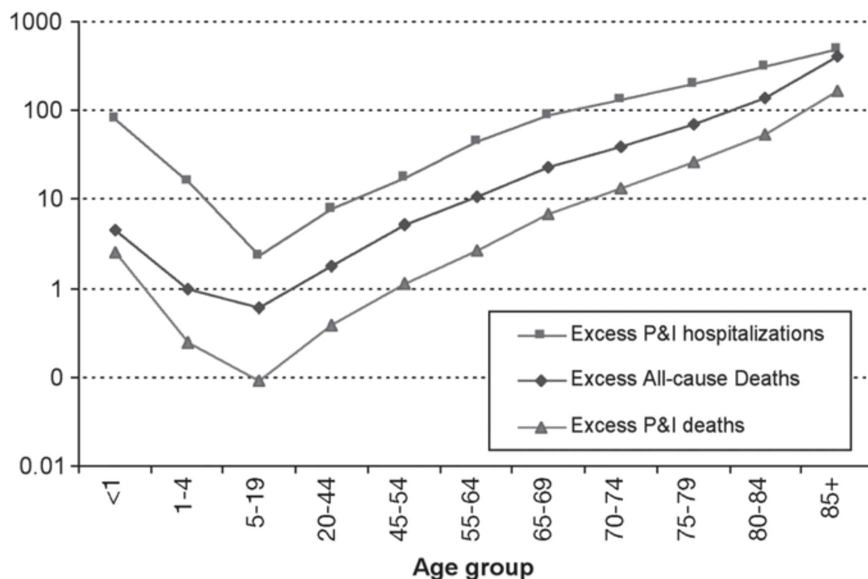

Figure 2. Average age-specific rates of influenza-related excess deaths and hospitalizations for ten seasons during 1990–2001 in the US (estimated from Serfling regression models).
Note the characteristic U shape of severe disease burden by age that characterizes seasonal influenza. Data source: Vital Statistics from the National Center for Health Statistics (NCHS) and hospital discharge data from Agency for Health Care Research and Quality (AHRQ).

(sub)tropical locations are similar to those in temperate countries [40–43]. In Hong Kong, as in many other countries, the impact of influenza is seen not only on pneumonia and influenza outcomes, but also on a wide range of chronic health conditions such as diabetes and cardiovascular diseases [43]. In addition, influenza-related hospitalization rates in Hong-Kong vary with age as a U-shaped curve [42], in which young infants and elderly people are at highest risk of severe outcomes, reminiscent of the age pattern of inter-pandemic influenza in the US and other temperate countries.

The spread of influenza in the tropics has also proven to be an enigma. Influenza seasonality in the southernmost temperate regions is 6 months out of phase with the Northern hemisphere. Most recently, a study from Brazil found seasonal influenza activity starting early in remote, less densely populated equatorial regions of the North (March-April) and traveling in ~3 months to the more temperate areas of the South during their winter season (June–July) [28]. This finding was contrary to what was expected, given that the larger, well-connected, densely populated cities are located in the South. If population movements were a driving factor like in the US [14], then the opposite traveling wave would have been expected. This study has inspired further studies to investigate the circulation of specific influenza virus subtypes during a season based on analysis of viral genomics data.

Finding firm evidence of this unusual circulation pattern suggested from analysis of regional mortality data also bears on considerations of use of Southern or Northern hemisphere vaccine formulation and timing. Because of this study, the Brazilian ministry of health is currently considering changing the timing of vaccination in the North of Brazil to accommodate the early occurrence of influenza in that area.

The burden of influenza in infants and young children

For age groups other than those over 65 years of age, it can be difficult to measure the relatively low seasonal impact of influenza mortality above the expected baseline. But for occasional severe seasons, a surge in P&I deaths can often be seen in children and young adults. For example, the 2003–2004 season was dominated by a new antigenic variant of A/H3N2 viruses (A/Fujian/2003) and was unusually severe; in the US, 153 children with documented influenza infections died of primary or secondary pneumonia and sepsis [56]. Surprisingly, 47% of the children who died had no known underlying risk conditions. The reason for this unusual epidemic of pediatric deaths has not been resolved. As a result of this experience, the CDC enhanced their influenza surveillance system with a reporting system for children hospitalized with laboratory-confirmed influenza (http://www.cdc.gov/flu/ accessed 12 October 2007).

The impact of influenza on morbidity

Very few quantitative data on mild influenza morbidity with known population denominators are available. The most careful studies using the longest existing time series come from the Royal Network of General Practitioners in the UK, which has reported influenza-like-illnesses on a weekly basis since 1966 [53, 57]. Such long-term morbidity records are unique and have allowed the study of the 1968–1969 influenza pandemic transmission patterns based on case data [58]. In addition to the UK, several countries have national sentinel surveillance systems in place (US, France, Netherlands, Australia, and New Zealand are examples). These are used to detect the onset and peak timing of influenza epidemics, as well as the magnitude of morbidity impact relative to surrounding seasons. In the US, emergency room visit time series are now being analyzed in the context of biodefense research and have shed light on inter-annual and age-specific variability in influenza impact [25, 59].

In contrast, quantitative burden studies using samples of national hospital discharge data and estimation approaches similar to those used for excess mortality are more widely available, in particular since the 1970s [11, 50, 60, 61]. The patterns of excess hospitalizations are quite similar to those

of excess mortality, with a U-shaped incidence reflecting the highest values in young children and seniors (Fig. 2).

The relative contribution of influenza and RSV

One controversy in the literature concerns the relative contributions of influenza and RSV to the winter increase in respiratory hospitalizations and deaths, especially among seniors. The current CDC modeling approach simultaneously estimates the influenza and RSV burden by correlating periods of excess mortality with their respective period and magnitude of viral activity [5]. In the overall US population, the CDC investigators estimate the average seasonal RSV burden is approximately one-third that of influenza for all seasons during the 1990s. However, the relative contribution of RSV and influenza varies greatly with age.

For US infants of <12 months of age, the RSV contribution to mortality is more than 2-fold greater than that of influenza (5.5 *versus* 2.2 deaths per 100,000) based on the CDC model [5]. Past age 5, the influenza burden becomes predominant in the US data, in agreement with the age pattern of respiratory deaths in the UK [62]. For seniors over age 65, the CDC model puts the average seasonal RSV burden at ~10,000 deaths, which is about 3-fold lower than the estimated deaths attributed to influenza over the same period. But others disagree; several observational studies set in the UK by Fleming and colleagues [63, 64] have argued that RSV has replaced influenza as the major cause of respiratory mortality and hospitalization, in particular in the elderly. Further, in a recent laboratory-based study set in a large cohort of seniors hospitalized with pneumonia, twice as many hospitalizations were attributed to RSV as influenza [65]. But because influenza-related pneumonia is most often due to secondary bacterial infections (quite distinct from primary RSV pneumonia) that occur long after the triggering influenza infection has been cleared, it is possible that this study substantially underestimated the influenza burden [66].

Two recent studies carefully delineated the relative burden of influenza and RSV in children, using seasonality in pediatric respiratory hospitalizations and focusing the analysis on seasons when the influenza and RSV epidemics occurred at different times [40, 67]. The authors subtracted hospitalization rates during periods of high influenza circulation from baseline "peri-influenza" winter periods when neither influenza nor RSV was circulating (Fig. 3). Using this approach, the authors attributed a similar number of hospitalizations to RSV and influenza in children under 5 in the US [67]. In a parallel study from Hong Kong, investigators attempted to tease out the burden of RSV, influenza and other respiratory pathogens in various age groups in this sub-tropical setting with less clear seasonality [40]. Although influenza burden estimates in Hong-Kong were similar to those of the US in most age groups [27], the specific subgroup of children under 5 appeared

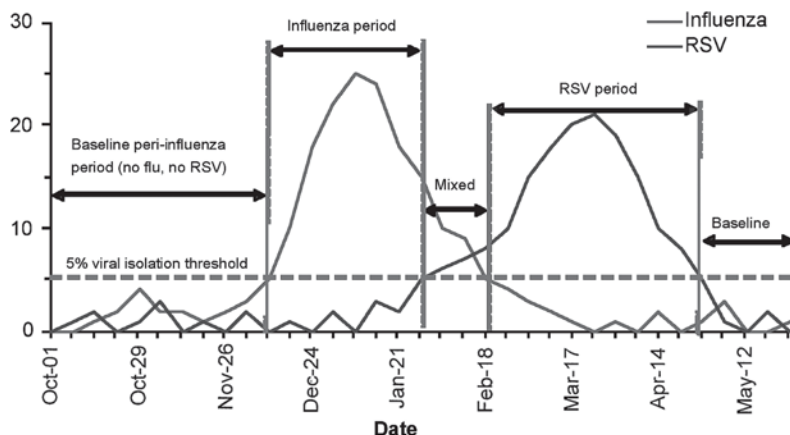

Figure 3. An analytic approach to estimate influenza-related hospitalization rates in US and Hong-Kong children [40, 67].
This method relies on identifying the precise periods of influenza and RSV viral circulation for each season studied. Influenza-related excess rates were calculated as the difference in observed rates between periods of influenza and RSV circulation and those with low circulation of both influenza and RSV.

to have ~10-fold higher rates of hospitalization in Hong-Kong than in the US [40]. Such large discrepancies may reflect true geographical differences in influenza transmission and impact, although they perhaps more likely result from differences in access to hospital care. Indeed, young children in Hong-Kong tend to be rushed to the hospital when they have respiratory symptoms (Malik Peiris, personal communication).

Finally, there is a large body of literature on respiratory virus isolates from children hospitalized with respiratory symptoms in tropical and subtropical settings. These papers, which have been nicely summarized by Weber et al. [68], attribute a substantial proportion of pediatric respiratory hospitalizations to influenza A and B viruses. Unfortunately, it is difficult to compare findings across studies because they are often done using different laboratory techniques and are set in different study years, seasons, and settings (hospital, outpatient clinic, etc.). These studies frequently present a systematic age pattern that suggests that RSV is more important in infancy, with a gradual shift to influenza by about age 5 as the pathogen more likely to cause severe respiratory illness.

Observational transmission studies

The transmission patterns of influenza were carefully documented in classic virus surveillance studies that meticulously followed all respiratory ill-

ness episodes in a large number of families in Cleveland, Ohio, Tecumseh, Michigan and Seattle, Washington in the 1950s through the 1970s [36, 69, 70]. Unfortunately, such careful studies have not been repeated in contemporary populations, so little is known about the consequences of increasing population movements and changing intra-familial interactions. The result is that the current mathematical models employed to "forecast" the likely patterns and spread of a future pandemic influenza virus largely depend on parameter values of transmission and age group dynamics that are decades old and may not reflect modern realities.

In parallel to careful family studies tracking the infection status of each individual, time series mortality data aggregated at the scale of cities, regions or countries can also be used as a proxy to estimate the transmissibility of influenza [16, 19, 20, 23, 24, 71–74]. Two crucial factors, the basic reproductive number R0 and the effective reproductive number, R, have been estimated for pandemic and epidemic influenza. R0 measures the average number of secondary infections per primary case for a new pathogen invading a fully susceptible population (e.g., a pandemic influenza virus); while R measures a similar quantity for a recurrent pathogen re-invading a partially susceptible population (e.g., seasonal influenza virus). Current estimates of R0 and R are in the range of 1.7–5.4 for pandemics and 1.0–2.1 for seasonal influenza epidemics. While these estimates of transmissibility are not as high as for other respiratory viruses (e.g. for measles R is ~15), the generation time for influenza is very short, on the order of 2–4 days. Consequently, in a 2-month period there could be $R^{(60/4)}$ to $R^{(60/2)}$ infections.

Overall, the use of time series of population-level data (hospitalizations, mortality) in large populations has provided a more complete picture of the transmissibility of influenza through space and time. One study correlated mortality peaks in US influenza seasons for the last 30 years with daily transportation data and found that epidemics spread across the country in an average of about 6 weeks, and that transmission was correlated with adult work-travel patterns [14].

Syndromic surveillance and its contributions to influenza epidemiology

Use of real-time syndromic surveillance data is another area with substantial promise in influenza epidemiology. Information technology now allows for the rapid compilation and analysis of electronic health records from emergency rooms, inpatient hospitals and outpatient clinics. Such studies can provide real-time data that can help identify unusual events that may indicate the presence of emerging pathogens.

Syndromic surveillance efforts have already provided a new level of insight into age and geographic patterns of impact of influenza epidemics. In particular, a recent study that combined time series analysis of age-specific emergency room visits with laboratory-confirmed timing of influenza and

RSV periods in New York City demonstrated that the burden of a contemporary influenza epidemic varies greatly at the level of age cohorts in children and adults, perhaps as a consequence of different historical exposures to influenza [25].

A higher resolution: genomics and molecular epidemiology of influenza

A new generation of phylogenetic and antigenic studies of influenza viruses has increased our understanding of the emergence and spread of new influenza drift variants both locally and globally. Begun in 2004, the Influenza Genome Sequencing Project (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/request.cgi accessed 6 March 2006, http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html Accessed 25 May 2007) as well as an increased number of sequences published by other contributors, has resulted in the publication of ~2400 complete influenza virus genomes (mostly human isolates). These data have led directly to important advances in molecular influenza epidemiology [31]. A handful of new studies emerging from this project have demonstrated a high frequency of gene segment reassortment in A/H3N2 viruses, perhaps more frequent around the time of transition to new antigenic variants [30]. Specifically, one possible mechanism leading to the emergence of antigenic novelty is reassortment between dominant and sub-dominant lineages of past seasons. Further, each A/H3N2-dominated season features multiple genetically distinct co-circulating lineages that may or may not have similar antigenic properties [32]. Studies of recent epidemics of A/H3N2 in New York City and New Zealand have shown that next season's viruses are seeded by importation from either the opposite global hemisphere or from the tropics, and that there is no preferred hemisphere leading the circulation of viruses [75]. This rapidly emerging area of molecular influenza epidemiology has enormous potential to increase our understanding of viral circulation patterns around the globe, and the genesis and spread of drift variants.

Pandemic influenza: Lessons from historical data and modeling

Historical experience with pandemics, which have occurred regularly over several centuries, points to the inevitability of pandemics to come – although the timing and severity are unknown [76]. The three pandemics of the 20th century – 1918 Spanish Influenza A/H1N1, 1957 Asian Influenza A/H2N2 and 1968 Hong Kong Influenza A/H3N2 – were highly variable in terms of mortality impact (Tab. 1). The catastrophic 1918 pandemic resulted in 0.2% to as much as 8% mortality in various countries around the world, and an estimated global mortality of ~50 million people [77]. The relatively mild 1968 pandemic, however, was not appreciably worse than other severe

Table 1. Mortality impact and patterns of three most recent pandemics, compared to the contemporary impact of seasonal influenza.

Pandemic and virus subtype	Evolutionary history (segments involved)	Approximate global mortality impact	Proportion of deaths in persons <65 years of age
1918–1919 A(H1N1)	All avian (all 8 segments)	~50 M	~95%
1957–1958 A(H2N2)	Reassortant HA+NA+PB1	~1–2 M	~40%
1968–1969 A(H3N2)	Reassortant HA + PB1	~0.5–1 M	~50%
Contemporary H3N2 seasons	No shift – only gradual genetic drift	~0.5–1 M	~10%

HA, hemagglutinin; NA, neuraminidase; PB1, polymerase; M, million

epidemics in terms of total influenza-related deaths, while the 1957 A/H2N2 pandemic was moderately severe [15, 18].

History lessons from the field of archaeo-epidemiology

Recent efforts to re-examine and re-discover the forgotten epidemiology of the 1918 Spanish influenza pandemic [78], as well as that of later pandemics, has allowed for a more comprehensive view of pandemics and highlighted their diversity in time and space. Historical vital statistics data have also allowed for the quantitative analysis of the last century's three pandemics. For each of these pandemics, there was a quantitative and qualitative change in the mortality patterns, as compared to seasonal influenza epidemics. The shift of the mortality burden to younger ages has been a "signature" of each pandemic, and stands in marked contrast to the low mortality burden among young people during typical influenza epidemics [15, 79]. This age shift was most pronounced in the 1918 pandemic, but occurred in all three pandemics for which age group mortality data have been studied (Tab. 1, Fig. 4).

Sero-archaeology studies of collections of serum from blood donors have been informative about pre-existing influenza antibodies, and therefore shed light on the circulation of historical pandemic viruses, even in tropical populations. These studies provide interesting pieces of the puzzle, but have unfortunately fallen out of fashion lately. For example, one collection of serum gathered before the 1968 pandemic showed that people born before 1892 had antibodies to the hemagglutinin A/H3 antigen [80]; this may partially explain the fact that seniors older than 77 years were only at a moderate risk during that pandemic [15, 81]. In another example, a sero-epidemiology study looking at influenza antibodies in a population of

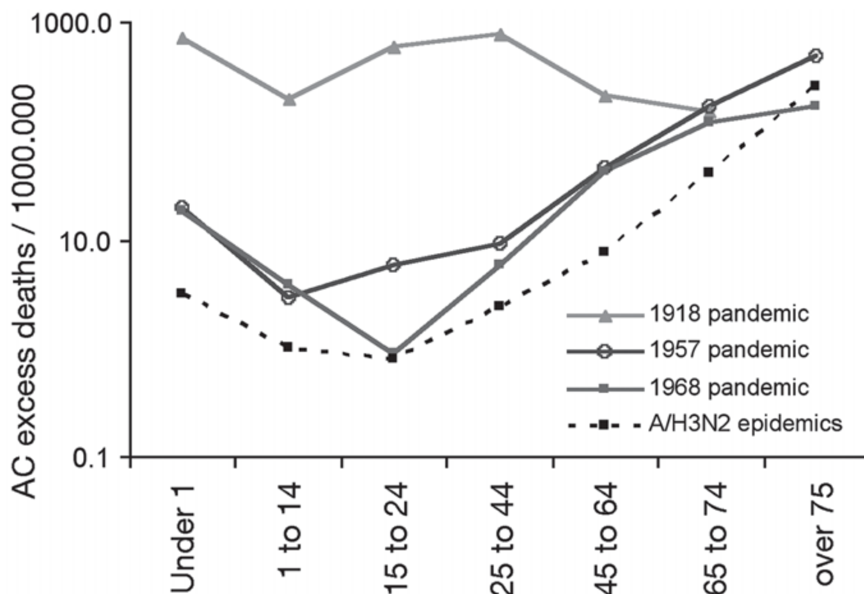

Figure 4. Age-specific mortality impact of three historical pandemics contrasted with the average impact of recent A/H3N2 epidemics in the 1990s.
Based on a Serfling model applied to US all-cause excess mortality data (and presented on a logarithmic scale).

women in Ghana following the 1968 pandemic showed that also in the tropics, most had become infected 5 years after the emergence of the A/H3N2 subtype [82].

It is not clear whether previous exposure to H1 antigen could explain the extreme case of mortality age shift observed in the 1918 A/H1N1 pandemic. In the 1918 pandemic, seniors in New York City were completely spared, in stark contrast to the extreme mortality impact in the young adults (Fig. 5) [17, 79]. This was further confirmed in an additional study of age-detailed mortality time series from Copenhagen [22]. This phenomenon could be explained by immune protection conferred by prior exposure (recycling) of an H1Nx virus in the late 19th century. Alternatively, the atypical mortality spike in young adults in the 1918 pandemic may be explained by an unusual immune dysfunction causing a "cytokine storm" [83–85], which primarily affected young adults. These two possibilities – recycling and immune pathology – cannot be resolved without further experimental and epidemiological studies. This unfortunately leaves us with a great unknown when planning for a future pandemic: if that pandemic is caused by a virus with a hemagglutinin antigen that has not previously circulated in human populations – such as the current avian A/H5N1 virus in Asia – then the recycling

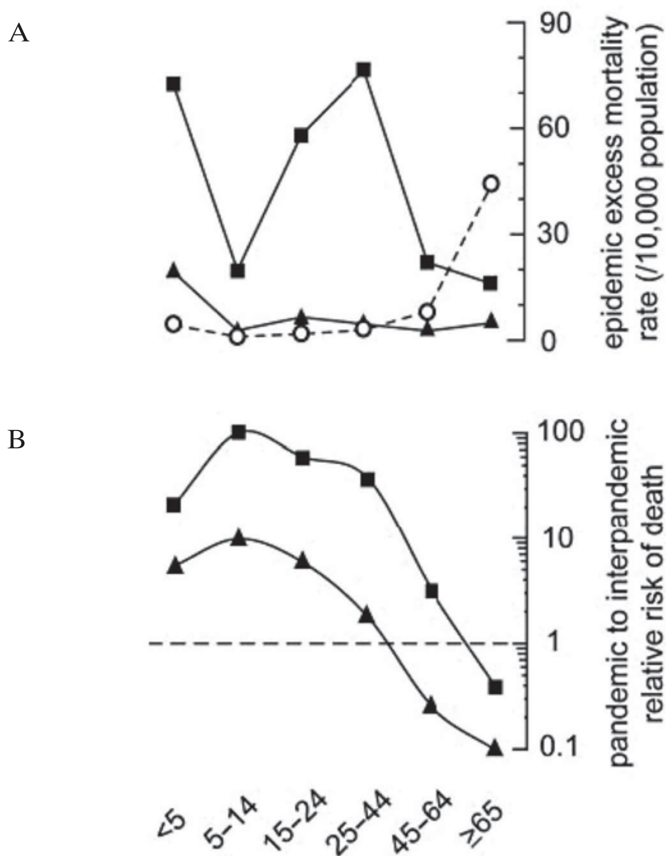

Figure 5. Comparison of age mortality patterns during the 1918 pandemic and a severe inter-pandemic season, New-York City.
(A) Influenza-season-attributable excess deaths are plotted for the 1915–1916 inter-pandemic seasons (○), the pandemic herald wave (epidemic months March and April 1918; ▲), and the main fall pandemic wave (September 1918 to April 1919, ■). (B) Relative risk of death is plotted by age group on a log10 scale for the herald and fall pandemic waves, relative to the severe inter-pandemic season. Adapted from Olson et al. [17].

hypothesis would suggest seniors could be at great risk, as suggested by one author [86]. In contrast, were the immune pathology hypothesis correct, the relative immune senescence in seniors may mitigate the full impact of a pandemic virus and the young adults could be at highest risk of dying.

Comparative studies of pandemic influenza in multiple countries have revealed many interesting insights. For example, a recent study used annual mortality data from multiple countries to estimate the mortality burden of the 1918–1920 influenza pandemic and uncovered substantial geographical

differences in influenza-related mortality rates. The percentage of the population that died varied from 0.2% in Scandinavia to 8% in some areas of India, representing a 40-fold difference in mortality risk in these settings [77]. The underlying reasons for this substantial variability are not well understood, but might be revealed by additional historical pandemic studies.

In a second example, analysis of excess mortality data from several countries put a surprising spin on the 1968 pandemic [18]. An unexpected pattern of a "smoldering" mortality impact in European and some Asian countries was revealed – a relatively mild first wave of the emerging virus in the 1968–1969 season, followed by a very severe 1969–1970 season. This is different from the classical impression based on the North American experience that most of the impact occurs with the first exposure to pandemic strains. It may be more common than previously thought that the first wave of a pandemic virus results in low mortality, only to be followed by a more dramatic impact a few months later. Indeed, this intriguing pattern was not only observed in some countries during the mild 1968 pandemic, but it is also consistent with the herald wave experience in New York City and Scandinavia during the catastrophic 1918 pandemic [17, 22]. Further, historical mortality data from the less-studied 1889–1892 pandemic in England also suggest a pattern of successive pandemic waves where the first encounter was not the most lethal [87]. The reasons for this "smoldering" (or herald wave) pattern are still unknown and may be partly related to on-going adaptation in newly emerged pandemic viruses and pre-existing population immunity.

Transmission models used to predict future pandemic scenarios

Large-scale mathematical transmission models have been employed to simulate in detail the possible spread of a future pandemic virus in a susceptible human population (see for example [71, 72, 88, 89]). These models seek to predict the spatio-temporal dynamic of a hypothetical future pandemic virus and effectiveness of possible intervention strategies, including vaccination before an outbreak with a partially matched, low-efficacy vaccine, distribution of antivirals for prophylaxis or treatment, school closure, case isolation or household quarantine. These models generally agree that a combination of measures, if implemented early and with sufficient compliance, might bring about a meaningful level of mitigation and substantially slow geographic spread. Subsequent studies found that early, targeted, and layered use of non-pharmaceutical interventions could greatly reduce the overall pandemic attack rate, provided the intrinsic transmissibility (basic reproductive number, Ro) of the emerging virus is not greater than 2 [90–92]. Mathematical models can be useful to estimate the levels of drug stockpiles needed to buy time, how and when to modify vaccines, whom to target with vaccines and drugs, and when to enforce quarantine measures. But the simulation models currently used for pandemic planning still need

to be tested against real disease data, and for this we must continue to gather data on influenza morbidity, mortality, and viral genetic sequences, not just for exceptional scenarios but also for seasonal influenza [93].

Predicting the impact of a future pandemic in light of the A/H5N1 threat

Currently, pandemic concerns are focused on the highly pathogenic variant of A/H5N1 influenza that emerged in Hong Kong in 1997 and remerged in 2003. A/H5N1 has now spread to avian populations in more than 30 countries. It is present endemically in South-East Asia, causing regular die-offs in poultry and wild birds, and occasionally affects humans. As of 31 August 2007, the World Health Organization (WHO) had counted 327 laboratory-confirmed H5N1 cases and noted a very high case fatality of ~61% (http://www.who.int/topics/avian_influenza/en/ 2007). While H5N1 continues to be an important economic problem in Asia, Africa, Europe and the Middle East (so far sparing the Americas), the critical question for public health is whether it will gain the ability to effectively transmit among humans. This could occur in one of two ways: by gradual mutations of purely avian H5N1 viruses, or by reassortment with circulating human influenza A viruses (H3N2 or H1N1), in humans or other animal "mixing vessels." Several comprehension discussions at the threat of an avian influenza pandemic have been published (see for example [86, 94–97]).

There are still many uncertainties about the pandemic potential of the circulating avian H5N1 virus, including its potential to effectively transmit between humans and the evolutionary mechanisms that may concurrently affect its virulence. The classical belief is that extremely pathogenic viruses are not well adapted to their hosts – moribund patients do not transmit viruses as easily as those who remain mobile. Further, the pathogenesis of novel pandemic viruses remains unclear, in particular the proportions of severe disease caused by immune-mediated pathological responses, secondary bacterial infections (for which treatments exist) and exacerbation of chronic illnesses. Modern medicine can mitigate some of the pathological mechanisms and control secondary bacterial infections to a certain extent; however, there is undoubtedly a different proportion of persons living with chronic co-morbid conditions now than was the case during previous pandemics. Finally, we do not know the degree of cross protection afforded by early exposure to other influenza virus antigens [98]. If one simply applies the 1918 mortality experience to today's population, anywhere from 0.2% to 8% of a country's population could die, and the highest burden would be suffered by developing countries [77].

Epidemiology and the control of influenza

Influenza vaccines were originally developed for use by the military and have been shown to be highly effective in preventing infection in healthy adults [99]. Most countries that use seasonal influenza vaccine have adopted a policy of targeting influenza vaccination efforts to those at "high risk" of severe outcomes, including those age 65 and older, persons with certain chronic diseases and their close contacts. Although current policy continues to emphasize vaccination of seniors, the "gold standard" evidence that this strategy effectively reduces influenza-related mortality in that age group is not strong [100]. It has recently become evident that influenza-related mortality has not decreased in at least some countries despite major gains in vaccination coverage among people at highest risk [5, 12, 55, 101]. Because "gold standard" evidence from randomized clinical trials is scarce, epidemiological tools and studies constitute the vast majority of the evidence base for whether vaccination programs are beneficial. Paradoxically, observational studies have consistently argued that about 50% of all winter deaths in seniors are preventable with influenza vaccination despite the relatively low immune response to vaccine in this population [102].

The scarce evidence from clinical trials

Langmuir, who originally formulated the policy of targeting seniors and high risk population for vaccination, questioned whether the vaccine would really be effective in seniors who respond less vigorously to the vaccine than younger adults [103]. Only a single randomized placebo-controlled clinical trial set in young healthy seniors is available. It showed that vaccination effectively prevents influenza illness in seniors aged 60–69 years but could not document significant benefits in seniors ≥70 years [104]. The authors expressed concern that their nonsignificant finding of 23% efficacy in seniors >70 years old indicated immune senescence (a decline in immune response with age), although they also noted limitations on the statistical power of their study to address this question. As both T cell and B cell responses are impaired in older individuals, it is plausible that the vaccine antibody response to the drifting influenza viruses and vaccine components is less vigorous in seniors [105]. Consequently, immunologists have long perceived a need for more effective vaccine formulations for this population, including the need for adjuvants and a move back to whole-cell vaccine products. The recent emergence of novel avian strains and development of vaccines against them has reopened many of the discussions of immunogenicity and correlates of protection.

Evidence from observational studies

In the near-absence of randomized clinical trials, these cohort studies have long provided the evidence base that supports influenza vaccine policy. Paradoxically, the concerns about immune senescence and vaccine failure have existed in parallel with cohort studies reporting extraordinarily large mortality benefits in vaccinated seniors [106–108]. In these studies, comparison of vaccinated and unvaccinated seniors indicates that vaccination could prevent fully 50% of all deaths among during winter months, implying that influenza causes half of all winter deaths among seniors. Instead, meta-analyses consolidated the findings and produced estimates with tight confidence intervals. But only about 5% of all winter deaths can be attributed to influenza in an average season according to excess mortality studies [5, 12, 55]. Even in the 1968 A/H3N2 pandemic and in more recent seasons such as 1997–1998, when the vaccine was completely mismatched to the new circulating variant of A/H3N2, the proportion of all deaths attributed to influenza never exceeded 10% of all winter deaths among seniors [12].

A few researchers subsequently addressed this paradox directly, and investigated the possibility that unrecognized bias has led the majority of cohort studies to systematically overestimate influenza vaccine benefits. In 2006, two published reports clearly demonstrated that the senior cohort study findings are largely a result of systematic mis-measurements [109, 110]. First, they showed that the greatest mortality reductions occurred in early winter before influenza ever circulated, and were not specifically associated with the peak influenza period. Second, they showed that the analytical adjustment techniques typically used in cohort studies actually magnified the mis-measurement rather than reducing it. The authors concluded that the magnitude of the unadjusted bias detected was sufficient to account entirely for the observed benefit of 50% mortality reduction during the entire winter period. This problem in the evidence base was also highlighted in a recent Cochrane review and an editorial [107, 111]. The source of bias may be a subset of frail seniors who are undervaccinated in the fall months for that season and subsequently contribute substantially to mortality in the early winter months [100]. Studies have substantiated that frail elderly are indeed vaccinated less often than their healthy peers [112, 113]. Controlling for these biases yields far more modest estimates of mortality reductions [114].

In summary, the emerging picture is a mixture of that residual selection bias, counter-productive adjustment efforts, and low-specificity endpoints has led to systematic overestimation in virtually all cohort studies published over the last decades. Adjustments for selection bias may be possible, but only if high specificity endpoints are studied. Beyond that, a commonly agreed set of standards for carrying out and reporting observational studies that includes a framework for detection of bias would be helpful. Also, previously published observational studies could undergo re-analysis, guided

by such expectations as that vaccine benefits should be highest in peak influenza periods and for well-matched influenza vaccines. We have recently proposed such a framework [100].

Revisiting the evidence base supporting strategies for protecting populations with vaccine

If we discount the biased cohort studies, the remaining studies suggest the vaccine benefits are in fact much lower than previously thought – probably lower than 30% in seniors >70 years of age. This assessment is based on the "gestalt" of results from the randomized placebo-controlled clinical trial described earlier [104], a nested case-control study using laboratory-confirmed endpoints from an RSV study [65, 66], and the excess mortality studies showing little decline in mortality as vaccine coverage rose [12, 55]. None of these studies are conclusive; but if these findings hold up in future studies, then there is ample room for improvement of influenza vaccines, including better vaccine formulations, adjuvants, or higher doses or combinations of live and killed vaccine doses [115–117].

Japan is the single country that has implemented a policy of vaccinating school children, with a strategy of reducing transmission in the community and thereby indirectly protecting high-risk populations. Although Japan abandoned this policy in 1994, an excess mortality study found evidence that it was associated with substantially reduced excess mortality in elderly people for the decades it was in place [118] (Fig. 6). Other studies have examined the value of inducing greater herd immunity based on local community trials or mathematical models [119–121], but unfortunately none have thus far proved conclusive enough to extend the policy of school children vaccination nationally. To fully investigate the indirect benefits of a school children vaccination program, it would be necessary to conduct a large cluster-randomized study across the country; such a study has been proposed but has not yet been undertaken [122].

Vaccines for the control of pandemic influenza

Policy planners are anticipating that an effective vaccine can be developed, manufactured, and distributed rapidly enough help limit the impact of the next influenza pandemic (http://www.hhs.gov/nvpo/pandemicplan accessed 22 March 2004, http://www.hhs.gov/pandemicflu/plan/appendixd.html accessed 11 December 2006, http://www.hhs.gov/pandemicflu/plan/sup6.html accessed 11 December 2006). A great deal of effort has already been expended to develop and clinically test several types of vaccines against H5N1 influenza, including inactivated, live attenuated, and DNA vaccine preparations. Several countries have stockpiled million doses of "pre-pan-

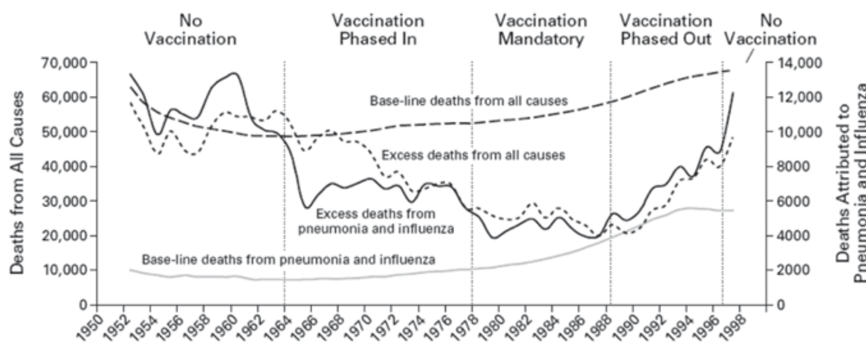

Figure 6. Herd immunity and influenza vaccination
Encouraging evidence from the Japanese experience of vaccinating school children between 1964 and 1996. The graph compares the different phases of the vaccination program with base-line total death rates, rates of excess deaths from all causes and pneumonia and influenza, in Japan, 1950–1998. Adapted from Reichert et al. [118, 123].

demic" inactivated vaccines based on H5N1 strains. National planning documents have set forth priorities for how to deploy an effective vaccine as it becomes available, and detailed logistical plans have been laid for vaccine distribution. The World Health Organization and national pandemic plans are reviewed in Uscher-Pines et al. [124].

But uncertainties abound, and it is unknown how much warning we will have before the full-blown impact of the next pandemic. Nor is it known which age groups will be most at risk; although a shift in mortality toward younger people is very likely, whether this shift will put young adults at greatest absolute risk (as was the case in 1918–1919), or relative risk (as in 1957–1958 and 1968–1969) cannot be predicted. While the chances are good that an effective vaccine will eventually be developed and manufactured in large quantities, it is unknown how long production and distribution will take. In fact, the fear among resource-poor countries that they will only be able to obtain vaccine after wealthy countries have covered their own populations has already exacerbated tensions over sharing of H5N1 data and samples [125]. For all these reasons, it is not clear that planners' hopes that vaccines will play a major role in limiting the global impact of the next pandemic will be realized.

Conclusion

Many unsolved yet intriguing mysteries about influenza epidemiology remain [87, 126–128]. Solving these riddles will depend on the successful integration of many separate fields, including immunology, phylogenetics,

virology, and clinical ascertainment. Exciting progress has recently been made in areas where mathematical modelers and phylogenetic researchers have entered the influenza field [29, 31, 129, 130]. This cross-fertilization has, for example, produced useful new findings in molecular influenza epidemiology, which may in turn lead to improved tools for the selection of vaccine strains [131].

Regarding pandemic influenza, the enormous variability of experience with past pandemics – which have ranged from mild to devastating – makes predictions about a future pandemic unreliable. Will it be the young and healthy who die at the highest rate (as was the case in 1918), or will it be "business as usual" similar to severe seasonal influenza epidemics where elderly people and other high-risk populations are at highest risk? What will be the effect of the presence of large numbers of immunosuppressed people, including people with HIV infection? And perhaps most important, will the next pandemic merely have a moderate impact (like 1968) or be catastrophic (like 1918)?

The fact is, we cannot know the answers to these questions in advance. The age distributions of the ~300 human cases and deaths from A/H5N1 influenza most closely resemble those of the 1918 pandemic, with highest impact among the young (http://www.who.int/topics/avian_influenza/en/ 2007, 132–133). But that could change considerably as the virus gains the ability to transmit effectively in human populations. Furthermore, the next pandemic may not be caused by an A/H5N1 virus.

Whatever the scenario, the epidemiological characteristics of a future pandemic directly affect the ethical principles that should be invoked when allocating limited vaccine doses [134, 135]. For that reason, it is absolutely essential that real-time surveillance data from the early phase of the next pandemic be freely shared and rapidly interpreted, to determine who is at risk and where scarce resources such as pandemic vaccine and antivirals could best be used. Moreover, pandemic planners should build sufficient flexibility into their plans to allow rapid shifts in planned control strategies, as key epidemiological insights hopefully become available in the early pandemic phase. Continued influenza surveillance efforts in temperate and tropical regions, combined with international sharing of epidemiological and viral sequence data are our best hope for limiting the impact of the next influenza pandemic.

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References

1 Nicholson K, Hay A (1998) *Textbook of influenza*. Blackwell, Oxford
2 Schoenbaum S (1996) Impact of influenza in persons and populations. In: L Brown, A Hampson, R Webster (eds): *Options for the Control of Influenza III*. Elsevier Science Publishers, Amsterdam, 17–25
3 Reichert TA, Simonsen L, Sharma A, Pardo SA, Fedson DS, Miller MA (2004) Influenza and the winter increase in mortality in the United States, 1959–1999. *Am J Epidemiol* 160: 492–502
4 Simonsen L (1999) The global impact of influenza on morbidity and mortality. *Vaccine* 17 (Suppl 1): S3–10
5 Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K (2003) Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* 289: 179–186
6 Serfling R (1963) Methods for current statistical analysis of excess pneumonia-influenza deaths. *Public Health Rep* 78: 494–506
7 Choi K, Thacker SB (1981) An evaluation of influenza mortality surveillance, 1962–1979. I. Time series forecasts of expected pneumonia and influenza deaths. *Am J Epidemiol* 113: 215–226
8 Carrat F, Valleron AJ (1995) Influenza mortality among the elderly in France, 1980–90: How many deaths may have been avoided through vaccination? *J Epidemiol Community Health* 49: 419–425
9 Simonsen L, Clarke MJ, Williamson GD, Stroup DF, Arden NH, Schonberger LB (1997) The impact of influenza epidemics on mortality: Introducing a severity index. *Am J Public Health* 87: 1944–1950
10 Simonsen L, Fukuda K, Schonberger LB, Cox NJ (2000) The impact of influenza epidemics on hospitalizations. *J Infect Dis* 181: 831–837
11 Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, Fukuda K (2004) Influenza-associated hospitalizations in the United States. *JAMA* 292: 1333–1340
12 Simonsen L, Reichert TA, Viboud C, Blackwelder WC, Taylor RJ, Miller MA (2005) Impact of influenza vaccination on seasonal mortality in the US elderly population. *Arch Intern Med* 165: 265–272
13 Dushoff J, Plotkin JB, Viboud C, Earn DJ, Simonsen L (2006) Mortality due to influenza in the United States – An Annualized Regression approach using Multiple-Cause Mortality Data. *Am J Epidemiol* 163: 181–187
14 Viboud C, Bjornstad ON, Smith DL, Simonsen L, Miller MA, Grenfell BT (2006) Synchrony, waves, and spatial hierarchies in the spread of influenza. *Science* 312: 447–451
15 Simonsen L, Olson D, Viboud C, Miller M (2004) Pandemic influenza and mortality: Past evidence and projections for the future. In: S Knobler, K Oberholtzer (eds): *Forum on microbial threats. Pandemic influenza: Assessing capabilities for prevention and response*. Institute of Medicine, The National Academy of Sciences, Washington, DC
16 Mills CE, Robins JM, Lipsitch M (2004) Transmissibility of 1918 pandemic influenza. *Nature* 432: 904–906
17 Olson DR, Simonsen L, Edelson PJ, Morse SS (2005) Epidemiological evidence

of an early wave of the 1918 influenza pandemic in New York City. *Proc Natl Acad Sci USA* 102: 11059–11063
18 Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L (2005) Multinational impact of the 1968 Hong Kong influenza pandemic: Evidence for a smoldering pandemic. *J Infect Dis* 192: 233–248
19 Chowell G, Ammon CE, Hengartner NW, Hyman JM (2006) Transmission dynamics of the great influenza pandemic of 1918 in Geneva, Switzerland: Assessing the effects of hypothetical interventions. *J Theor Biol* 241: 193–204
20 Viboud C, Tam T, Fleming D, Handel A, Miller MA, Simonsen L (2006) Transmissibility and mortality impact of epidemic and pandemic influenza, with emphasis on the unusually deadly 1951 epidemic. *Vaccine* 24: 6701–6707
21 Viboud C, Tam T, Fleming D, Miller MA, Simonsen L (2006) 1951 influenza epidemic, England and Wales, Canada, and the United States. *Emerg Infect Dis* 12: 661–668
22 Andreasen V, Viboud C, Simonsen L (2007) Epidemiologic characterization of the summer wave of the 1918 influenza pandemic in Copenhagen: Implications for pandemic control strategies. *J Infect Dis* 197: 270–278
23 Chowell G, Miller MA, Viboud C (2007) Seasonal influenza in the United States, France, and Australia: Transmission and prospects for control. *Epidemiol Infect* 2: 1–13
24 Chowell G, Nishiura H, Bettencourt LM (2007) Comparative estimation of the reproduction number for pandemic influenza from daily case notification data. *J R Soc Interface* 4: 155–166
25 Olson DR, Heffernan RT, Paladini M, Konty K, Weiss D, Mostashari F (2007) Monitoring the impact of influenza by age: Emergency Department fever and respiratory complaint surveillance in New York City. *PLoS Med* 4: e247
26 Dushoff J, Plotkin JB, Levin SA, Earn DJ (2004) Dynamical resonance can account for seasonality of influenza epidemics. *Proc Natl Acad Sci USA* 101: 16915–16916
27 Viboud C, Alonso WJ, Simonsen L (2006) Influenza in tropical regions. *PLoS Med* 3: e89
28 Alonso WJ, Viboud C, Simonsen L, Hirano EW, Daufenbach LZ, Miller MA (2007) Seasonality of influenza in Brazil: A traveling wave from the Amazon to the subtropics. *Am J Epidemiol* 165: 1434–1442
29 Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, Mumford JA, Holmes EC (2004) Unifying the epidemiological and evolutionary dynamics of pathogens. *Science* 303: 327–332
30 Holmes EC, Ghedin E, Miller N, Taylor J, Bao Y, St George K, Grenfell BT, Salzberg SL, Fraser CM, Lipman DJ, Taubenberger JK (2005) Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses. *PLoS Biol* 3: e300
31 Nelson MI, Holmes EC (2007) The evolution of epidemic influenza. *Nat Rev Genet* 8: 196–205
32 Nelson MI, Simonsen L, Viboud C, Miller MA, Taylor J, George KS, Griesemer SB, Ghedin E, Sengamalay NA, Spiro DJ et al (2006) Stochastic Processes Are Key Determinants of Short-Term Evolution in Influenza A Virus. *PLoS Pathog* 2: e125

34 Cox NJ, Subbarao K (2000) Global epidemiology of influenza: Past and present. *Annu Rev Med* 51: 407–421

35 Glezen WP (1982) Serious morbidity and mortality associated with influenza epidemics. *Epidemiol Rev* 4: 25–44

36 Monto AS (2002) Epidemiology of viral respiratory infections. *Am J Med* 112 (Suppl 6A): 4S-12S

37 Noble G (1982) *Epidemiological and clinical aspects of influenza*. CRC Press, Boca Raton

38 Stuart-Harris C (1979) Epidemiology of influenza in man. *Br Med Bull* 35: 3–8

39 Glezen WP, Taber LH, Frank AL, Gruber WC, Piedra P (1997) Influenza virus infections in infants. *Pediatr Infect Dis J* 16: 1065–1068

40 Chiu SS, Lau YL, Chan KH, Wong WH, Peiris JS (2002) Influenza-related hospitalizations among children in Hong Kong. *N Engl J Med* 347: 2097–2103

41 Chow A, Ma S, Ling AE, Chew SK (2006) Influenza-associated deaths in tropical Singapore. *Emerg Infect Dis* 12: 114–121

40 Chiu SS, Lau YL, Chan KH, Wong WH, Peiris JS (2002) Influenza-related hospitalizations among children in Hong Kong. *N Engl J Med* 347: 2097–2103

41 Chow A, Ma S, Ling AE, Chew SK (2006) Influenza-associated deaths in tropical Singapore. *Emerg Infect Dis* 12: 114–121

42 Wong CM, Yang L, Chan KP, Leung GM, Chan KH, Guan Y, Lam TH, Hedley AJ, Peiris JS (2006) Influenza associated weekly hospitalization in a subtropical city. *PLoS Med* 3: e89

43 Wong CM, Chan KP, Hedley AJ, Peiris JS (2004) Influenza-associated mortality in Hong Kong. *Clin Infect Dis* 39: 1611–1617

44 Assaad F, Cockburn WC, Sundaresan TK (1973) Use of excess mortality from respiratory diseases in the study of influenza. *Bull World Health Organ* 49: 219–233

45 Rizzo C (2007) Trends for influenza-related deaths during pandemic and epidemic seasons, Italy, 1969–2001. *Emerg Infect Dis* 13: 694–699

46 Rocchi G, Ragona G, De Felici A, Muzzi A (1974) Epidemiological evaluation of influenza in Italy. *Bull World Health Organ* 50: 401–406

47 Viboud C, Boelle PY, Pakdaman K, Carrat F, Valleron AJ, Flahault A (2004) Influenza epidemics in the United States, France, and Australia, 1972–1997. *Emerg Infect Dis* 10: 32–39

48 Imaz MS, Eimann M, Poyard E, Savy V (2006) [Influenza associated excess mortality in Argentina: 1992–2002]. *Rev Chilena Infectol* 23: 297–306

49 Stroup DF, Thacker SB, Herndon JL (1988) Application of multiple time series analysis to the estimation of pneumonia and influenza mortality by age 1962–1983. *Stat Med* 7: 1045–1059

50 Barker WH (1986) Excess pneumonia and influenza associated hospitalization during influenza epidemics in the United States, 1970–78. *Am J Public Health* 76: 761–765

51 Simonsen L, Taylor R, Viboud C, Dushoff J, Miller M (2006) US flu mortality estimates are based on solid science. *Br Med J* 332: 177–178

52 Thompson W, Weintraub E, Cheng P et al (2007) Comparing methods for estimating influenza-associated deaths in the United States: 1976/1977 through

2002/2003 respiratory seasons. In: *Options for the Control of Influenza VI*. Toronto, Canada

53 Fleming DM (2000) The contribution of influenza to combined acute respiratory infections, hospital admissions, and deaths in winter. *Commun Dis Public* Health 3: 32–38

54 Schanzer DL, Tam TW, Langley JM, Winchester BT (2007) Influenza-attributable deaths, Canada 1990–1999. *Epidemiol Infect* 135: 1109–1116

55 Rizzo C, Viboud C, Montomoli E, Simonsen L, Miller MA (2006) Influenza-related mortality in the Italian elderly: No decline associated with increasing vaccination coverage. *Vaccine* 24: 6468–6475

56 Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos AM, Posey DL, Klimov A, Lindstrom SE et al (2005) Influenza-associated deaths among children in the United States, 2003–2004. *N Engl J Med* 353: 2559–2567

57 Elliot AJ, Fleming DM (2006) Surveillance of influenza-like illness in England and Wales during 1966–2006. *Eur Surveill* 11: 249–250

58 Hall IM, Gani R, Hughes HE, Leach S (2007) Real-time epidemic forecasting for pandemic influenza. *Epidemiol Infect* 135: 372–385

59 Brownstein JS, Kleinman KP, Mandl KD (2005) Identifying pediatric age groups for influenza vaccination using a real-time regional surveillance system. *Am J Epidemiol* 162: 686–693

60 Crighton EJ, Elliott SJ, Moineddin R, Kanaroglou P, Upshur RE (2007) An exploratory spatial analysis of pneumonia and influenza hospitalizations in Ontario by age and gender. *Epidemiol Infect* 135: 253–261

61 Fleming DM, Zambon M, Bartelds AI, de Jong JC (1999) The duration and magnitude of influenza epidemics: A study of surveillance data from sentinel general practices in England, Wales and the Netherlands. *Eur J Epidemiol* 15: 467–473

62 Fleming DM, Pannell RS, Cross KW (2005) Mortality in children from influenza and respiratory syncytial virus. *J Epidemiol Community Health* 59: 586–590

63 Fleming DM, Cross KW (1993) Respiratory syncytial virus or influenza? *Lancet* 342: 1507–1510

64 Fleming DM, Elliott AJ, Cross KW (2007) Is routine seasonal influenza vaccination of elderly people an effective community policy? In: *Options for the Control of Influenza VI*. Toronto, Canada

65 Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE (2005) Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med* 352: 1749–1759

66 Simonsen L, Viboud C (2005) Respiratory syncytial virus infection in elderly adults. *N Engl J Med* 353: 422–423

67 Izurieta HS, Thompson WW, Kramarz P, Shay DK, Davis RL, DeStefano F, Black S, Shinefield H, Fukuda K (2000) Influenza and the rates of hospitalization for respiratory disease among infants and young children. *N Engl J Med* 342: 232–239

68 Weber MW, Mulholland EK, Greenwood BM (1998) Respiratory syncytial virus infection in tropical and developing countries. *Trop Med Int Health* 3: 268–280

69 Monto AS (1994) Studies of the community and family: Acute respiratory illness and infection. *Epidemiol Rev* 16: 351–373
70 Monto AS, Cavallaro JJ (1971) The Tecumseh study of respiratory illness. II. Patterns of occurrence of infection with respiratory pathogens, 1965–1969. *Am J Epidemiol* 94: 280–289
71 Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS (2006) Strategies for mitigating an influenza pandemic. *Nature* 442: 448–452
72 Ferguson NM, Cummings DAT, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S, Burke DS (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437: 209–214
73 Spicer CC (1979) The mathematical modelling of influenza epidemics. *Br Med Bull* 35: 23–28
74 Spicer CC, Lawrence CJ (1984) Epidemic influenza in Greater London. *J Hyg (Camb)* 93: 105–112
75 Nelson MI, Simonsen L, Viboud C, Miller MA, Holmes EC (2007) Phylogenetic analysis reveals the global migration of seasonal influenza a viruses. *PLoS Pathog* 3: 1220–1228
76 Kilbourne ED (1997) Perspectives on pandemics: A research agenda. *J Infect Dis* 176 (Suppl 1): S29–S31
77 Murray CJ, Lopez AD, Chin B, Feehan D, Hill KH (2006) Estimation of potential global pandemic influenza mortality on the basis of vital registry data from the 1918–20 pandemic: A quantitative analysis. *Lancet* 368: 2211–2218
78 Barry J (2004) *The great influenza: The epic story of the deadliest plague in history.* Viking Penguin, New York
79 Simonsen L, Clarke MJ, Schonberger LB, Arden NH, Cox NJ, Fukuda K (1998) Pandemic *versus* epidemic influenza mortality: A pattern of changing age distribution. *J Infect Dis* 178: 53–60
80 Masurel N, Marine WM (1973) Recycling of Asian and Hong Kong influenza A virus hemagglutinins in man. *Am J Epidemiol* 97: 44–49
81 Simonsen L, Reichert TA, Miller M (2003) The virtues of antigenic sin: Consequences of pandemic recycling on influenza-associated mortality. In: Y Kawaoka, (ed): *Options for the Control of Influenza V*. International Congress Series 1263, Elsevier, Okinawa, 791–794
82 McGregor IA, Schild GC, Billewicz WZ, Williams K (1979) The epidemiology of influenza in a tropical (Gambian) environment. *Br Med Bull* 35: 15–22
83 Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, Thomas MJ, Basler CF, Palese P, Taubenberger JK, García-Sastre A et al (2006) Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* 443: 578–581
84 Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M et al (2007) Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445: 319–323
85 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solórzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, García-Sastre A (2005) Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310: 77–80
86 Palese P (2004) Influenza: Old and new threats. *Nat Med* 10: S82–S87

87 Stuart-Harris CH (1970) Pandemic influenza: An unresolved problem in prevention. *J Infect Dis* 122: 108–115
88 Germann TC, Kadau K, Longini IM Jr, Macken CA (2006) Mitigation strategies for pandemic influenza in the United States. *Proc Natl Acad Sci USA* 103: 5935–5940
89 Longini IM, Jr., Nizam A, Xu S, Ungchusak K, Hanshaoworakul W, Cummings DA, Halloran ME (2005) Containing pandemic influenza at the source. *Science* 309: 1083–1087
90 Bootsma MC, Ferguson NM (2007) From the cover: The effect of public health measures on the 1918 influenza pandemic in U.S. cities. *Proc Natl Acad Sci USA* 104: 7588–7593
91 Glass K, Barnes B (2007) How much would closing schools reduce transmission during an influenza pandemic? *Epidemiology* 18: 623–628
92 Glass RJ, Glass LM, Beyeler WE, Min HJ (2006) Targeted social distancing design for pandemic influenza. *Emerg Infect Dis* 12: 1671–1681
93 Smith DJ (2006) Predictability and preparedness in influenza control. *Science* 312: 392–394
94 Peiris JS, de Jong MD, Guan Y (2007) Avian influenza virus (H5N1): A threat to human health. *Clin Microbiol Rev* 20: 243–267
95 Subbarao K, Luke C (2007) H5N1 viruses and vaccines. *PLoS Pathog* 3: e40
96 Taubenberger JK, Morens DM, Fauci AS (2007) The next influenza pandemic: Can it be predicted? *JAMA* 297: 2025–2027
97 Webster RG, Hulse-Post DJ, Sturm-Ramirez KM, Guan Y, Peiris M, Smith G, Chen H (2007) Changing epidemiology and ecology of highly pathogenic avian H5N1 influenza viruses. *Avian Dis* 51: 269–272
98 Bermejo-Martin JF, Kelvin DJ, Guan Y, Chen H, Perez-Breña P, Casas I, Arranz E, de Lejarazu RO (2007) Neuraminidase antibodies and H5N1: Geographic-dependent influenza epidemiology could determine cross-protection against emerging strains. *PLoS Med* 4: e212
99 Demicheli V, Rivetti D, Deeks JJ, Jefferson TO (2004) Vaccines for preventing influenza in healthy adults. *Cochrane Database Syst Rev* 3: CD001269
100 Simonsen L, Taylor RJ, Viboud C, Miller MA, Jackson LA (2007) Mortality benefits of influenza vaccination in elderly people: An ongoing controversy. *Lancet Infect Dis* 7: 658–666
101 Reichert TA, Pardo SA, Valleron AJ et al (2007) National vaccination programs and trends in influenza-attributable mortality in four countries. In: *Options for the Control of Influenza VI.* Toronto, Canada
102 Goodwin K, Viboud C, Simonsen L (2005) Antibody response to influenza vaccination in the elderly: A quantitative review. *Vaccine* 24: 1159–1169
103 Langmuir AD, Henderson DA, Serfling RE (1964) The epidemiological basis for the control of influenza. *Am J Public Health Nations Health* 54: 563–571
104 Govaert TM, Thijs CT, Masurel N, Sprenger MJ, Dinant GJ, Knottnerus JA (1994) The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial. *JAMA* 272: 1661–1665
105 Vallejo AN (2007) Immune remodeling: Lessons from repertoire alterations during chronological aging and in immune-mediated disease. *Trends Mol Med* 13: 94–102

106 Gross PA, Hermogenes AW, Sacks HS, Lau J, Levandowski RA (1995) The efficacy of influenza vaccine in elderly persons. A meta-analysis and review of the literature. *Ann Intern Med* 123: 518–527
107 Jefferson T, Rivetti D, Rivetti A, Rudin M, Di Pietrantonj C, Demicheli V (2005) Efficacy and effectiveness of influenza vaccines in elderly people: A systematic review. *Lancet* 366: 1165–1174
108 Vu T, Farish S, Jenkins M, Kelly H (2002) A meta-analysis of effectiveness of influenza vaccine in persons aged 65 years and over living in the community. *Vaccine* 20: 1831–1836
109 Jackson LA, Jackson ML, Nelson JC, Neuzil KM, Weiss NS (2006) Evidence of bias in estimates of influenza vaccine effectiveness in seniors. *Int J Epidemiol* 35: 337–344
110 Jackson LA, Nelson JC, Benson P, Neuzil KM, Reid RJ, Psaty BM, Heckbert SR, Larson EB, Weiss NS (2006) Functional status is a confounder of the association of influenza vaccine and risk of all cause mortality in seniors. *Int J Epidemiol* 35: 345–352
111 Jefferson T (2006) Influenza vaccination: Policy *versus* evidence. *Br Med J* 333: 912–915
112 Bratzler DW, Houck PM, Jiang H, Nsa W, Shook C, Moore L, Red L (2002) Failure to vaccinate Medicare inpatients: A missed opportunity. *Arch Intern Med* 162: 2349–2356
113 Fedson DS, Wajda A, Nicol JP, Roos LL (1992) Disparity between influenza vaccination rates and risks for influenza-associated hospital discharge and death in Manitoba in 1982–1983. *Ann Intern Med* 116: 550–555
114 Örtqvist Å, Granath F, Askling J, Hedlund J (2007) Influenza vaccination and mortality: Prospective cohort study of the elderly in a large geographical area. *Eur Respir J* 30: 414–422
115 Keitel WA, Atmar RL, Cate TR, Petersen NJ, Greenberg SB, Ruben F, Couch RB (2006) Safety of high doses of influenza vaccine and effect on antibody responses in elderly persons. *Arch Intern Med* 166: 1121–1127
116 Minutello M, Senatore F, Cecchinelli G, Bianchi M, Andreani T, Podda A, Crovari P (1999) Safety and immunogenicity of an inactivated subunit influenza virus vaccine combined with MF59 adjuvant emulsion in elderly subjects, immunized for three consecutive influenza seasons. *Vaccine* 17: 99–104
117 Treanor JJ, Mattison HR, Dumyati G, Yinnon A, Erb S, O'Brien D, Dolin R, Betts RF (1992) Protective efficacy of combined live intranasal and inactivated influenza A virus vaccines in the elderly. *Ann Intern Med* 117: 625–633
118 Reichert TA, Sugaya N, Fedson DS, Glezen WP, Simonsen L, Tashiro M (2001) The Japanese experience with vaccinating schoolchildren against influenza. *N Engl J Med* 344: 889–896
119 Glezen WP (2006) Herd protection against influenza. *J Clin Virol* 37: 237–243
120 Longini IM Jr, Halloran ME (2005) Strategy for distribution of influenza vaccine to high-risk groups and children. *Am J Epidemiol* 161: 303–306
121 Monto AS, Davenport FM, Napier JA, Francis T Jr (1970) Modification of an outbreak of influenza in Tecumseh, Michigan by vaccination of schoolchildren. *J Infect Dis* 122: 16–25

122 Halloran ME, Longini IM Jr (2006) Public health. Community studies for vaccinating schoolchildren against influenza. *Science* 311: 615–616
123 Reichert TA, Sugaya N, Fedson DS, Glezen WP, Simonsen L, Tashiro M (2001) Vaccinating Japanese schoolchildren against influenza: Author reply. *N Engl J Med* 344: 1948
124 Uscher-Pines L, Omer SB, Barnett DJ, Burke TA, Balicer RD (2006) Priority setting for pandemic influenza: An analysis of national preparedness plans. *PLoS Med* 3: e436
125 Enserink M (2007) Data sharing. New Swiss influenza database to test promises of access. *Science* 315: 923
126 Earn D, Dushoff J, Levin S (2002) Ecology and evolution of the flu. *Trends Ecol Evol* 37: 334–340
127 Hope-Simpson RE (1992) *The transmission of epidemic influenza*. Plenum Press, New York
128 Thacker SB (1986) The persistence of influenza A in human populations. *Epidemiol Rev* 8: 129–142
129 Ferguson NM, Galvani AP, Bush RM (2003) Ecological and immunological determinants of influenza evolution. *Nature* 422: 428–433
130 Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA (2004) Mapping the antigenic and genetic evolution of influenza virus. *Science* 305: 371–376
131 Plotkin JB, Dushoff J, Levin SA (2002) Hemagglutinin sequence clusters and the antigenic evolution of influenza A virus. *Proc Natl Acad Sci USA* 99: 6263–6268
132 Sedyaningsih ER, Isfandari S, Setiawaty V, Rifati L, Harun S, Purba W, Imari S, Giriputra S, Blair PJ, Putnam SD, Uyeki TM, Soendoro T (2007) Epidemiology of cases of H5N1 virus infection in Indonesia, July 2005–June 2006. *J Infect Dis* 196: 522–527
133 Smallman-Raynor M, Cliff AD (2007) Avian influenza A (H5N1) age distribution in humans. *Emerg Infect Dis* 13: 510–512
134 Emanuel EJ, Wertheimer A (2006) Public health. Who should get influenza vaccine when not all can? *Science* 312: 854–855
135 Gostin LO (2006) Medical countermeasures for pandemic influenza: Ethics and the law. *JAMA* 295: 554–556

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Influenza and influenza vaccination in children

Kathryn M. Edwards

Vanderbilt University School of Medicine, Department of Pediatrics, Pediatric Clinical Research Office, Nashville, TN 37232, USA

Abstract

Ecological and active population-based surveillance studies have clearly shown the large burden of influenza disease in children, both in hospital and outpatient settings. Mortality and encephalitis due to influenza have also been reported. Two vaccines are licensed for use in children; trivalent inactivated and live attenuated vaccines. Both have been shown to be efficacious for the prevention of clinically and laboratory-confirmed influenza. In recent comparative trials in young children, live attenuated vaccines were shown to be more effective than trivalent inactivated vaccines for the prevention of laboratory-confirmed influenza. However, episodes of wheezing were increased in the youngest children receiving live attenuated vaccine. Trivalent inactivated influenza vaccine has an excellent safety profile and has been mainly associated with local pain and tenderness at the injection site. Vaccine efficacy for the inactivated vaccine has been shown to be greater in older children. Increased use of either influenza vaccine has the potential to reduce the disease burden in children and to extend herd protection to those who are not vaccinated.

Introduction

Over the past several years a number of ecological studies have demonstrated the excessive burden of influenza disease in children [1, 2]. Izurieta et al. [1] used local viral surveillance to define periods when the circulation of influenza viruses predominated over that of respiratory syncytial virus (RSV) and calculated rates of hospitalization for acute respiratory disease in children younger than 18 years of age enrolled in two large health maintenance organizations (HMO). Among children without high-risk conditions, hospitalization rates in children younger than 2 years of age were 231 per 100,000 person-months in one HMO and 193 per 100 000 person-months in the other. In children 5–17 years of age, rates were 19 per 100,000 person-months in one HMO and 16 per 100,000 person-months in the other. Finally, among high-risk children 5–17 years of age, hospitalization rates were 386

per 100,000 person-months and 216 per 100,000 person-months in the two HMOs, respectively.

In another ecological study, Neuzil et al. [2] assessed the influenza burden in a large cohort of children less than 15 years of age enrolled in the Tennessee Medicaid program. Over a period of 19 years and a total of 2,035,143 person-years of observation, the average number of hospitalizations each year for cardiopulmonary conditions attributable to influenza was 10.4 per 1000 children younger than 6 months of age, 5.0 per 1000 for those 6–12 months, 1.9 per 1000 for those 1–3 years, 0.9 per 1000 for those 3–5 years, and 0.4 per 1000 for those 5–15 years. In addition, for every 100 children there were an average of 6 to 15 outpatient visits and 3 to 9 courses of antibiotics attributable to influenza disease each year [2].

Recently rates observed in these ecological studies were confirmed through an active, prospective, population-based surveillance network [3–5]. Children younger than 5 years of age residing in three United States counties were enrolled during hospitalizations or either outpatient or emergency department visits for acute respiratory tract infections or fever. Nasal and throat swabs were tested for the influenza virus by viral culture and polymerase chain reaction assay and epidemiological data were collected [5]. Combining data from four influenza seasons, the average annual hospitalization rates associated with influenza were 0.9 per 1000 children (Tab. 1). The rates were 4.5 per 1000 children less than 6 months of age, 0.9 per 1000 children 6–23 months of age, and 0.3 per 1000 children 24–59 months of age. The estimated burden of outpatient and emergency department visits associated with influenza was even greater and depended upon the severity of the influenza season (Tab. 2). During 2 years of outpatient surveillance there were between 50 and 95 clinic visits and 6–27 emergency department visits per 1000 children per year. Remarkably, only 28% of the hospitalized children with laboratory confirmed influenza and only 17% of those seen in the outpatient settings with confirmed influenza were diagnosed with influenza by their treating physician. This is despite the usefulness of rapid diagnostic tests for the confirmation of influenza in young children [6–9].

Population-based estimates from other US studies have provided comparable rates using different study years, populations, and study methods [10–16]. Additional studies of influenza burden in children have also been conducted in other countries. Montes et al. [17] determined the incidence of virologically confirmed influenza-related hospitalizations in children aged <5 years in southern Spain during three study years. Their average yearly hospitalization rates were 4.1 per 1000 children less than 6 months of age, 0.8 per 1000 children aged 6–11 months of age, 0.7 per 1000 children 12–23 months of age, and 0.5 per 1000 children aged 24–59 months. These rates are nearly identical to those reported by Poehling et al. [5]. In a retrospective, population-based study, Chiu et al. [18] determined the annual laboratory-confirmed influenza-associated hospitalization rates among children 15 years old or younger who lived in Hong Kong. The adjusted rates of

Table 1. Rate of hospitalizations attributable to influenza per 1000 children, according to age group and study year[a]

Age group	2000–2001	2001–2002	2002–2003	2003–2004	2000–2004
0–5 months					
Rate (95% CI)	2.4 (1.0–3.9)	4.3 (2.2–6.6)	2.3 (0.9–3.8)	7.2 (5.3–9.2)	4.5 (3.4–5.5)
6–23 months					
Rate (95% CI)	0.6 (0.2–1.2)	0.9 (0.4–1.3)	0.4 (0.1–0.7)	1.5 (1.0–2.1)	0.9 (0.7–1.2)
24–59 months					
Rate (95% CI)	0.2 (0.1–0.4)	0.3 (0.1–0.6)	0.04 (0.00–0.13)	0.6 (0.3–0.9)	0.3 (0.2–0.5)
0–59 months					
Rate (95% CI)	0.6 (0.3–0.8)	0.9 (0.6–1.2)	0.4 (0.2–0.6)	1.5 (1.2–1.9)	0.9 (0.8–1.1)

[a]Numbers are combined rates for three sites in the New Vaccine Surveillance Network. CI denotes confidence interval. Counts were weighted for days of surveillance and proportion of eligible children enrolled.
Modified from [5].

Table 2. Outpatient visits for acute respiratory tract infection or fever associated with confirmed influenza.

Age group	Visits for acute respiratory tract infection or fever associated with confirmed influenza		Mean rate of visits for acute respiratory tract infection or fever, 1998–2002[a]	Estimated rate of visits attributable to influenza[b]	
	2002–2003	2003–2004			
	% (95% CI)		No./1000 children (95% CI)		
Outpatient clinics					
0–59 months	10.2 (7.5–13.6)	19.4 (16.0–23.1)	489 (387–591)	50 (35–71)	95 (72–125)
Emergency departments					
0–59 months	5.9 (3.7–8.9)	28.8 (25.0–32.7)	94 (78–110)	6 (4–9)	27 (22–33)

[a]The mean rate of visits for acute respirator tract infection or fever per 1000 children was calculated from the National Ambulatory Medical Care Survey/National Hospital Ambulatory Medical Care Survey.
[b]Rates were calculated by multiplying the proportions of visits for acute respiratory tract infection or fever associated with confirmed influenza (columns 2 and 3) by the mean rate of visits for acute respiratory tract infections or fever, 1998–2002 (column 4).
Modified from [5].

Table 3. Effect of winter illness on school and family[a]

Variable	Events during non-influenza winter season (44 school days)	Events during influenza season (37 school days)			
		Expected	Observed	Relative risk (95% CI)[b]	Influenza-attributable events per 100 children[c]
Illness episodes	194	163	250	1.5 (1.3–1.9)	27.8
Days missed	343	288	485	1.7 (1.5–1.9)	62.9
Febrile illnesses	95	80	168	2.1 (1.6–2.7)	28.1
Health care visits	54	45	58	1.3 (0.9–1.9)	4.2
Days of work missed by parent	116	97	159	1.6 (1.3–2.1)	19.8
Household members ill within 3 days after absence	69	58	126	2.2 (1.6–2.9)	21.7

[a]Study involved 313 schoolchildren monitored from 1 December 2000 to 13 April 2001.
[b]Values indicate the comparison of the rate of events during influenza season with the rate of events during the non-influenza winter season.
[c]Values were calculated by subtracting the expected outcomes from the observed outcomes during the influenza season. An excess event rate per 100 children was generated by dividing by the total number of children in the cohort (n=313), then multiplying by 100.
Modified from [23].

hospitalization attributable to influenza were 28 per 1000 children less than 1 year of age, 21 per 1000 children 1–2 years of age; 9 per 1000 children 2–5 years of age; 4 per 1000 children 5–10 years of age; and nearly 1 per 1000 children 10–15 years of age. These rates are considerably higher than those reported from either Spain or the United States [5, 17].

In addition, mortality due to influenza has been reported in children. During the 2003–2004 influenza season, 153 influenza-associated deaths in children were reported to the Centers for Disease Control and Prevention (CDC) [19]. The median age of those who died was 3 years, 96 children were younger than 5 years old, and the highest mortality rate was noted in those less than 6 months of age; 47 of the children died outside a hospital setting, 45 died within 3 days of illness onset, and bacterial co-infections were identified in 24% of the children. Only 33% of the children had underlying medical conditions associated with increased influenza risk.

Influenza-associated encephalopathy has also been described in Asian children, and less commonly reported in European and American children [20]. With a fatality rate of nearly 30%, and nearly one third of the survivors left with permanent disability, influenza-associated encephalopathy occurs early in the influenza illness and is manifested by confusion, seizures, and progressive coma. Imaging studies reveal uniform cerebral edema with

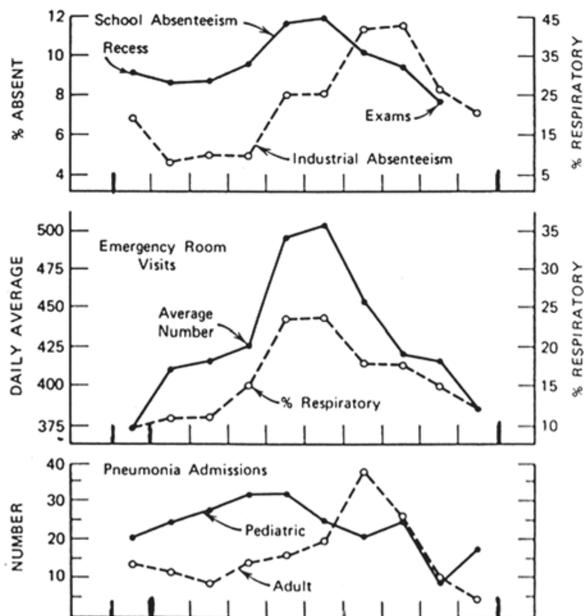

Figure 1. Influenza morbidity in children precedes that in adults – Houston, 1978 (from [24]).

necrosis of the thalamus and other deep brain structures seen in 10–20% of victims. Elevated levels of pro-inflammatory cytokines are noted and apoptosis of brain cells has been postulated to contribute to disease pathogenesis [20, 21].

Children spread influenza disease

A number of studies have shown that children shed high titers of influenza virus in the nasopharynx during illness and that they act as effective disseminators of infection [6, 22]. These observations were recently confirmed in a study conducted in an elementary school, where the effect of influenza on illness episodes, school absenteeism, medication use, parental absenteeism from work, and the occurrence of secondary illness in other family members was assessed [23]. For every 100 school children enrolled during the 37 school days of the influenza season, there were 28 illness episodes and 63 missed school days attributable to influenza (Tab. 3). In addition, for every 100 children followed, influenza accounted for an estimated 20 days of work missed by their parents and 22 secondary illness episodes among other family members. These findings support earlier observations made during an

interpandemic influenza period in Houston in 1978 [24]. As can be see in Figure 1, school absenteeism in Houston preceded industrial absenteeism by several weeks, indicating that children have a central role in the transmission of influenza to older family members within a community.

Influenza vaccination in children

There are two seasonal influenza vaccines licensed for use in children, the TIV given by intramuscular injection and the LAIV administered intranasally. The TIV is licensed for use in all children 6 months of age and older; the LAIV is licensed for use in children, without a history of asthma, 2 years of age and older. Both of these vaccines have been studied in a number of safety, immunogenicity, and efficacy studies conducted in children of various ages. Because many other respiratory viruses mimic the symptoms of influenza, influenza vaccine efficacy trials that use only clinical outcomes generally have lower estimates of vaccine efficacy since they include a number of non-influenza cases. Efficacy trials that assess laboratory-confirmed influenza are regarded as the gold standards by which the vaccines are most appropriately judged. The results of such vaccine trials are summarized in the next sections.

Efficacy of TIV

Many studies of seasonal TIV in children have been conducted, but a number of them have been of relatively small sample size and have used both clinically defined and laboratory-confirmed influenza disease as the efficacy outcomes of interest. Several recent reviews and meta-analyses of these trials provide a comprehensive assessment of the published literature [25–29]. Three reports discussing TIV efficacy are highlighted here [26, 29, 30].

A recently published comprehensive meta-analysis conducted by Manzoli et al. [29] evaluated all the published randomized clinical studies of TIV for the prevention of naturally occurring influenza in healthy children and adolescents less than 18 years of age. Each trial was assessed for the quality of randomization, concealment of group allocation, and double blinding; studies judged to be inadequate were excluded. Including data from 9 randomized clinical studies of TIV in children and using clinically diagnosed illness as the study endpoint, overall vaccine efficacy was determined to be 45% [95% confidence interval (CI): 33–55%]. Including data from 11 trials of TIV and using laboratory confirmed influenza as the study endpoint, overall vaccine efficacy was 62% (95% CI: 45–75%). In addition, TIV efficacy for both clinically defined and laboratory-confirmed influenza improved with increasing age of the children. These authors also attempted to determine vaccine efficacy for children less than 2 years of age but found

only three studies of relatively small sample size [29]. One of these trials assessing clinically defined cases showed a statistically significant efficacy, but two additional trials evaluating culture confirmed influenza did not demonstrate significant efficacy [29]. Additionally, three studies that evaluated the impact of vaccine on acute otitis also showed no overall benefit of vaccine [29, 31, 32]. These authors concluded that in children younger than 2 years of age, "the scarcity of data available suggests that any conclusion should be avoided until further studies are published."

Zangwill and Belshe [26] also assessed the overall vaccine efficacy of TIV in their review and came to much the same conclusions. When results from five clinical studies in children <9 years of age receiving two doses of TIV and using laboratory-confirmed influenza as the efficacy criteria, vaccine efficacy was 63% (95% CI: 45–70%). They also made several general conclusions that mirror those of Manzoli et al.; protective efficacy increased with age of the child and the range of vaccine efficacy in children <5 years of age was broad and limited by the small sample size of the few existing studies.

From 1985 to 1990, a randomized, placebo-controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease in individuals 1–65 years of age was conducted at Vanderbilt University [33]. Data from a subset of patients younger than 16 years were evaluated to determine TIV efficacy based on culture-positive illness and seroconversion [30]. During the 5 years of the study, 791 children younger than 16 years received 1809 doses of either inactivated or cold-adapted vaccine or placebo. In these children TIV was 91.4% and 77.3% efficacious in preventing symptomatic, culture-positive influenza A H1N1 and H3N2 illness, respectively. The efficacy of the inactivated vaccine based on hemagglutination inhibition assay seroconversion for H1N1 and H3N2 serotypes was 67.1% and 65.5%, respectively. There were no statistically significant differences in vaccine efficacy between the TIV and LAIV for the prevention of laboratory-confirmed disease in this study. The conclusion from that study was that TIV was efficacious in the prevention of influenza disease in children 1–16 years old.

Safety of TIV

Two large studies have assessed the safety of TIV in children and provide assurance that the vaccine is well tolerated in this age group [34, 35]. Hambidge et al. [34] conducted a retrospective chart review of significant medically attended events at eight managed care organizations in the United States that participate in the CDC-funded Vaccine Safety Datalink. All children in this cohort who were 6–23 months old and who received TIV between 1 January 1991, and 31 May 2003 were assessed. This represented 45 356 children who received a total of 69 359 TIV vaccinations. Any medi-

cally attended event associated with TIV was evaluated in four risk windows; 0–3 days, 1–14 days, 1–42 days, and 15–42 days after vaccination and compared with two control periods, one before vaccination and the second after the risk window. The results of this study indicate that there were very few medically attended events, none were serious, and none were significantly associated with the vaccine. This study provides additional evidence supporting the safety of immunizing children 6–23 months old with TIV.

In another study from the CDC-funded Vaccine Safety Datalink, France et al. [35] evaluated children younger than 18 years of age who received TIV. Medical visits in the 2 weeks after TIV vaccination were compared with those in two control periods. Children vaccinated from 1 January 1993 to 31 December 1999 were randomly divided into two equal groups. In group 1, risks of outpatient, emergency department, and inpatient visits during the 14 days after vaccination were compared with the risks of visits in the control periods. Significant medically attended events identified in group 1 were then evaluated in group 2, using the same two control periods. Medically attended events significant in both groups were considered potentially associated with vaccination and were assessed by medical record review. A total of 251 600 vaccination episodes were assessed. Study participants incurred 1165, 230, and 489 different diagnoses during the 14 days after vaccination according to the outpatient, emergency department, and inpatient data, respectively. After medical record review, impetigo in 9 children 6–23 months of age was significantly more common after vaccination. The conclusion of this large safety study was that TIV was well tolerated in the entire age range of pediatric vaccinations.

Efficacy safety of LAIV

A number of studies of LAIV have been published using monovalent, bivalent, and trivalent experimental and manufacturing lot preparations of LAIV. One of the largest was a multicenter, double-blind, placebo-controlled trial of trivalent LAIV conducted in children 15–71 months old in the late 1990s [36]. In this pivotal study, 1314 children were assigned to receive two doses of live attenuated intranasal vaccine and 288 children were assigned to receive one dose of either live attenuated vaccine or placebo. The strains included in the live attenuated vaccine were antigenically equivalent to those in the contemporary inactivated influenza virus vaccine. Ill subjects were evaluated with viral cultures for influenza during the subsequent influenza season. A case of influenza was defined as illness associated with isolation of wild-type influenza virus from respiratory secretions. The intranasal vaccine was well tolerated with no serious adverse events reported. Among children who were initially seronegative, fourfold titer rises were noted in 61–96% of the subjects, depending on the influenza strain. Cases of influenza were significantly less common in the vaccine group than the

placebo group and vaccine efficacy against culture-confirmed influenza illness was 93% (95% CI: 88–96%). In addition, the one-dose regimen had 89% efficacy against culture-confirmed disease.

To determine the safety of cold-adapted trivalent intranasal influenza virus vaccine a randomized, double-blind, placebo-controlled safety trial was conducted in nearly 10,000 healthy children age 12 months to 17 years of age given live vaccine or placebo in a 2:1 randomization scheme [37]. Children <9 years of age received two doses of either vaccine or placebo 28–42 days after the first dose. Enrolled children were then followed for 42 days after each vaccination for all medically attended events. Acute respiratory tract events, systemic bacterial infections, acute gastrointestinal tract events and rare events potentially associated with wild-type influenza were assessed and none were found to be associated with vaccine. However, a statistically significant increase in the relative risk for reactive airway disease [4.06 (90% CI: 1.29–17.86)] was observed in children 18–35 months of age. Based on the high efficacy rates obtained in the Belshe et al. [36] study, but tempered by the safety concerns associated with wheezing in this large study, the LAIV was licensed for use in children over 5 years of age without a previous history of wheezing.

Given concerns over these reactive airway findings in the study of Bergen et al. [37], another study comparing the efficacy and safety of LAIV with inactivated influenza vaccine in children 6–71 months of age with a history of recurrent respiratory tract infections was conducted [38]. Children were randomized to receive two doses of either LAIV ($n=1101$) or inactivated vaccine ($n=1086$) before the 2002–2003 influenza season. Participants were followed for culture-confirmed influenza illness and vaccine safety. Overall, there were 52.7% (95% CI: 21.6–72.2%) fewer cases of confirmed influenza caused by antigenically similar strains in the live attenuated than in the inactivated vaccine groups. There were no differences between groups in the incidence of wheezing after vaccination.

To further compare the safety and efficacy of the LAIV and TIV in asthmatic children, Fleming et al. [39] randomized over 2000 asthmatic children 6–17 years of age, to either TIV or LAIV in an open-label study during the 2002–2003 influenza season. Participants were assessed for culture-confirmed influenza illness and vaccine safety. When the incidence of culture-confirmed influenza illness was compared between the two vaccine groups, the LAIV had significantly greater relative efficacy 34.7% (95% CI: 3.9–56.0%). No significant differences were noted between the two vaccine groups in the incidence of asthma exacerbations, mean peak expiratory flow rate findings, asthma symptom scores, or nighttime awakening scores. Runny nose and nasal congestion were more common in the recipients of the LAIV and more injection site reactions were noted for the TIV recipients.

Another trial to assess vaccine efficacy of LAIV was recently conducted in Asia. Tam et al. [40] evaluated the efficacy and safety of LAIV against

culture-confirmed influenza in a placebo-controlled trial during two influenza seasons. In year 1, 3174 children 12–36 months of age were randomized to receive two doses of LAIV or placebo. In year 2, 2947 subjects were again randomized to receive one dose of LAIV or placebo. Vaccine efficacy in year 1 was 72.9% (95% CI: 62.8–80.5%) against antigenically similar influenza subtypes, and 70.1% (95% CI: 60.9-77.3%) against any strain. In year 2, LAIV was effective against antigenically similar (84.3%; 95% CI: 70.1–92.4%) and any (64.2%; 95% CI: 44.2–77.3%) influenza strains. No increase in wheezing episodes was noted in vaccine recipients in either study year.

In the most recent comparative efficacy study, Belshe et al. [41] compared the safety and efficacy of intranasally administered LAIV with that of inactivated vaccine in infants and young children. Children 6–59 months of age, without a recent episode of wheezing illness or severe asthma, were randomly assigned in a 1:1 ratio to receive either cold-adapted trivalent LAIV or TIV in a double-blind manner. Influenza-like illness was assessed with cultures during the 2004–2005 influenza season and safety was carefully monitored. Overall, there were 54.9% fewer cases of cultured-confirmed influenza in the LAIV recipients than in the TIV recipients (153 vs. 338 cases, $p < 0.001$). The better efficacy of live attenuated vaccine was seen for both antigenically well-matched and drifted viruses. Among previously unvaccinated children, wheezing within 42 days of administration of dose one of LAIV was more common than with TIV. Among children 6–11 months of age, 12 more episodes of wheezing were noted within 42 days after receipt of dose one among recipients of LAIV (3.8%) than among recipients of TIV (2.1%, $p = 0.076$). Rates of hospitalization for any cause during the 180 days after vaccination were higher among the recipients of LAIV who were 6–11 months of age (6.1%) than among the recipients of TIV in this age group (2.6%, $p = 0.002$). Based on these results, LAIV is licensed down to 2 years of age in children without a previous history of wheezing or asthma.

Finally, review of a very large open-label, nonrandomized, community-based trial of a LAIV was conducted by Piedra et al. [42] and provides some of the most comprehensive safety data available in the literature for the live vaccine. Medical records of all children who received LAIV were surveyed for serious adverse events (SAEs) and health care utilization 6 weeks after vaccination. In the four study years, 18 780 doses of LAIV were administered to 11 096 children. A total of 4529, 7036, and 7215 doses of LAIV-T were administered to children who were 18 months to 4 years, 5–9 years, and 10–18 years of age, respectively. During the four study years 42 SAEs were identified, but none were attributed to LAIV-T. Compared with the prevaccination period, there were no increases in medically attended acute respiratory infections from 0 to 14 and 15 to 42 days after vaccination in children who were 18 months to 4 years, 5–9 years, and 10–18 years of age. A relative risk of 2.85 (95% CI: 1.01–8.03) for asthma events 15–42 days after

vaccination was detected in children who were 18 months to 4 years of age during one study year, but was not significantly increased for the other three vaccine years [vaccine year 2, RR: 1.42 (95% CI: 0.59–3.42); vaccine year 3, RR: 0.47 (95% CI: 0.12–1.83); vaccine year 4, RR: 0.20 (95% CI: 0.03–1.54)]. They concluded that LAIV was safe in children receiving the vaccine [42].

Can herd immunity for influenza be achieved?

There are a number of highly contagious infections, such as measles and varicella, where immunization of a portion of the population confers protection to unimmunized individuals by decreasing the circulation of the pathogen, a concept called herd immunity. Several years ago a study in Japan assessed the impact of influenza immunization of school children on influenza mortality in elderly persons and others at high risk [43]. From 1962 to 1987, Japanese school children were mandated to receive TIV, and most were vaccinated; in 1987 the laws were relaxed and in 1994 they were repealed. The study looked at influenza vaccination rates and death rates spanning this time period in Japan, and compared them to data from the U.S. (Fig. 2).

After the vaccination program for school children was initiated in Japan, excess mortality rates dropped from values three to four times those in the U.S. to values similar to those in the U.S. Routine vaccination of Japanese children was estimated to have prevented 37 000–49 000 deaths per year, or about 1 death for every 420 children vaccinated. As the vaccination mandate was relaxed, vaccination rates dropped and excess mortality rates increased. In contrast, excess mortality rates in the U.S were nearly constant over the same period of time. The data from Japan suggested that vaccinating school children against influenza reduced influenza mortality among older persons, confirming that herd immunity was occurring with influenza vaccine [43].

A similar study was recently reported from the U.S., where school children were vaccinated with LAIV and its impact was assessed in their households and community [44]. Eleven demographically similar clusters of elementary schools in four states were chosen. Within each cluster, one school was selected to receive vaccination (intervention school) and one or two schools in that cluster did not participate (control schools). During a predicted week of peak influenza activity in each state, all households with children in the intervention and control schools were asked about influenza vaccination and influenza-like illness. Persons living in intervention school households had significantly fewer influenza-like symptoms and outcomes during the recall week than those in control school households, even though they themselves might not have been immunized. This suggests that vaccinating children protects their unimmunized contacts, the essential mechanism of herd immunity.

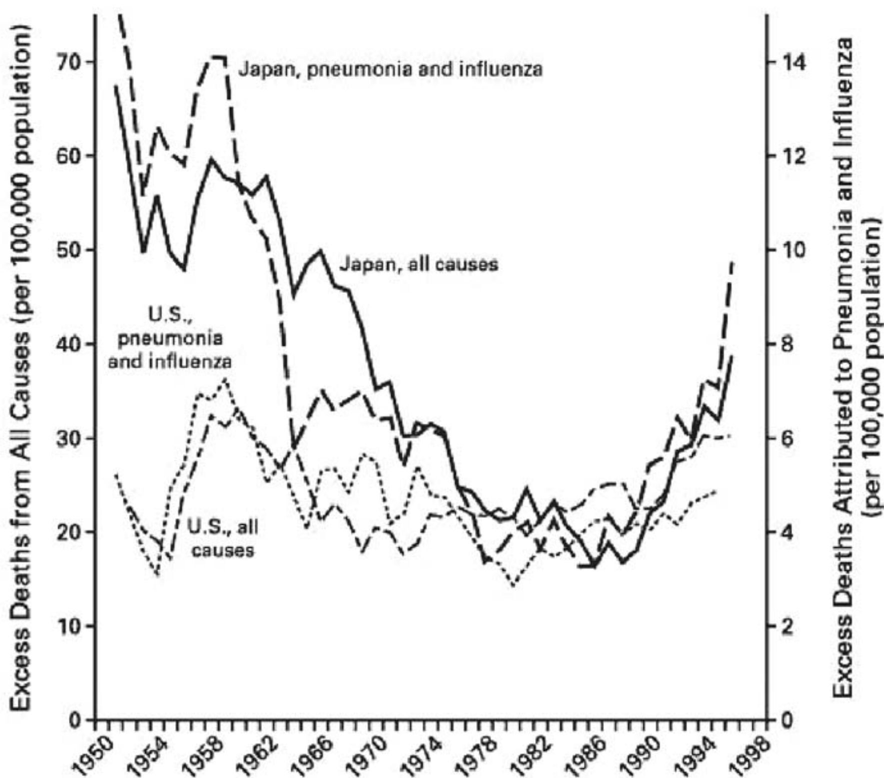

Figure 2. Excess deaths attributed to both pneumonia and influenza and all causes, spanning the years when the Japanese school immunization program was dismantled (from [43]).

Practical implications for influenza vaccination of children

For many years, all children with high-risk conditions associated with influenza have been recommended to receive annual influenza vaccination. These conditions include asthma or other chronic pulmonary diseases, significant cardiac disease, immunosuppressive disorders, human immunodeficiency virus infection, sickle cell anemia, long-term aspirin therapy, chronic renal disease, chronic metabolic disorders, and children with neurological disorders. Beginning with the 2006–2007 influenza season in the United States, all children between the ages of 6 months and 5 years were recommended to receive annual TIV to reduce the burden of both hospitalization and outpatient visits associated with influenza. With the universal recommendations for influenza vaccine in young children, studies have been conducted to monitor vaccine uptake. Data from the National Immunization Survey measured vaccination rates in children 6–23 months

of age the year after the universal influenza recommendations were issued. Although influenza immunization rates varied widely among the different states, overall 33.4% of children between 6 and 23 months of age received one dose of vaccine and 17.8% received two doses [45]. Continued assessment of influenza vaccination rates in this population is needed. It is hoped that immunization rates will increase with each year that passes after a new recommendation.

Considerable discussion has surrounded the question whether routine influenza vaccination of all school children might reduce disease in both the children and other community members, as discussed earlier. A study conducted through the CDC-funded Vaccine Safety Datalink addressed the simple question of whether two doses of TIV could be delivered to children <9 years of age who had not previously received vaccine [46]. A total of 125,928 children 6 months to 8 years of age were evaluated. Among children 6–23 months of age, a fairly high proportion of first time-vaccinated children also received a second vaccination, with rates of 44% in 2001–2002, 54% in 2002–2003, and 29% in 2003–2004 (a season with vaccine shortages). In contrast, among children 2–8 years of age, the corresponding rates were only 15%, 24%, and 12%. The fact that the majority of children who required two doses of vaccine did not receive them highlights some of the difficulties that may be encountered in implementing universal vaccination of all school children in the primary care setting.

Might school-based vaccine delivery circumvent some of these problems? A recent report describing on-site administration of LAIV to all students in a large, metropolitan public school system demonstrated that large numbers of school children could be effectively immunized [47]. There were 53,420 students in the system; 56% of the elementary school students, 45% of the middle school students, and 30% of the high school students were immunized. This experience clearly highlights that a vaccination campaign in a large public school system can achieve relatively high coverage levels; however, considerable effort by the local health department was expended in the process. Analyses of studies assessing the effectiveness of the school-based influenza immunization programs are in progress.

Conclusion

Given the clear evidence that both live and inactivated influenza vaccines can prevent influenza disease, influenza vaccination should be offered to all children who wish to reduce their burden of influenza. The recent evidence of improved vaccine efficacy for LAIV in young children and the likely approval of the vaccine for a younger age group, also suggests that it might be a better alternative to TIV in young children without a history of asthma.

Also, given that influenza disease is so rarely specifically diagnosed [5] and that it can mimic other respiratory viral infections, it is imperative that laboratory-based surveillance be conducted to assess vaccine efficacy as influenza vaccine is utilized more broadly. The future for influenza prevention is bright, but continued attention to measuring vaccine effect is needed to sustain this effort.

References

1 Izurieta HS, Thompson WW, Kramarz P, Shay DK, Davis RL, DeStefano F, Black S, Shinefield H, Fukuda K (2000) Influenza and the rates of hospitalization for respiratory disease among infants and young children. *N Engl J Med* 342: 232–239

2 Neuzil KM, Mellen BG, Wright PF, Mitchel EF Jr, Griffin MR (2000) The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. *N Engl J Med* 342: 225–231

3 Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA, Coulen C, Poehling KA, Shone LP, Balter S et al (2004) New Vaccine Surveillance Network. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. *Pediatrics* 113: 1758–1764

4 Griffin MR, Walker FJ, Iwane MK, Weinberg GA, Staat MA, Erdman DD (2004) Epidemiology of respiratory infections in young children: Insights from the New Vaccine Surveillance Network. *Pediatr Infect Dis J* 23 (Suppl): S188–S192

5 Poehling KA, Edwards KM, Weinberg GA, Szilagyi P, Staat MA, Iwane MK, Bridges CB, Grijalva CG, Zhu Y, Bernstein DI et al (2006) New Vaccine Surveillance Network. The under-recognized burden of influenza in young children. *N Engl J Med* 355: 31–40

6 Weinberg GA, Erdman DD, Edwards KM, Hall CB, Walker FJ, Griffin MR, Schwartz B, New Vaccine Surveillance Network Study Group (2004) Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. *J Infect Dis* 189: 706–710

7 Poehling KA, Griffin MR, Dittus RS, Tang YW, Holland K, Li H, Edwards KM (2002) Bedside diagnosis of influenza virus infections in hospitalized children. *Pediatrics* 110: 83–88

8 Bonner AB, Monroe KW, Talley LI, Klasner AE, Kimberlin DW (2003) Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: Results of a randomized, prospective, controlled trial. *Pediatrics* 112: 363–367

9 Sharma V, Dowd MD, Slaughter AJ, Simon SD (2002) Effect of rapid diagnosis of influenza virus type A on the emergency department management of febrile infants and toddlers. *Arch Pediatr Adolesc Med* 156: 41–43

10 Schrag SJ, Shay DK, Gershman K, Thomas A, Craig AS, Schaffner W, Harrison LH, Vugia D, Clogher P, Lynfield R et al (2006) Emerging Infections Program

Respiratory Diseases Activity. Multistate surveillance for laboratory-confirmed, influenza-associated hospitalizations in children: 2003–2004. *Pediatr Infect Dis J* 25: 395–400

11 Grijalva CG, Craig AS, Dupont WD, Bridges CB, Schrag SJ, Iwane MK, Schaffner W, Edwards KM, Griffin MR (2006) Estimating influenza hospitalizations among children. *Emerg Infect Dis* 12: 103–109

12 Mullooly JP, Barker WH (1982) Impact of type A influenza on children: A retrospective study. *Am J Public Health* 72: 1008–1016

13 Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, Fukuda K (2004) Influenza-associated hospitalizations in the United States. *JAMA* 292: 1333–1340

14 O'Brien MA, Uyeki TM, Shay DK, Thompson WW, Kleinman K, McAdam A, Yu XJ, Platt R, Lieu TA (2204) Incidence of outpatient visits and hospitalizations related to influenza in infants and young children. *Pediatrics* 113: 585–593

15 Neuzil KM, Zhu Y, Griffin MR, Edwards KM, Thompson JM, Tollefson SJ, Wright PF (2002) Burden of interpandemic influenza in children younger than 5 years: A 25-year prospective study. *J Infect Dis* 185: 147–152

16 Glezen WP, Greenberg SB, Atmar RL, Piedra PA, Couch RB (2000) Impact of respiratory virus infections on persons with chronic underlying conditions. *JAMA* 283: 499–505

17 Montes M, Vicente D, Pérez-Yarza EG, Cilla G, Pérez-Trallero E (2005) Influenza-related hospitalisations among children aged less than 5 years old in the Basque Country, Spain: A 3-year study (July 2001–June 2004). *Vaccine* 23: 4302–4306

18 Chiu SS, Lau YL, Chan KH, Wong WH, Peiris JS (2002) Influenza-related hospitalizations among children in Hong Kong. *N Engl J Med*. 347: 2097–2103

19 Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos AM, Posey DL, Klimov A, Lindstrom SE et al (2005) Influenza Special Investigations Team. Influenza-associated deaths among children in the United States, 2003–2004. *N Engl J Med* 353: 2559–2567

20 Morishima T, Togashi T, Yokota S, Okuno Y, Miyazaki C, Tashiro M, Okabe N (2002) Collaborative Study Group on Influenza-Associated Encephalopathy in Japan. Encephalitis and encephalopathy associated with an influenza epidemic in Japan. *Clin Infect Dis* 35: 512–517

21 Surtees R, DeSousa C (2006) Influenza virus associated encephalopathy. *Arch Dis Child* 91: 455–456

22 Frank AL, Taber LH, Wells CR, Wells JM, Glezen WP, Paredes A (1981) Patterns of shedding of myxoviruses and paramyxoviruses in children. *J Infect Dis* 144: 433–441

23 Neuzil KM, Hohlbein C, Zhu Y (2002) Illness among schoolchildren during influenza season: Effect on school absenteeism, parental absenteeism from work, and secondary illness in families. *Arch Pediatr Adolesc Med* 156: 986–991

24 Glezen WP, Couch RB (1978) Interpandemic influenza in the Houston area, 1974–76. *N Engl J Med* 298: 587–592

25 Ruben FL (2004) Inactivated influenza virus vaccines in children. *Clin Infect Dis*. 38: 678–688
26 Zangwill KM, Belshe RB (2004) Safety and efficacy of trivalent inactivated influenza vaccine in young children: A summary for the new era of routine vaccination. *Pediatr Infect Dis J* 23: 189–197
27 Jefferson T, Smith S, Demicheli V, Harnden A, Rivetti A, Di Pietrantonj C (2005) Assessment of the efficacy and effectiveness of influenza vaccines in healthy children: Systematic review. *Lancet* 365: 773–780
28 Negri E, Colombo C, Giordano L, Groth N, Apolone G, La Vecchia C (2005) Influenza vaccine in healthy children: A meta-analysis. *Vaccine* 23: 2851–2861
29 Manzoli L, Schioppa F, Boccia A, Villari P (2007) The efficacy of influenza vaccine for healthy children: A meta-analysis evaluating potential sources of variation in efficacy estimates including study quality. *Pediatr Infect Dis J* 26: 97–106
30 Neuzil KM, Dupont WD, Wright PF, Edwards KM (2001) Efficacy of inactivated and cold-adapted vaccines against influenza A infection, 1985 to 1990: The pediatric experience. *Pediatr Infect Dis J* 20: 733–740
31 Hoberman A, Greenberg DP, Paradise JL, Rockette HE, Lave JR, Kearney DH, Colborn DK, Kurs-Lasky M, Haralam MA, Byers CJ et al (2003) Effectiveness of inactivated influenza vaccine in preventing acute otitis media in young children: A randomized controlled trial. *JAMA* 290: 1608–1616
32 Clements DA, Langdon L, Bland C, Walter E (1995) Influenza A vaccine decreases the incidence of otitis media in 6- to 30-month-old children in day care. *Arch Pediatr Adolesc Med* 149: 1113–1117
33 Edwards KM, Dupont WD, Westrich MK, Plummer WD Jr, Palmer PS, Wright PF (1994) A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J Infect Dis* 169: 68–76
34 Hambidge SJ, Glanz JM, France EK, McClure D, Xu S, Yamasaki K, Jackson L, Mullooly JP, Zangwill KM, Marcy SM et al (2006) Safety of trivalent inactivated influenza vaccine in children 6 to 23 months old. *JAMA* 296: 1990–1997
35 France EK, Glanz JM, Xu S, Davis RL, Black SB, Shinefield HR, Zangwill KM, Marcy SM, Mullooly JP, Jackson LA, Chen R (2004) Safety of the trivalent inactivated influenza vaccine among children: A population-based study. *Arch Pediatr Adolesc Med* 158: 1031–1036
36 Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, Bernstein DI, Hayden FG, Kotloff K, Zangwill K et al (1998) The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N Engl J Med* 338: 1405–1412
37 Bergen R, Black S, Shinefield H, Lewis E, Ray P, Hansen J, Walker R, Hessel C, Cordova J, Mendelman PM (2004) Safety of cold-adapted live attenuated influenza vaccine in a large cohort of children and adolescents. *Pediatr Infect Dis J* 23: 138–144
38 Ashkenazi S, Vertruyen A, Arístegui J, Esposito S, McKeith DD, Klemola T, Biolek J, Kühr J, Bujnowski T, Desgrandchamps D et al (2006) Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections. *Pediatr Infect Dis J* 25: 870–879

39 Fleming DM, Crovari P, Wahn U, Klemola T, Schlesinger Y, Langussis A, Øymar K, Garcia ML, Krygier A, Costa H et al (2006) Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. *Pediatr Infect Dis J* 25: 860–869
40 Tam JS, Capeding MR, Lum LC, Chotpitayasunondh T, Jiang Z, Huang LM, Lee BW, Qian Y, Samakoses R, Lolekha S et al (2007) Efficacy and safety of a live attenuated, cold-adapted influenza vaccine, trivalent against culture-confirmed influenza in young children in Asia. *Pediatr Infect Dis J* 26: 619–628
41 Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, Kemble G, Connor EM, CAIV-T Comparative Efficacy Study Group (2007) Live attenuated *versus* inactivated influenza vaccine in infants and young children. *N Engl J Med* 356: 685–696
42 Piedra PA, Gaglani MJ, Riggs M, Herschler G, Fewlass C, Watts M, Kozinetz C, Hessel C, Glezen WP (2005) Live attenuated influenza vaccine, trivalent, is safe in healthy children 18 months to 4 years, 5 to 9 years, and 10 to 18 years of age in a community-based, nonrandomized, open-label trial. *Pediatrics* 116: e397–407
43 Reichert TA, Sugaya N, Fedson DS, Glezen WP, Simonsen L, Tashiro M (2001) The Japanese experience with vaccinating schoolchildren against influenza. *N Engl J Med* 344: 889–896
44 King JC Jr, Stoddard JJ, Gaglani MJ, Moore KA, Magder L, McClure E, Rubin JD, Englund JA, Neuzil K, King JC et al (2006) Effectiveness of school-based influenza vaccination. *N Engl J Med* 355: 2523–2532
45 http: //www.cdc.gov/mmwr/preview/mmwrhtml/mm5539a1.htm
46 Jackson LA, Neuzil KM, Baggs J, Davis RL, Black S, Yamasaki KM, Belongia E, Zangwill KM, Mullooly J, Nordin J et al (2006). Compliance with the recommendations for 2 doses of trivalent inactivated influenza vaccine in children less than 9 years of age receiving influenza vaccine for the first time: A Vaccine Safety Datalink study. *Pediatrics* 118: 2032–2037
47 Carpenter LR, Lott J, Lawson BM, Hall S, Craig AS, Schaffner W, Jones TF (2007) Mass distribution of free, intranasally administered influenza vaccine in a public school system. *Pediatrics* 120: e172–178

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The immune response to influenza A viruses

Justine D. Mintern[1], Carole Guillonneau[1], Stephen J. Turner[1] and Peter C. Doherty[1,2]

[1]Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia; [2]Department of Immunology, St Jude Children's Research Hospital, Memphis, TN 38105, USA

Abstract

The influenza A viruses are dangerous pathogens with the potential to provoke devastating disease. The challenge for the medical research community is to design preventive measures and therapeutic interventions that will limit the severe consequences of pandemic influenza A virus infections. Vaccines have long been available, but there is considerable scope for improvement as they target only the prevailing influenza A virus strains, do not give broad immunity and work poorly in the elderly, the target group that is most at risk of fatal disease. Improved vaccines will only emerge if the development strategy is based on a firm understanding of the host immune response to the virus. Here, we summarize the research to date that details immune mechanisms participating in the control and elimination of influenza A viruses.

Introduction

The influenza viruses are Orthomyxoviruses with an eight-segmented, negative-sense single-stranded (ss) RNA genome. There are three types: influenza A, B and C. The influenza A viruses that cause the most serious problems in humans are the subject of this review. These pathogens are classified according to their two major surface glycoproteins: hemagglutinin (HA, or H) and neuraminidase (NA or N). Infecting both mammalian and avian species, the highly contagious influenza A viruses are responsible for widespread morbidity and mortality [1]. In mammals, infection is established in the upper and lower respiratory tracts, provoking an illness that is associated with fever, myalgia, congestion, pharyngitis and, in severe cases, pneumonia. Early on, some of the very virulent influenza A viruses can induce a "cytokine shock" syndrome mediated *via* the innate immune response pathway. Fortunately, infection also elicits potent adaptive immunity and long-term memory, although the virus can mutate readily allowing strains with variant HA molecules to cause successive pandemics. The

current killed, or subunit vaccines induce effective antibody responses in normal adults, although they do not promote a virus-specific $CD8^+$ T cell response and memory and they are poorly immunogenic in those who are even marginally immunologically compromised. The major task for immunologists interested in the problem that influenza virus poses, is to develop better vaccines. Most of our detailed knowledge about immunity to the influenza A viruses is derived from the murine model that allows rigorous analysis due to the availability of an extensive panel of defined analytical reagents. Here, we provide a comprehensive summary of a large body of research examining the immune mechanisms that act to control influenza A virus infection (Fig. 1). This information should provide a useful basis for the informed design of novel, next generation influenza A virus vaccines.

Detection of influenza A virus

Invading influenza A viruses are detected in the host environment by "pattern recognition receptors" (PRR) [2]. Previously, the molecular target was considered to be double-stranded viral RNA (dsRNA) recognized by the PRR, toll-like receptor 3 (TLR3) [3, 4]. A role for TLR3 was questioned, however, given that the concentration of dsDNA is unlikely to be sufficient to signal TLR3 [5]. It is now considered that influenza A virus infection does not generate dsRNA at all [6]. Instead, the influenza A virus polymerase generates ssRNA with an uncapped, 5'-phosphate that serves as the molecular signature identified by the immune system [6]. The cytoplasmic RNA helicase, RIG-1 [6, 7], but not MDA5 [6, 8], is responsible for influenza A virus recognition, which occurs independently of viral replication [7]. In addition to RIG-1, TLR7 is implicated in influenza A virus detection. Expressed in the endosomal compartments of plasmacytoid dendritic cells (DCs) and B cells, TLR7 detects influenza A virus ssRNA [9, 10]. The participation of multiple PRR in the surveillance of influenza A virus may reflect cell type-specific roles [11]. Once influenza A virus is recognized, PRR initiate multiple signaling cascades that facilitate both innate and adaptive immunity to enable viral eradication.

Innate immunity and the influenza A viruses

Innate immunity directed against influenza A virus provides an immediate and rapid response to the pathogen. The pulmonary infiltrate of innate immune cells is comprised mainly of natural killer (NK) cells, neutrophils and macrophages. The NK cell represents the major innate response element and are detected in the infected lung as early as 48 h following influenza A virus infection [12, 13]. Protection is thought to be mediated by both cytokine production [interferon (IFN)-γ and tumor necrosis factor

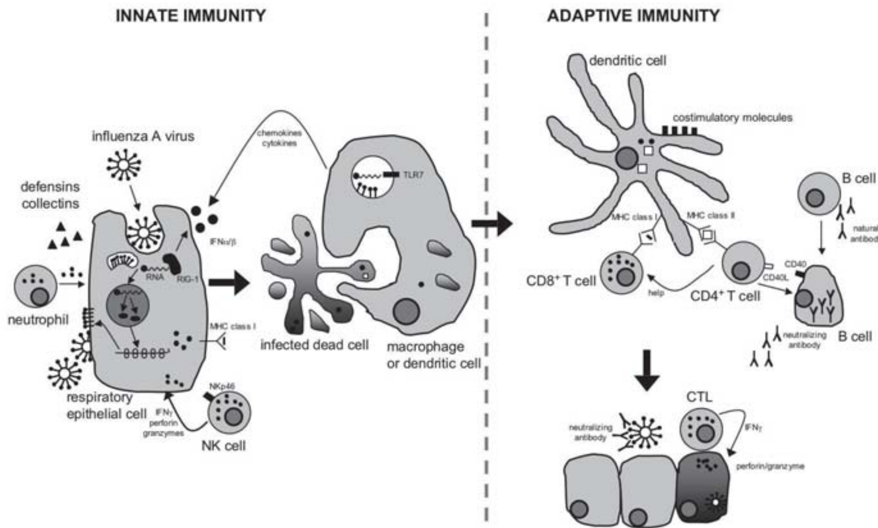

Figure 1. Summary of the host immune response to influenza A virus

(TNF-α)] and direct cytotoxicity of virus-infected cells [14]. Influenza A virus–infected cells are recognized by NKp46 [15] and NKp44 [16] interaction with HA. The critical role for this pathway in influenza control is illustrated by the fatal infection that occurs in mice that lack NKp46 [17]. Together with NK cells, neutrophils also contribute to influenza A virus clearance through the secretion of an array of pro-inflammatory molecules that serve to limit viral replication [18–20]. Finally, alveolar macrophages (AM) are also present in the innate pulmonary infiltrate, although initially they form only a small contribution, but are recruited in large numbers later by the T cell response. AM represent the major phagocytic cell type resident in the lung [21], acting to scavenge influenza A virus-derived antigen [22]. In addition, AM secrete pro-inflammatory cytokines including TNF-α, interleukin (IL)-1β, IL-6 and IFN-α/β) [23, 24], together with the chemokines macrophage inflammatory protein (MIP)-1a, monocyte chemotactic protein (MCP)-1, RANTES and IFN-inducible protein (IP)-10 [18, 23, 25–27]. The AM can also modulate adaptive T cell immunity to influenza A viruses [28]. Present in the lung during active viral replication, AM are fully susceptible to influenza A virus infection [23]. Unlike in epithelial cells, however, the infection is non-productive with little, if any, virion release [23, 29], although it does lead to subsequent apoptosis [29]. Depletion of macrophages during influenza A virus infection results in elevated viral titers and increased morbidity and mortality, illustrating their participation in the response [18]. Therefore, multiple immune cell types provide immediate innate defense against the influenza A viruses.

The pulmonary infiltrate releases a torrent of innate immune molecules that are considered to limit influenza A virus infection. A long list of cytokines and chemokines are potentially involved. A major player is type I IFN, representing the most potent cytokine attack against the virus [30]. So potent is the IFN response that the influenza A viruses encode a protein (NS2) to disable this pathway (described in *Influenza A Virus Escape* below). Nasal and pulmonary IFN-α and -β rise rapidly following influenza A virus infection [31] and act to directly limit viral replication and induce further cytokines and/or chemokine secretion that enhances recruitment and activation of multiple immune cell types. Type I IFN serves to enhance macrophage function, promote antigen presentation by antigen-presenting cells (APC) and to modulate adaptive immunity. The importance of this pathway is exemplified by the severe pulmonary disease that develops following influenza A virus infection of mice with disrupted type I IFN signaling [32, 33]. Plasmacytoid DCs are the major producers of type I IFN in response to many viruses, including influenza A virus [34–37]. Other cytokines implicated in influenza A virus immunity include TNF-α [38], IL-6 [39, 40], IL-1 [41], IL-18 [42] and IL-12 [43, 44]. In contrast, mice that lack functional IFN-γ can efficiently clear influenza A viruses, suggesting only a minor or redundant role for IFN-γ in the response [45–47]. Chemokines with defined roles in influenza A virus immunity include MIP-1α [48] and CCR5 [49], as illustrated by the elevated disease burden following infection of the chemokine-deficient mice. Finally, while cytokines and chemokines are important in the immune control of influenza A virus infections, their contribution can be detrimental as they elicit potentially fatal 'cytokine shock' [50]. Recent studies dramatically illustrate the devastating impact of increased inflammatory infiltrates upon viral-induced pathology. In animal models, infection with the reconstructed 1918 influenza A virus promotes massive inflammatory infiltrates with significantly higher levels of cytokines (IFN-γ, TNF-α, IL-1, IL-6, IL-12, IL-18 and granulocyte-colony stimulating factor) and chemokines (MIP-2, MIP-1α/β, MCP-1) [18, 51–53]. Therefore, particularly early on, potent inflammatory anti-viral activity may be dangerous, rather than protective, to the host due to the deleterious impact upon lung pathology.

Collectins are collagen-like lectins that participate in innate immunity to viral pathogens [54]. Collectin family members, the surfactant proteins A (SP-A) and SP-D, are constitutively present in the fluids that line the respiratory tract [55]. Together with the mannan binding lectin (MBL), SP-A and SP-D contribute to influenza A virus clearance *via* a number of mechanisms. Hemagglutination and viral infectivity is inhibited by SP-A [56, 57], SP-D [56, 58] and MBL [56, 59, 60]. In addition, complement-mediated lysis of influenza A virus-infected cells is enhanced by MBL [61], while SP-A and SP-D promote the binding and uptake of influenza A viruses by neutrophils [56, 62] and SP-A promotes opsonization and phagocytosis of influenza A virus by the AM population [63]. The sensitivity of different influenza A

viral strains to collectin-mediated defense correlates with the degree of glycosylation of the HA glycoprotein [61, 64].

Defensins are cationic peptides produced by both leukocytes and epithelial cells. Defensins can exert direct microbial activity or promote immunity by acting as chemotactic agents. Examples of defensin-mediated anti-influenza A virus activity include retrocyclin-2 (θ-defensin) and human β defensin 3 inhibition of HA-mediated membrane fusion [65]. The human neutrophil peptide (HNP) 1 (α-defensin) directly inactivates influenza A virus by an unknown mechanism [60].

Humoral immunity and the influenza A viruses

Humoral immunity provides host defense through B lymphocyte secretion of antibody. Protective antibodies target antigenic structures exposed on the pathogen surface. Antibody-mediated immunity contributes to defense against the influenza A viruses [66–69], but is not always essential for optimal viral clearance [70, 71]. In any case, the influenza A viruses elicit a diverse spectrum of anti-viral antibody responses. Natural antibodies present the first line of antibody-mediated defense [72]. These are low-affinity antibodies that restrict early virus dissemination [72] and promote the recruitment of viral antigen to the secondary lymphoid organs [73]. Natural antibodies reduce the overall load of influenza A virus and, as such, are required for optimal specific IgG antibody responses [69, 74]. Secretion of natural antibodies requires the transcriptional repressor Blimp-1: mice with Blimp-1-deficient B cells are more susceptible to influenza A virus infection [75]. Although natural antibodies are involved in the primary response to influenza A viruses, they are not required for optimal protection from secondary challenge [76]. While natural antibodies clearly display anti-viral properties, effective virus clearance requires the induction of neutralizing antibody. Such neutralizing antibodies can be rapidly induced and possess high affinity (or avidity) for viral antigen. Mostly, virus neutralization is thought to be optimally achieved *via* antibody-mediated interference with viral binding to the host receptors required for cell entry or egress. Consequently, the influenza virus HA is heavily targeted by neutralizing antibodies [77]. Crystallographic examination of HA in complex with neutralizing antibodies, reveals that antibody binding can occur at the same site as host receptor binding [78], or in distal regions where receptor binding is obstructed by steric hindrance [79]. Similar to HA, NA is also targeted by neutralizing antibodies [80]. Neutralizing antibodies represent the major target of current influenza A virus vaccine strategies. While most neutralizing antibody strategies target HA or NA [81], the matrix protein 2 (M2) represents an interesting potential vaccine candidate [82]. M2 is a transmembrane protein expressed at the infected cell surface [83], but in contrast to HA and NA, is highly conserved amongst influenza A virus strains. Unfortunately, thus far,

M2-targeted vaccine strategies have elicited only weak immunity that does not protect mice from lethal challenge [84].

$CD4^+$ T helper cells contribute to humoral immunity by promoting B cell differentiation into immunoglobulin class-switched, antibody-secreting cells. In most studies, the production of anti-influenza A virus antibody is $CD4^+$ T cell dependent [68, 85–87], although exceptions are reported [67, 68]. Classically, $CD4^+$ T cell help involves (i) the recognition of viral antigen, and (ii) the delivery of an activation signal to the B cell *via* the TNFR family member, CD40. Mice deficient in CD40 generate significantly impaired influenza A virus-specific antibody responses [85, 88]. Of interest, $CD4^+$ T cells can help B lymphocytes by non-cognate interactions that do not require specific influenza A virus antigen recognition [85].

T immunity and the influenza A viruses

Dendritic cells

DCs enable pathogen-derived antigens to be presented in a context that facilitates successful T cell immunity [89]. Specialized in antigen presentation, the DCs facilitate: (i) the acquisition of antigen, (ii) processing and presentation of antigenic peptides in the context of host major histocompatibility complex (MHC) molecules and (iii) the provision of costimulatory signals. Immunity to influenza A virus infection requires DC for both primary [90] and secondary T cell responses [91, 92]. DC can control the magnitude of influenza A virus-specific T cell immunity *via* Fas ligand (FasL)-mediated apoptosis [93]. In the respiratory tract, an extensive network of DC populations is present both in the lung [94] and the draining lymph node [95]. Furthermore, pulmonary infection recruits additional DC populations into the lung [96–98]. To acquire influenza A virus antigen, DC may simply be directly infected with the virus. Infection induces the maturational changes (up-regulation of costimulatory molecules and MHC class II) that are necessary for DC stimulation of T cells [99–101]. Infection can result in the expression of influenza NA at the DC surface, with NA-mediated removal of sialic acids serving to both enhance, and inhibit, DC function depending on the multiplicity of infection [102, 103]. DCs can also acquire influenza A virus-derived antigen released following the apoptotic lysis of infected respiratory cells [104, 105]. Once antigen is acquired, lung DCs migrate to the lymph node that drains the respiratory tract [96, 106, 107]. Migration occurs early after infection (24–48 h), after which the DC display a refractory state to further inflammatory stimuli [96]. The lymph node also contains a resident DC set that has no direct access to the airways. Despite this, these resident DCs can also present influenza A virus-derived antigen [106]. Therefore, antigen transfer between the resident and migratory lung DC subsets must occur [108, 109]. Most experiments indicate that MHC class I presentation

of influenza A virus-derived antigen in the lung draining lymph node ceases beyond 12–14 days [110, 111], although recently it has been suggested that antigen presentation can occur for up to 2 months following infection [112]. MHC class II presentation is also reported to persist for as long as 4 weeks after infection [113]. This is surprising given that infectious virus is cleared by day 10 [114]. Therefore, it has been postulated that the respiratory tract DCs can serve as a reservoir for antigen, with a depot being maintained well beyond the clearance of pathogen from the infected respiratory tissue [112, 115]. This however, remains to independently verified.

Costimulation

The participation of DCs in adaptive immunity is critical due to the rich array of costimulatory molecules expressed at the cell surface. A growing list of costimulatory molecules has been identified, most of which belong to either the CD28/B7 [116] or TNFR [117] families. Costimulation serves to enhance the antigen-specific signals that are delivered through the T cell receptor (TCR). As such, costimulation is required for optimal T cell immunity in many viral infections [118]. The major pathway of costimulation is *via* the CD28/B7 interaction that plays an important role in influenza A virus immunity. This signal contributes to the generation of influenza A virus-specific T cell immunity at multiple levels. For $CD8^+$ T cells, CD28/B7 contributes to expansion [119–121], cytotoxicity and/or effector cytokine production [119, 122, 123], recruitment to the infected airways [122] and survival [123]. In contrast, the hierarchy of T cell response magnitude to individual influenza A virus-derived epitopes (a phenomenon termed immunodominance [124, 125]) is not altered in the absence of CD28/B7 signaling [126]. Mice deficient in CD28/B7 also display impaired influenza-specific neutralizing antibody responses [121]. While CD28/B7 plays a prominent part early in response to influenza A virus infection, 41BB/41BBL is important for sustained $CD8^+$ T cell expansion and is critical for optimal recall responses [119, 121, 127]. Effective $CD4^+$ T cell immunity during influenza A virus infection also requires CD28/B7 [121], OX40/OX40L [128] and ICOS/ICOSL [129]-mediated costimulation. The accumulation of T cells in influenza A virus-infected lungs depends on CD27/CD70 signaling [120, 130]. This is due to its impact on T cell survival and/or migration to the infected respiratory tract [120]. Together, multiple costimulatory signals are delivered *via* the DCs to promote optimal adaptive immunity and, in turn, influenza A virus elimination.

$CD8^+$ T cells

Effector $CD8^+$ T cells, also known as cytolytic T lymphocytes (CTL), are important in the normal clearance of influenza A viruses [131]. Mice defi-

cient in $CD8^+$ T cells show delayed influenza A virus clearance, although they eventually control infection with all but the most virulent viruses [132]. The influenza A virus-specific $CD8^+$ T cell response has been extensively characterized utilizing murine models of infection, particularly with the HKx31 (H3N2) and PR/8 (H1N1) influenza A viruses. $CD8^+$ T cells are primed, activated and expand in the lung draining lymph nodes during the first week or so after primary infection [110, 133]. Activated $CD8^+$ T cells then traffic to the respiratory airways and the infected lung to mediate viral clearance [134]. The trafficking [135] and retention of $CD8^+$ T cells in the lung [136], is dependent on LFA-1 expression. At the site of infection, $CD8^+$ T cells target virus-infected cells that express peptide derived from influenza A virus protein associated with MHC class I. An array of epitopes is recognized in the C57BL/6 (B6) mouse model, with the dominant epitopes (in terms of response magnitude) seen by $CD8^+$ T cells being provided by the viral polymerase A ($PA_{224-233}$) [137] and nucleoprotein ($NP_{366-374}$) [138, 139]. Subdominant epitopes are derived from the basic polymerase subunit 1 ($PB1_{703-711}$) [140], the mitochondrial protein PB1-$F2_{62-70}$ [140, 141], non-structural protein 2 ($NS2_{114-121}$) [139] and matrix protein 1 ($M1_{128-135}$) [142]. In the absence of the dominant epitopes, subdominant epitope-specific $CD8^+$ T cells account for a compensatory response, although a slight delay in viral clearance is observed [143, 144]. Depending on the experimental model, 30–90% of $CD8^+$ T cells recovered from the respiratory tract are influenza A virus specific at the peak of the primary response, illustrating their enrichment in the pneumonic lung [125, 139, 140, 145]. Epitope-specific $CD8^+$ T cells can be found widely dispersed throughout various body organs, including the lung, spleen, bone marrow, blood, liver and non-draining lymph nodes [145, 146]. Once their target antigen is recognized, $CD8^+$ T cells exert multiple effectors functions. Cytokines such as IFN-γ, TNF-α and IL-2 are secreted by influenza A virus-specific $CD8^+$ T cells [147]. In addition, $CD8^+$ T cells mediate direct cytolysis of influenza A virus-infected target cells by the exocytosis of cytolytic granules that contain perforin and granzymes [148, 149] and/or through the expression of FasL [150–152].

Following influenza A virus clearance, virus-specific $CD8^+$ T cells decrease in number until a plateau is reached approximately 2 months following infection [111, 145]. After primary infection, the co-dominant $D^bNP_{366-374}$ and $D^bPA_{224-233}$-specific $CD8^+$ T cell populations contract at the same rate [145] to memory pools that are approximately equivalent in number and represents 10% of the population at the peak of the response [153]. Influenza A virus-specific $CD8^+$ T cells persist as a stable population for the life of a laboratory mouse [145, 154, 155]. Retention of memory $CD8^+$ T cells in non-lymphoid tissue such as the lung, is mediated by T cell expression of VLA-1 [156]. Secondary challenge recruits the memory $CD8^+$ T cells that expand in the lymph nodes and promote viral clearance

approximately 2 days earlier than after primary infection [145]. During secondary infection, the $NP_{366–374}$ $CD8^+$ T cell population is clearly dominant, representing up to 80% of the virus-specific CTL responses [111, 125, 139, 140]. This dominance is maintained in the memory populations that persist following the peak of the secondary response (day 8) [111]. The skewed immunodominance hierarchy observed in secondary *versus* primary influenza A virus infection was initially thought to be largely a consequence of differential antigen presentation [157], although it is now considered that T cell precursor frequency and antigen dose are likely to be important determining variables [158].

$CD4^+$ T cells

Virus-specific $CD4^+$ T cells are important participants in influenza immunity [159, 160]. Although, acting alone, these cells do not normally eliminate virus [161], they exert distinct roles in both humoral immunity (as discussed) and $CD8^+$ T cell responses. A vigorous, heterogeneous $CD4^+$ T cell response is elicited following influenza A virus infection [160]. Again, the process of clonal expansion and differentiations is initiated in the lung draining lymph node, with the peak response in the respiratory airways occurring 6–7 days following infection [160]. This is dominated by producers of the Th1 cytokines, such as IL-2, IFN-γ and TNF-α, while Th2 cells do not provide any protection [162]. Following influenza A virus clearance, $CD4^+$ T cells demonstrate increased contraction in the respiratory tract compared to influenza A virus-specific $CD8^+$ T cells [163, 164]. A major role for $CD4^+$ T cells is the provision of 'help' for optimal $CD8^+$ T cell immunity. Although $CD4^+$ T cells are not required for primary influenza-specific $CD8^+$ T cell responses, presumably due to the direct activation of DC by viral infection [165–167], they are critical for the optimal establishment of $CD8^+$ T cell memory. The absence of $CD4^+$ T cells during primary influenza A virus infections leads to a significant reduction in the size and magnitude of the secondary response and impaired viral clearance [71, 165]. Activation of $CD4^+$ T cells requires antigen-specific signaling *via* TCR recognition of antigens presented in the context of MHC class II molecules. Until recently, the spectrum of influenza A virus $CD4^+$ T cell epitopes was much less well characterized than the panel known for the $CD8^+$ subset. Recently, however, 20–30 peptides were identified for the influenza-specific $CD4^+$ T cell response in C57BL/6 mice, with the majority being derived from the NP and HA proteins [168]. There is some evidence that influenza MHC class II epitopes persist for a substantial interval after the virus has been cleared from the host [113]. Overall, the adaptive immune response to the influenza A viruses involves complex interactions between a spectrum of functionally different cell types.

Influenza A virus escape

The major influenza A virus escape mechanism rests in the inherent genetic variation of these RNA viruses, combined with the selective pressure exerted by HA-specific neutralizing antibody [169–171]. This process is known as "antigenic drift". Lacking proof reading capacity, the influenza A virus RNA polymerase promotes the accumulation of nucleotide point mutations. Such mutations generate approximately 3.5 amino acid substitutions per year [172]. Circulating viral subtypes are then selected where substitutions have occurred that maintain viral fitness [173], but abrogate immune recognition. For example, virus escape mutants are poorly recognized by neutralizing antibody due to: (i) introduced steric interference with antibody binding [78], (ii) virus conformational changes that render antibody binding energetically unfavorable [79], or (iii) the introduction of new oligosaccharide attachment sites to surface glycoproteins that obscure antibody binding [174, 175]. Retention of amino acid substitutions at the HA membrane distal surface, an area targeted by antibodies, is favored over those buried within the protein [77]. Virus-specific CTL immunity can also be targeted by antigenic drift [176]. Here, viruses are selected with mutations that interfere with epitope binding to MHC class I, or with epitopes that are no longer recognized by the TCR. The $NP_{388–391}$ [177, 178] and $NP_{418–426}$ [179, 180] CTL peptides have both shown evidence of antigenic drift. Hypervariability within a CTL epitope correlates with the functional avidity of the TCR [181]. Such antigenic drift can function to limit cross protective immunity against multiple influenza A virus strains and, as a consequence, contribute to seasonal epidemics.

While antigenic drift represents a subtle mode of immune escape, influenza A viruses can also undergo major antigenic variation to outmaneuver the immune system. This takes place by "antigenic shift", where infection of the same cell with two distinct influenza A virus strains allows reassortment of the viral genomic segments, generating a new hybrid influenza A virus. Reassortment can occur following infection with different species-adapted viruses. For example, pigs can be infected with both human and avian influenza A viruses. Simultaneous infection may thereby generate a reassortment virus where the "human" pathogen acquires an "avian" virus HA or NA gene. In this case, for the HA and NA in particular, there would be no prevailing immunity in the human population leading to the possibility of a human pandemic [182, 183]. Such antigenic shift involving avian and human strains has been implicated in two of the influenza A virus pandemics that have occurred in the 20th century; the 1957 H2N2 [184, 185] and 1968 H3N2 [172, 185] infections. Of interest, the influenza A virus that provoked the 1918 pandemic did not arise through antigenic shift. Instead, the 1918 H1N1 virus, that was responsible for millions of deaths world wide, is believed to be an entirely avian viral strain that mutated in a way that allowed it to infect humans [186, 187].

The nonstructural protein 1 (NS1) encoded by influenza A virus, provides a mode of immune escape that does not require manipulation of the genome. NS1 inhibits the host cell IFN-α/β response [188, 189], a major pathway of immune defense against the virus (as discussed). Type I IFN induction is antagonized by NS1-mediated suppression of IFN-induced proteins dsRNA-activated protein kinase, 2'-5-oligo (A) synthetase [190–192], the transcription factors NFκB [193] and the IFN regulatory factor-3 [194]. Containing an RNA-binding domain at its N terminus [193], it was previously considered that NS1 sequestered influenza A virus dsRNA [195]. Instead, NS1 forms a complex with RIG-1, the cellular sensor of influenza A virus uncapped ssRNA [6]. Therefore, NS1 acts to disable the host mechanism for detection of virus-derived RNA and the induction of the IFN response. Influenza A viruses lacking the NS1 protein are good vaccine candidates, as the absence of this immunomodulatory protein greatly enhances the immunogenicity of the virus [196].

Heterotypic influenza A virus immunity

Heterotypic immunity in this system is defined by cross-reactive, protective responses between serologically different (HA-distinct) influenza A viruses. It would obviously be advantageous if, for example, prior infection with a human influenza A virus could generate immune memory that provides at least some resistance to a highly pathogenic avian virus that suddenly adapted to transmit between people [197, 198]. Clearly, promoting heterotypic immunity is a desirable strategy for influenza A virus vaccine development. Described many decades ago [199], heterotypic immunity has now been shown for many influenza A virus combinations [200–203]. At least in mice, heterotypic immunity can both be long lasting and provide protection against otherwise lethal virus challenge. The best understood component of such responses is CTL immunity directed at generally conserved, internal viral proteins [200, 202, 203]. However, there is also evidence for the retention of a measure of heterotypic immunity in mice lacking $CD8^+$ T cells [201, 204]. In addition to the $CD8^+$ T effectors, $CD4^+$ T cells, non-neutralizing IgA antibody, NKT cells and γδ T cells have all been considered as possible players [202]. Immunization with a low dose of a cold-adapted, attenuated influenza A virus provides one vaccination strategy that has the potential to induce at least some degree of long-term, heterotypic immunity [205]. The promotion of such responses is clearly a worthwhile focus for future vaccination strategies.

Conclusion

The influenza A viruses are dangerous pathogens with the potential to provoke devastating disease. The challenge for the medical research com-

munity is to design preventive measures and therapeutic interventions that will limit the severe consequences of pandemic influenza A virus infections [206]. Vaccines have long been available, but there is considerable scope for improvement as they target only the prevailing influenza A virus strains, do not give broad immunity and work poorly in the elderly, the target group that is most at risk of fatal disease. Improved vaccines will only emerge if the development strategy is based on a firm understanding of the host immune response to the virus. Moving beyond the currently available products will depend on exploiting our understanding of immune defense mechanisms against this important, and potentially very dangerous group of human pathogens. Here, we have briefly summarized a current view of how these viruses are controlled by elements of both the innate and adaptive host response, together with the escape strategies that influenza A viruses exploit to survive in nature and to maintain transmission at the species level. An ideal vaccine could be thought to induce high levels of neutralizing antibody and CTL memory. This might optimally be achieved by promoting more effective DC vaccination, perhaps *via* the pathway of driving the innate response in ways that enhance T cell immunity. An important caveat is, though, that much of our understanding of (particularly) the innate and T cell responses to the influenza A viruses is based on mouse experiments. As we go forward to develop vaccine candidates, it is important that the analysis of influenza virus cell-mediated immunity, in particular, should be greatly extended in human subjects.

References

1 Lewis DB (2006) Avian flu to human influenza. *Annu Rev Med* 57: 139–154

2 Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. *Annu Rev Immunol* 20: 197–216

3 Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M (2006) Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* 2: e53

4 Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M (2005) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 280: 5571–5580

5 Edelmann KH, Richardson-Burns S, Alexopoulou L, Tyler KL, Flavell RA, Oldstone MB (2004) Does Toll-like receptor 3 play a biological role in virus infections? *Virology* 322: 231–238

6 Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314: 997–1001

7 Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S,

Conzelmann KK, Schlee M et al (2006) 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994–997
8 Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S (2005) Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23: 19–28
9 Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004) Innate antiviral responses by means of TLR7–mediated recognition of single-stranded RNA. *Science* 303: 1529–1531
10 Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A Flavell RA (2004) Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci USA* 101: 5598–5603
11 Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ et al (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441: 101–105
12 Leung KN, Ada GL (1981) Induction of natural killer cells during murine influenza virus infection. *Immunobiology* 160: 352–366
13 Stein-Streilein J, Bennett M, Mann D, Kumar V (1983) Natural killer cells in mouse lung: Surface phenotype, target preference, and response to local influenza virus infection. *J Immunol* 131: 2699–2704
14 Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP (1999) Natural killer cells in antiviral defense: Function and regulation by innate cytokines. *Annu Rev Immunol* 17: 189–220
15 Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Davis DM, Strominger JL, Yewdell JW, Porgador A (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055–1060
16 Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* 31: 2680–2689
17 Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, Achdout H, Hanna J, Qimron U, Landau G, Greenbaum E et al (2006) Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. *Nat Immunol* 7: 517–523
18 Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ, Schultz-Cherry S, Solorzano A, Van Rooijen N, Katz JM, Basler CF (2005) Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: Functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J Virol* 79: 14933–14944
19 Fujisawa H (2001) Inhibitory role of neutrophils on influenza virus multiplication in the lungs of mice. *Microbiol Immunol* 45: 679–688
20 Ratcliffe DR, Nolin SL, Cramer EB (1988) Neutrophil interaction with influenza-infected epithelial cells. *Blood* 72: 142–149
21 Sibille Y, Reynolds HY (1990) Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* 141: 471–501
22 Fujimoto I, Pan J, Takizawa T, Nakanishi Y (2000) Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages. *J Virol* 74: 3399–3403
23 Hofmann P, Sprenger H, Kaufmann A, Bender A, Hasse C, Nain M, Gemsa D

(1997) Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. *J Leukoc Biol* 61: 408–414

24 Gong JH, Sprenger H, Hinder F, Bender A, Schmidt A, Horch S, Nain M, Gemsa D (1991) Influenza A virus infection of macrophages. Enhanced tumor necrosis factor-alpha (TNF-alpha) gene expression and lipopolysaccharide-triggered TNF-alpha release. *J Immunol* 147: 3507–3513

25 Kaufmann A, Salentin R, Meyer RG, Bussfeld D, Pauligk C, Fesq H, Hofmann P, Nain M, Gemsa D, Sprenger H (2001) Defense against influenza A virus infection: Essential role of the chemokine system. *Immunobiology* 204: 603–613

26 Sprenger H, Meyer RG, Kaufmann A, Bussfeld D, Rischkowsky E, Gemsa D (1996) Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection. *J Exp Med* 184: 1191–1196

27 Bussfeld D, Kaufmann A, Meyer RG, Gemsa D, Sprenger H (1998) Differential mononuclear leukocyte attracting chemokine production after stimulation with active and inactivated influenza A virus. *Cell Immunol* 186: 1–7

28 Wijburg OL, DiNatale S, Vadolas J, van Rooijen N, Strugnell RA (1997) Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection. *J Virol* 71: 9450–9457

29 Fesq H, Bacher M, Nain M, Gemsa D (1994) Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. *Immunobiology* 190: 175–182

30 Theofilopoulos AN, Baccala R, Beutler B, Kono DH (2005) Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu Rev Immunol* 23: 307–336

31 Wyde PR, Wilson MR, Cate TR (1982) Interferon production by leukocytes infiltrating the lungs of mice during primary influenza virus infection. *Infect Immun* 38: 1249–1255

32 Durbin JE, Fernandez-Sesma A, Lee CK, Rao TD, Frey AB, Moran TM, Vukmanovic S, Garcia-Sastre A, Levy D (2000). Type I IFN modulates innate and specific antiviral immunity. *J Immunol* 164: 4220–4228

33 Garcia-Sastre A, Durbin RK, Zheng H, Palese P, Gertner R, Levy DE, Durbin JE (1998) The role of interferon in influenza virus tissue tropism. *J Virol* 72: 8550–8558

34 Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, Colonna M (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* 5: 919–923

35 Nakano H, Yanagita M, Gunn MD (2001) CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* 194: 1171–1178

36 Bruno L, Seidl T, Lanzavecchia A (2001) Mouse pre-immunocytes as non-proliferating multipotent precursors of macrophages, interferon-producing cells, CD8alpha(+) and CD8alpha(–) dendritic cells. *Eur J Immunol* 31: 3403–3412

37 O'Keeffe M, Hochrein H, Vremec D, Caminschi I, Miller JL, Anders EM, Wu L, Lahoud MH, Henri S, Scott B et al (2002) Mouse plasmacytoid cells: Long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *J Exp Med* 196: 1307–1319

38 Seo SH, Webster RG (2002) Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. *J Virol* 76: 1071–1076
39 Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J (2003) Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 19: 225–234
40 Lee SW, Youn JW, Seong BL, Sung YC (1999) IL-6 induces long-term protective immunity against a lethal challenge of influenza virus. *Vaccine* 17: 490–496
41 Schmitz N, Kurrer M, Bachmann MF, Kopf M (2005) Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. *J Virol* 79: 6441–6448
42 Denton AE, Doherty PC, Turner SJ, La Gruta NL (2007) IL-18, but not IL-12, is required for optimal cytokine production by influenza virus-specific CD8(+) T cells. *Eur J Immunol* 37: 368–375
43 Bhardwaj N, Seder RA, Reddy A, Feldman MV (1996) IL-12 in conjunction with dendritic cells enhances antiviral $CD8^+$ CTL responses *in vitro*. *J Clin Invest* 98: 715–722
44 Monteiro JM, Harvey C, Trinchieri G (1998) Role of interleukin-12 in primary influenza virus infection. *J Virol* 72: 4825–4831
45 Nguyen HH, van Ginkel FW, Vu HL, Novak MJ, McGhee JR, Mestecky J (2000) Gamma interferon is not required for mucosal cytotoxic T-lymphocyte responses or heterosubtypic immunity to influenza A virus infection in mice. *J Virol* 74: 5495–5501
46 Bot A, Bot S, Bona CA (1998) Protective role of gamma interferon during the recall response to influenza virus. *J Virol* 72: 6637–6645
47 Baumgarth N, Kelso A (1996) *In vivo* blockade of gamma interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue. *J Virol* 70: 4411–4418
48 Cook DN, Beck MA, Coffman TM, Kirby SL, Sheridan JF, Pragnell IB, Smithies O (1995) Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* 269: 1583–1585
49 Dawson TC, Beck MA, Kuziel WA, Henderson F, Maeda N (2000) Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. *Am J Pathol* 156: 1951–1959
50 La Gruta NL, Kedzierska K, Stambas J, Doherty PC (2007) A question of self-preservation: Immunopathology in influenza virus infection. *Immunol Cell Biol* 85: 85–92
51 Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M et al (2007) Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445: 319–323
52 Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, Suzuki H, Nishimura H, Mitamura K, Sugaya N et al (2004) Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature* 431: 703–707
53 Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, Thomas MJ, Basler CF, Palese P, Taubenberger JK, Garcia-Sastre A et al (2006) Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* 443: 578–581

54 Holmskov U, Thiel S, Jensenius JC (2003) Collections and ficolins: Humoral lectins of the innate immune defense. *Annu Rev Immunol* 21: 547–578
55 Crouch E, Hartshorn K, Ofek I (2000) Collectins and pulmonary innate immunity. *Immunol Rev* 173: 52–65
56 Hartshorn KL, White MR, Shepherd V, Reid K, Jensenius JC, Crouch EC (1997) Mechanisms of anti-influenza activity of surfactant proteins A and D: Comparison with serum collectins. *Am J Physiol* 273: L1156–1166
57 Benne CA, Kraaijeveld CA, van Strijp JA, Brouwer E, Harmsen M, Verhoef J, van Golde LM, van Iwaarden JF (1995) Interactions of surfactant protein A with influenza A viruses: Binding and neutralization. *J Infect Dis* 171: 335–341
58 Hartshorn K, Chang D, Rust K, White M, Heuser J, Crouch E (1996) Interactions of recombinant human pulmonary surfactant protein D and SP-D multimers with influenza A. *Am J Physiol* 271: L753–762
59 Hartshorn KL, Sastry K, White MR, Anders EM, Super M, Ezekowitz RA, Tauber A (1993) I. Human mannose-binding protein functions as an opsonin for influenza A viruses. *J Clin Invest* 91: 1414–1420
60 Daher KA, Selsted ME, Lehrer RI (1986) Direct inactivation of viruses by human granulocyte defensins. *J Virol* 60: 1068–1074
61 Reading PC, Hartley CA, Ezekowitz RA, Anders EM (1995) A serum mannose-binding lectin mediates complement-dependent lysis of influenza virus-infected cells. *Biochem Biophys Res Commun* 217: 1128–1136
62 Hartshorn KL, Reid KB, White MR, Jensenius JC, Morris SM, Tauber AI, Crouch E (1996) Neutrophil deactivation by influenza A viruses: Mechanisms of protection after viral opsonization with collectins and hemagglutination-inhibiting antibodies. *Blood* 87: 3450–3461
63 Benne CA, Benaissa-Trouw B, van Strijp JA, Kraaijeveld CA, van Iwaarden JF (1997) Surfactant protein A, but not surfactant protein D, is an opsonin for influenza A virus phagocytosis by rat alveolar macrophages. *Eur J Immunol* 27: 886–890
64 Hartley CA, Reading PC, Ward AC, Anders EM (1997) Changes in the hemagglutinin molecule of influenza type A (H3N2) virus associated with increased virulence for mice. *Arch Virol* 142: 75–88
65 Leikina E, Delanoe-Ayari H, Melikov K, Cho MS, Chen A, Waring AJ, Wang W, Xie Y, Loo JA, Lehrer RI, Chernomordik LV (2005) Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. *Nat Immunol* 6: 995–1001
66 Graham MB, Braciale TJ (1997) Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J Exp Med* 186: 2063–2068
67 Lee BO, Rangel-Moreno J, Moyron-Quiroz JE, Hartson L, Makris M, Sprague F, Lund FE, Randall TD (2005) CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection. *J Immunol* 175: 5827–5838
68 Mozdzanowska K, Furchner M, Zharikova D, Feng J, Gerhard W (2005) Roles of $CD4^+$ T-cell-independent and -dependent antibody responses in the control of influenza virus infection: Evidence for noncognate $CD4^+$ T-cell activities that enhance the therapeutic activity of antiviral antibodies. *J Virol* 79: 5943–5951

69 Kopf M, Brombacher F, Bachmann MF (2002) Role of IgM antibodies *versus* B cells in influenza virus-specific immunity. *Eur J Immunol* 32: 2229–2236
70 Topham DJ, Tripp RA, Hamilton-Easton AM, Sarawar SR, Doherty PC (1996) Quantitative analysis of the influenza virus-specific CD4+ T cell memory in the absence of B cells and Ig. *J Immunol* 157: 2947–2952
71 Riberdy JM, Christensen JP, Branum K, Doherty PC (2000) Diminished primary and secondary influenza virus-specific CD8(+) T-cell responses in CD4-depleted Ig(–/–) mice. *J Virol* 74: 9762–9765
72 Ochsenbein AF, Zinkernagel RM (2000) Natural antibodies and complement link innate and acquired immunity. *Immunol Today* 21: 624–630
73 Ochsenbein AF, Pinschewer DD, Odermatt B, Ciurea A, Hengartner H, Zinkernagel RM (2000) Correlation of T cell independence of antibody responses with antigen dose reaching secondary lymphoid organs: Implications for splenectomized patients and vaccine design. *J Immunol* 164: 6296–6302
74 Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J (2000) B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* 192: 271–280
75 Savitsky D, Calame K (2006) B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. *J Exp Med* 203: 2305–2314
76 Harada Y, Muramatsu M, Shibata T, Honjo T, Kuroda K (2003) Unmutated immunoglobulin M can protect mice from death by influenza virus infection. *J Exp Med* 197: 1779–1785
77 Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. *Annu Rev Biochem* 69: 531–569
78 Bizebard T, Gigant B, Rigolet P, Rasmussen B, Diat O, Bosecke P, Wharton SA, Skehel JJ, Knossow M (1995) Structure of influenza virus haemagglutinin complexed with a neutralizing antibody. *Nature* 376: 92–94
79 Fleury D, Barrere B, Bizebard T, Daniels RS, Skehel JJ, Knossow M (1999) A complex of influenza hemagglutinin with a neutralizing antibody that binds outside the virus receptor binding site. *Nat Struct Biol* 6: 530–534
80 Murphy BR, Kasel JA, Chanock RM (1972) Association of serum anti-neuraminidase antibody with resistance to influenza in man. *N Engl J Med* 286: 1329–1332
81 Belshe RB, Gruber WC, Mendelman PM, Cho I, Reisinger K, Block SL, Wittes J, Iacuzio D, Piedra P, Treanor J et al (2000) Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. *J Pediatr* 136: 168–175
82 Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers WA (1999) Universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med* 5: 1157–1163
83 Lamb RA, Zebedee SL, Richardson CD (1985) Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. *Cell* 40: 627–633
84 Jegerlehner A, Schmitz N, Storni T, Bachmann MF (2004) Influenza A vaccine based on the extracellular domain of M2: Weak protection mediated via antibody-dependent NK cell activity. *J Immunol* 172: 5598–5605

85 Sangster MY, Riberdy JM, Gonzalez M, Topham DJ, Baumgarth N, Doherty P (2003) C. An early CD4+ T cell-dependent immunoglobulin A response to influenza infection in the absence of key cognate T-B interactions. *J Exp Med* 198: 1011–1021
86 Scherle PA, Palladino G, Gerhard W (1992) Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J Immunol* 148: 212–217
87 Topham DJ, Tripp RA, Sarawar SR, Sangster MY, Doherty PC (1996) Immune CD4+ T cells promote the clearance of influenza virus from major histocompatibility complex class II-/- respiratory epithelium. *J Virol* 70: 1288–1291
88 Lee BO, Moyron-Quiroz J, Rangel-Moreno J, Kusser KL, Hartson L, Sprague F, Lund FE, Randall TD (2003) CD40, but not CD154, expression on B cells is necessary for optimal primary B cell responses. *J Immunol* 171: 5707–5717
89 Shortman K, Liu YJ (2002) Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2: 151–161
90 Sigal LJ, Rock KL (2000) Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. *J Exp Med* 192: 1143–1150
91 Belz GT, Wilson NS, Smith CM, Mount AM, Carbone FR, Heath WR (2006) Bone marrow-derived cells expand memory CD8+ T cells in response to viral infections of the lung and skin. *Eur J Immunol* 36: 327–335
92 Zammit DJ, Cauley LS, Pham QM, Lefrancois L (2005) Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* 22: 561–570
93 Legge KL, Braciale TJ (2005) Lymph node dendritic cells control CD8+ T cell responses through regulated FasL expression. *Immunity* 23: 649–659
94 Sung SS, Fu SM, Rose CE Jr, Gaskin F, Ju ST, Beaty SR (2006) A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J Immunol* 176: 2161–2172
95 Henri S, Vremec D, Kamath A, Waithman J, Williams S, Benoist C, Burnham K, Saeland S, Handman E, Shortman K (2001) The dendritic cell populations of mouse lymph nodes. *J Immunol* 167: 741–748
96 Legge KL, Braciale TJ (2003) Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity* 18: 265–277
97 McWilliam AS, Napoli S, Marsh AM, Pemper FL, Nelson DJ, Pimm CL, Stumbles PA, Wells TN, Holt PG (1996) Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *J Exp Med* 184: 2429–2432
98 Yamamoto N, Suzuki S, Shirai A, Suzuki M, Nakazawa M, Nagashima Y, Okubo T (2000) Dendritic cells are associated with augmentation of antigen sensitization by influenza A virus infection in mice. *Eur J Immunol* 30: 316–326
99 Nonacs R, Humborg C, Tam JP, Steinman RM (1992) Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J Exp Med* 176: 519–529
100 Macatonia SE, Taylor PM, Knight SC, Askonas BA (1989) Primary stimulation

by dendritic cells induces antiviral proliferative and cytotoxic T cell responses *in vitro*. *J Exp Med* 169: 1255–1264

101 Bhardwaj N, Bender A, Gonzalez N, Bui LK, Garrett MC, Steinman RM (1994) Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8$^+$ T cells. *J Clin Invest* 94: 797–807

102 Oh S, Eichelberger MC (1999) Influenza virus neuraminidase alters allogeneic T cell proliferation. *Virology* 264: 427–435

103 Oh S, McCaffery JM, Eichelberger MC (2000) Dose-dependent changes in influenza virus-infected dendritic cells result in increased allogeneic T-cell proliferation at low, but not high, doses of virus. *J Virol* 74: 5460–5469

104 Albert ML, Sauter B, Bhardwaj N (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392: 86–89

105 Wilson NS, Behrens GM, Lundie RJ, Smith CM, Waithman J, Young L, Forehan SP, Mount A, Steptoe RJ, Shortman KD et al (2006) Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat Immunol* 7: 165–172

106 Belz GT, Smith CM, Kleinert L, Reading P, Brooks A, Shortman K, Carbone FR, Heath WR (2004) Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci USA* 101: 8670–8675

107 Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA (2001) Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 193: 51–60

108 Carbone FR, Belz GT, Heath WR (2004) Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity. *Trends Immunol* 25: 655–658

109 Randolph GJ (2006) Migratory dendritic cells: Sometimes simply ferries? *Immunity* 25: 15–18

110 Lawrence CW, Braciale TJ (2004) Activation, differentiation, and migration of naive virus-specific CD8$^+$ T cells during pulmonary influenza virus infection. *J Immunol* 173: 1209–1218

111 Flynn KJ, Riberdy JM, Christensen JP, Altman JD, Doherty PC (1999) *In vivo* proliferation of naive and memory influenza-specific CD8(+) T cells. *Proc Natl Acad Sci USA* 96: 8597–8602

112 Zammit DJ, Turner DL, Klonowski KD, Lefrancois L, Cauley LS (2006) Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity* 24: 439–449

113 Jelley-Gibbs DM, Brown DM, Dibble JP, Haynes L, Eaton SM, Swain SL (2005) Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J Exp Med* 202: 697–706

114 Doherty PC, Christensen JP (2000) Accessing complexity: The dynamics of virus-specific T cell responses. *Annu Rev Immunol* 18: 561–592

115 Julia V, Hessel EM, Malherbe L, Glaichenhaus N, O'Garra A, Coffman RL (2002) A restricted subset of dendritic cells captures airborne antigens and remains able to activate specific T cells long after antigen exposure. *Immunity* 16: 271–283

116 Sharpe AH, Freeman GJ (2002) The B7-CD28 superfamily. *Nat Rev Immunol* 2: 116–126
117 Croft M (2003) Co-stimulatory members of the TNFR family: Keys to effective T-cell immunity? *Nat Rev Immunol* 3: 609–620
118 Bertram EM, Dawicki W, Watts TH (2004) Role of T cell costimulation in anti-viral immunity. *Semin Immunol* 16: 185–196
119 Halstead ES, Mueller YM, Altman JD, Katsikis PD (2002) *In vivo* stimulation of CD137 broadens primary antiviral $CD8^+$ T cell responses. *Nat Immunol* 3: 536–541
120 Hendriks J, Xiao Y, Borst J (2003) CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med* 198: 1369–1380
121 Bertram EM, Lau P, Watts TH (2002) Temporal segregation of 4-1BB *versus* CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. *J Immunol* 168: 3777–3785
122 Lumsden JM, Roberts JM, Harris NL, Peach RJ, Ronchese F (2000) Differential requirement for CD80 and CD80/CD86-dependent costimulation in the lung immune response to an influenza virus infection. *J Immunol* 164: 79–85
123 Liu Y, Wenger RH, Zhao M, Nielsen PJ (1997) Distinct costimulatory molecules are required for the induction of effector and memory cytotoxic T lymphocytes. *J Exp Med* 185: 251–262
124 Yewdell JW, Del Val M (2004) Immunodominance in $TCD8^+$ responses to viruses: Cell biology, cellular immunology, and mathematical models. *Immunity* 21: 149–153
125 Belz GT, Stevenson PG, Doherty PC (2000) Contemporary analysis of MHC-related immunodominance hierarchies in the $CD8^+$ T cell response to influenza A viruses. *J Immunol* 165: 2404–2409
126 Chen W, Bennink JR, Morton PA, Yewdell JW (2002) Mice deficient in perforin, $CD4^+$ T cells, or CD28–mediated signaling maintain the typical immunodominance hierarchies of CD8+ T-cell responses to influenza virus. *J Virol* 76: 10332–10337
127 DeBenedette MA, Wen T, Bachmann MF, Ohashi PS, Barber BH, Stocking KL, Peschon JJ, Watts TH (1999) Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J Immunol* 163: 4833–4841
128 Kopf M, Ruedl C, Schmitz N, Gallimore A, Lefrang K, Ecabert B, Odermatt B, Bachmann MF (1999) OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity* 11: 699–708
129 Bertram EM, Tafuri A, Shahinian A, Chan VS, Hunziker L, Recher M, Ohashi PS, Mak TW, Watts TH (2002) Role of ICOS *versus* CD28 in antiviral immunity. *Eur J Immunol* 32: 3376–3385
130 Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J (2000) CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol* 1: 433–440

131 Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG (1997) Effector CD4$^+$ and CD8$^+$ T-cell mechanisms in the control of respiratory virus infections. *Immunol Rev* 159: 105–117
132 Bender BS, Croghan T, Zhang L, Small PA Jr (1992) Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J Exp Med* 175: 1143–1145
133 Tripp RA, Sarawar SR, Doherty PC (1995) Characteristics of the influenza virus-specific CD8$^+$ T cell response in mice homozygous for disruption of the H-2lAb gene. *J Immunol* 155: 2955–2959
134 Cerwenka A, Morgan TM, Dutton RW (1999) Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: Homing properties rather than initial frequencies are crucial. *J Immunol* 163: 5535–5543
135 Galkina E, Thatte J, Dabak V, Williams MB, Ley K, Braciale TJ (2005) Preferential migration of effector CD8$^+$ T cells into the interstitium of the normal lung. *J Clin Invest* 115: 3473–3483
136 Thatte J, Dabak V, Williams MB, Braciale TJ, Ley K (2003) LFA-1 is required for retention of effector CD8 T cells in mouse lungs. *Blood* 101: 4916–4922
137 Belz GT, Xie W, Altman JD, Doherty PC (2000) A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. *J Virol* 74: 3486–3493
138 Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ (1986) The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44: 959–968
139 Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL, Doherty PC (1998) Virus-specific CD8$^+$ T cells in primary and secondary influenza pneumonia. *Immunity* 8: 683–691
140 Belz GT, Xie W, Doherty PC (2001) Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8$^+$ T cell responses. *J Immunol* 166: 4627–4633
141 Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P et al (2001) A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 7: 1306–1312
142 Vitiello A, Yuan L, Chesnut RW, Sidney J, Southwood S, Farness P, Jackson MR, Peterson PA, Sette A (1996) Immunodominance analysis of CTL responses to influenza PR8 virus reveals two new dominant and subdominant Kb-restricted epitopes. *J Immunol* 157: 5555–5562
143 Andreansky SS, Stambas J, Thomas PG, Xie W, Webby RJ, Doherty PC (2005) Consequences of immunodominant epitope deletion for minor influenza virus-specific CD8$^+$-T-cell responses. *J Virol* 79: 4329–4339
144 Webby RJ, Andreansky S, Stambas J, Rehg JE, Webster RG, Doherty PC, Turner SJ (2003) Protection and compensation in the influenza virus-specific CD8+ T cell response. *Proc Natl Acad Sci USA* 100: 7235–7240
145 Marshall DR, Turner SJ, Belz GT, Wingo S, Andreansky S, Sangster MY,

Riberdy JM, Liu T, Tan M, Doherty PC (2001) Measuring the diaspora for virus-specific CD8+ T cells. *Proc Natl Acad Sci USA* 98: 6313–6318
146 Turner SJ, Diaz G, Cross R, Doherty PC (2003) Analysis of clonotype distribution and persistence for an influenza virus-specific $CD8^+$ T cell response. *Immunity* 18: 549–559
147 La Gruta NL, Turner SJ, Doherty PC (2004) Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific $CD8^+$ T cell responses: Correlation of cytokine profile and TCR avidity. *J Immunol* 172: 5553–5560
148 Johnson BJ, Costelloe EO, Fitzpatrick DR, Haanen JB, Schumacher TN, Brown LE, Kelso A (2003) Single-cell perforin and granzyme expression reveals the anatomical localization of effector $CD8^+$ T cells in influenza virus-infected mice. *Proc Natl Acad Sci USA* 100: 2657–2662
149 Liu B, Mori I, Hossain MJ, Dong L, Chen Z, Kimura Y (2003) Local immune responses to influenza virus infection in mice with a targeted disruption of perforin gene. *Microb Pathog* 34: 161–167
150 Topham DJ, Tripp RA, Doherty PC (1997) $CD8^+$ T cells clear influenza virus by perforin or Fas-dependent processes. *J Immunol* 159: 5197–5200
151 Price GE, Huang L, Ou R, Zhang M, Moskophidis D (2005) Perforin and Fas cytolytic pathways coordinately shape the selection and diversity of $CD8^+$-T-cell escape variants of influenza virus. *J Virol* 79: 8545–8559
152 Fujimoto I, Takizawa T, Ohba Y, Nakanishi Y (1998) Co-expression of Fas and Fas-ligand on the surface of influenza virus-infected cells. *Cell Death Differ* 5: 426–431
153 Kedzierska K, La Gruta NL, Turner SJ, Doherty PC (2006) Establishment and recall of $CD8^+$ T-cell memory in a model of localized transient infection. *Immunol Rev* 211: 133–145
154 Hogan RJ, Usherwood EJ, Zhong W, Roberts AA, Dutton RW, Harmsen AG, Woodland DL (2001) Activated antigen-specific $CD8^+$ T cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* 166: 1813–1822
155 Wiley JA, Hogan RJ, Woodland DL, Harmsen AG (2001) Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection. *J Immunol* 167: 3293–3299
156 Ray SJ, Franki SN, Pierce RH, Dimitrova S, Koteliansky V, Sprague AG, Doherty PC, de Fougerolles AR, Topham DJ (2004) The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* 20: 167–179
157 Crowe SR, Turner SJ, Miller SC, Roberts AD, Rappolo RA, Doherty PC, Ely KH, Woodland DL (2003) Differential antigen presentation regulates the changing patterns of $CD8^+$ T cell immunodominance in primary and secondary influenza virus infections. *J Exp Med* 198: 399–410
158 La Gruta NL, Kedzierska K, Pang K, Webby R, Davenport M, Chen W, Turner SJ, Doherty PC (2006) A virus-specific $CD8^+$ T cell immunodominance hierarchy determined by antigen dose and precursor frequencies. *Proc Natl Acad Sci USA* 103: 994–999
159 Brown DM, Roman E, Swain SL (2004) CD4 T cell responses to influenza infection. *Semin Immunol* 16: 171–177

160 Roman E, Miller E, Harmsen A, Wiley J, Von Andrian UH, Huston G, Swain SL (2002) CD4 effector T cell subsets in the response to influenza: Heterogeneity, migration, and function. *J Exp Med* 196: 957–968
161 Mozdzanowska K, Furchner M, Maiese K, Gerhard W (1997) CD4$^+$ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. *Virology* 239: 217–225
162 Graham MB, Braciale VL, Braciale TJ (1994) Influenza virus-specific CD4$^+$ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J Exp Med* 180: 1273–1282
163 Powell TJ, Brown DM, Hollenbaugh JA, Charbonneau T, Kemp RA, Swain SL, Dutton RW (2004) CD8$^+$ T cells responding to influenza infection reach and persist at higher numbers than CD4$^+$ T cells independently of precursor frequency. *Clin Immunol* 113: 89–100
164 Homann D, Teyton L, Oldstone MB (2001) Differential regulation of antiviral T-cell immunity results in stable CD8$^+$ but declining CD4$^+$ T-cell memory. *Nat Med* 7: 913–919
165 Belz GT, Wodarz D, Diaz G, Nowak MA, Doherty PC (2002) Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J Virol* 76: 12388–12393
166 Bender A, Bui LK, Feldman MA, Larsson M, Bhardwaj N (1995) Inactivated influenza virus, when presented on dendritic cells, elicits human CD8$^+$ cytolytic T cell responses. *J Exp Med* 182: 1663–1671
167 Larsson M, Messmer D, Somersan S, Fonteneau JF, Donahoe SM, Lee M, Dunbar PR, Cerundolo V, Julkunen I, Nixon DF, Bhardwaj N (2000) Requirement of mature dendritic cells for efficient activation of influenza A-specific memory CD8$^+$ T cells. *J Immunol* 165: 1182–1190
168 Crowe SR, Miller SC, Brown DM, Adams PS, Dutton RW, Harmsen AG, Lund FE, Randall TD, Swain SL, Woodland DL (2006) Uneven distribution of MHC class II epitopes within the influenza virus. *Vaccine* 24: 457–467
169 Palese P, Young JF (1982) Variation of influenza A, B, and C viruses. *Science* 215: 1468–1474
170 Yewdell JW, Webster RG, Gerhard WU (1979) Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. *Nature* 279: 246–248
171 Webster RG, Laver WG, Air GM, Schild GC (1982) Molecular mechanisms of variation in influenza viruses. *Nature* 296: 115–121
172 Bean WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, Webster RG (1992) Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. *J Virol* 66: 1129–1138
173 Berkhoff EG, de Wit E, Geelhoed-Mieras MM, Boon AC, Symons J, Fouchier RA, Osterhaus AD, Rimmelzwaan GF (2005) Functional constraints of influenza A virus epitopes limit escape from cytotoxic T lymphocytes. *J Virol* 79: 11239–11246
174 Wiley DC, Wilson IA, Skehel JJ (1981) Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 289: 373–378
175 Caton AJ, Brownlee GG, Yewdell JW, Gerhard W (1982) The antigenic struc-

ture of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* 31: 417–427

176 Price GE, Ou R, Jiang H, Huang L, Moskophidis D (2000) Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. *J Exp Med* 191: 1853–1867

177 Rimmelzwaan GF, Boon AC, Voeten JT, Berkhoff EG, Fouchier RA, Osterhaus AD (2004) Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus Res* 103: 97–100

178 Voeten JT, Bestebroer TM, Nieuwkoop NJ, Fouchier RA, Osterhaus AD, Rimmelzwaan GF (2000) Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *J Virol* 74: 6800–6807

179 Boon AC, de Mutsert G, Graus YM, Fouchier RA, Sintnicolaas K, Osterhaus AD, Rimmelzwaan GF (2002) Sequence variation in a newly identified HLA-B35–restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *J Virol* 76: 2567–2572

180 Boon AC, de Mutsert G, van Baarle D, Smith DJ, Lapedes AS, Fouchier RA, Sintnicolaas K, Osterhaus AD, Rimmelzwaan GF (2004) Recognition of homo- and heterosubtypic variants of influenza A viruses by human $CD8^+$ T lymphocytes. *J Immunol* 172: 2453–2460

181 Boon AC, de Mutsert G, Fouchier RA, Osterhaus AD, Rimmelzwaan GF (2006) The hypervariable immunodominant NP418-426 epitope from the influenza A virus nucleoprotein is recognized by cytotoxic T lymphocytes with high functional avidity. *J Virol* 80: 6024–6032

182 Webby RJ, Webster RG (2003) Are we ready for pandemic influenza? *Science* 302: 1519–1522

183 Cox NJ, Subbarao K (2000) Global epidemiology of influenza: Past and present. *Annu Rev Med* 51: 407–421

184 Schafer JR, Kawaoka Y, Bean WJ, Suss J, Senne D, Webster RG (1993) Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *Virology* 194: 781–788

185 Kawaoka Y, Krauss S, Webster RG (1989) Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol* 63: 4603–4608

186 Reid AH, Taubenberger JK, Fanning TG (2004) Evidence of an absence: The genetic origins of the 1918 pandemic influenza virus. *Nat Rev Microbiol* 2: 909–914

187 Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG (2005) Characterization of the 1918 influenza virus polymerase genes. *Nature* 437: 889–893

188 Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T (1998) Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252: 324–330

189 Diebold SS, Montoya M, Unger H, Alexopoulou L, Roy P, Haswell LE, Al-Shamkhani A, Flavell R, Borrow P, Reis e Sousa C (2003) Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 424: 324–328

190 Bergmann M, Garcia-Sastre A, Carnero E, Pehamberger H, Wolff K, Palese P, Muster T (2000) Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol* 74: 6203–6206
191 Li S, Min JY, Krug RM, Sen GC (2006) Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 349: 13–21
192 Min JY, Krug RM (2006) The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc Natl Acad Sci USA* 103: 7100–7105
193 Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, Garcia-Sastre A (2000) Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol* 74: 11566–11573
194 Talon J, Horvath CM, Polley R, Basler CF, Muster T, Palese P, Garcia-Sastre A (2000) Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* 74: 7989–7996
195 Garcia-Sastre A (2001) Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology* 279: 375–384
196 Ferko B, Stasakova J, Romanova J, Kittel C, Sereinig S, Katinger H, Egorov A (2004) Immunogenicity and protection efficacy of replication-deficient influenza A viruses with altered NS1 genes. *J Virol* 78: 13037–13045
197 Jameson J, Cruz J, Terajima M, Ennis FA (1999) Human $CD8^+$ and $CD4^+$ T lymphocyte memory to influenza A viruses of swine and avian species. *J Immunol* 162: 7578–7583
198 Jameson J, Cruz J, Ennis FA (1998) Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J Virol* 72: 8682–8689
199 Schulman JL, Kilbourne ED (1965) Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. *J Bacteriol* 89: 170–174
200 Kreijtz JH, Bodewes R, van Amerongen G, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF (2007) Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. *Vaccine* 25: 612–620
201 Epstein SL, Lo CY, Misplon JA, Lawson CM, Hendrickson BA, Max EE, Subbarao K (1997) Mechanisms of heterosubtypic immunity to lethal influenza A virus infection in fully immunocompetent, T cell-depleted, beta2–microglobulin-deficient, and J chain-deficient mice. *J Immunol* 158: 1222–1230
202 Benton KA, Misplon JA, Lo CY, Brutkiewicz RR, Prasad SA, Epstein SL (2001) Heterosubtypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or gamma delta T cells. *J Immunol* 166: 7437–7445
203 Nguyen HH, Moldoveanu Z, Novak MJ, van Ginkel FW, Ban E, Kiyono H, McGhee JR, Mestecky J (1999) Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues. *Virology* 254: 50–60
204 Nguyen HH, van Ginkel FW, Vu HL, McGhee JR, Mestecky J (2001) Heterosubtypic immunity to influenza A virus infection requires B cells but not $CD8^+$ cytotoxic T lymphocytes. *J Infect Dis* 183: 368–376

205 Powell TJ, Strutt T, Reome J, Hollenbaugh JA, Roberts AD, Woodland DL, Swain SL, Dutton RW (2007) Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus. *J Immunol* 178: 1030–1038

206 Doherty PC, Turner SJ, Webby RG, Thomas PG (2006) Influenza and the challenge for immunology. *Nat Immunol* 7: 449–455

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Correlates of protection against influenza

Emanuele Montomoli

Department of Physiopathology, Experimental Medicine and Public Health, Laboratory of Molecular Epidemiology, University of Siena, Via Aldo Moro 3, 53100 Siena, Italy

Abstract

Correlates of protection for influenza virus has not been defined, but it is widely believed that protection against influenza can be conferred by serum hemagglutinin (HA) antibodies. The immune responses to injected influenza vaccines are routinely assessed using serological HA antibodies titration. It is generally accepted that neutralizing and HA antibodies, as well as antibodies to neuraminidase, can be detected in serum after 3–4 weeks post primary infection or vaccination.

Serological assays commonly used to quantify antibodies specific for influenza viruses include hemagglutination inhibition (HAI), single radial hemolysis (SRH), microneutralization (MN), ELISA and Western blot, the most widely used assays being HAI and SRH. Each method used for antibodies titration has different characteristics, and the validity index and specific use (seroepidemiology, serodiagnosis, response to vaccination, etc.) have to be considered to select the most suitable one. Recently ELISA and MN tests have been developed thanks to the discovery of the HA structure. While the number of data collected by conventional assays (HAI and SRH) has permitted a fairly good optimization, serological measures are used to characterize the number of antibodies before and after vaccination.

HAI is the assay used most frequently for influenza antibody titration; however, it has low sensitivity in detecting responses to avian viruses in mammalian sera and alternative serological tests are needed. SRH utilizes a complement-mediated hemolysis reaction to measure the amount of antibody. This test appears to be as sensitive as the MN assay. HAI and SRH assays are not functional tests for measuring immunity to influenza and suffers from several technical drawbacks.

Development of these assays will be a further step in preparation of new influenza vaccines, particularly for cell-derived products. Additional immunological assessments, such as cell-mediated immunity and the role of neuraminidase, need to be explored to give more insight into the overall effects of vaccination.

Introduction

The Centers for Disease Control and Prevention estimate that between 114,000 and 146,000 persons are hospitalized each year because of influ-

enza. Although the exact tabulations of illnesses and complications attributable to influenza virus injection are not available, the preceding estimates indicate that morbidity and mortality caused by influenza are major health problems.

The influenza virus belongs to the family of Orthomyxoviridae and is classified into three different types A, B and C on the basis of different epitopes, the antigenic differences in their respective nucleocapsid. Influenza A and B are the two types of influenza viruses that cause epidemic human disease. Influenza A viruses are further categorized into subtypes, e.g., H1N1, H2N2, and H3N2 on the basis of two surface antigens: hemagglutinin (HA) and neuraminidase (NA). Influenza B viruses are not categorized into subtypes. Since 1977, influenza A and B viruses have been in global circulation. Both are further separated into groups on the basis of antigenic characteristics. New influenza virus variants result from frequent antigenic change (i.e. antigenic drift) resulting from point mutations that occur during viral replication. Influenza B viruses undergo antigenic drift less rapidly than influenza A viruses.

Influenza is characterized by the occurrence of frequent unpredictable epidemics, and much less frequent worldwide pandemics. Epidemics arise because different strains of influenza are constantly generated through antigenic drift, and individuals are less or not at all protected in some years. A pandemic is responsible for higher morbidity and mortality than an epidemic because it affects a larger proportion of the population. The burden of epidemics, however, is cumulatively greater than that of pandemics. A worldwide pandemic is caused by the spread of a new influenza subtype arising from antigenic shift [1]. When such a subtype enters the population, there is no natural immunity and the new virus can easily infect exposed individuals.

An influenza pandemic occurs when a novel influenza virus emerges against which the vast majority of the world's population has no immunity. If such a virus demonstrates the ability to transmit efficiently from person to person, the result is a global outbreak of disease that affects a high percentage of individuals in a short period of time and is likely to cause substantially increased morbidity and mortality in all countries of the world. Over 50 million people are estimated to have succumbed to the most devastating influenza pandemic in 1918, the so-called "Spanish flu". "Asian flu" of 1957 has been responsible for about 70 000 deaths in the USA only.

Preliminary findings have identified the H2, H5, H6, H7 and H9 subtypes of influenza A virus as those most likely to be transmitted to humans, therefore presenting a potential pandemic threat [2].

Influenza viruses cause disease across all age groups. Rates of infection are highest among young children, but rates of serious illness and death are highest among persons aged ≥65 years and persons of any age who have medical conditions that place them at increased risk for complications from influenza [3]. Studies of morbidity and mortality associated with influenza

suggest that hospitalization rates for adults with medical conditions, which place them at high risk for influenza, often increase fivefold during epidemics, leading to an average of 172,000 excess hospitalizations during each epidemic [4]. This has important economic consequences, with annual productivity loss estimated at more than US$760 million and hospitalization costs in excess of US$300 million for each epidemic in the United States alone [5]. The total economic impact is considerable, and in industrialized countries total estimated costs (direct and indirect) may reach approximately US$10–60 million per million population.

In avian influenza virus, the HA characteristically has glutamine at position 226 and glycine at position 228 (human viruses have leucine at 226 and serine at 228), which form a narrow receptor binding pocket that preferentially binds to host cell receptors containing sialyloligosaccharides (SA) terminated by *N*-acetyl sialic acid linked to galactose with an α2,3 linkage (the major form in the avian trachea and intestine).

While a correlate of protection has not been defined, it is widely believed that protection against influenza can be conferred by serum antibodies. The immune responses to injectable influenza vaccines are routinely assessed using serological HA antibodies measurements. It is generally accepted that neutralizing HA antibodies, as well as antibodies to neuraminidase, can be detected in serum approximately 1–2 weeks after primary infection and peak at 3–4 weeks [6].

Influenza vaccines and criteria for licensure

Influenza surveillance information regarding the presence of influenza viruses in the community as well as diagnostic testing can aid clinical judgment and guide treatment decisions. Several commercial rapid diagnostic tests are available that can be used by laboratories in outpatient settings to detect influenza viruses in a few minutes. These rapid tests differ in the types of influenza virus they can detect.

Antiviral drugs are an adjunct to the vaccine for the control and prevention of influenza; however, these agents are not a substitute for vaccination. The decision to prescribe an antiviral drug for the prevention or treatment of influenza must be based on the certainty or the high probability that a person has been or will be exposed to the virus, or on a diagnosis of influenza. Four currently licensed antiviral agents against influenza are available in the US: amantadine, rimantadine, zanamivir, and oseltamivir.

Amantadine and rimantadine are chemically related antiviral drugs with activity against influenza A viruses only. Amantadine was approved in 1966 for prophylaxis of influenza A/H2N2 infection and was later approved also for the treatment and prophylaxis of influenza type A virus infections among adults and children aged ≥ 1 year. Rimantadine was approved in 1993 for treatment and prophylaxis of infection among adults, and for prophylax-

is among children. Neither antiviral drug has been used widely due to their spectrum of activity, the rapid onset of resistance, and the related adverse effects [7]. Zanamivir and oseltamivir are NA inhibitors with activity against both influenza A and B viruses. The site of enzyme activity of the influenza NA is highly constant between different types, subtypes and strains of influenza, and has therefore emerged as the target of this new class of antiviral agents effective in prevention and treatment. In the US, both drugs were approved in 1999 for the treatment of uncomplicated influenza infections. Zanamivir was approved for the treatment of patients aged ≥7 years, and oseltamivir was approved for treatment of patients aged ≥1 year and for the prophylaxis of persons of age ≥13 years. These antiviral drugs are only effective if started soon after the onset of disease.

Influenza vaccination is the primary method for preventing influenza and its severe complications. Vaccination is associated with a reduction in influenza-related respiratory illness and physician visits at all ages, in hospitalizations and deaths among high-risk persons, otitis media among children, and work absenteeism among adults. Vaccination with inactivated influenza virus currently represents the most important measure for reducing the impact of influenza.

The three current types of vaccines are inactivated vaccine, attenuated vaccine, and vector-based vaccines. Inactivated vaccines are produced by propagation of the virus in embryonated hen's eggs. The vaccine is available containing whole, split (chemically disrupted) and subunit (purified surface glycoproteins) virus. Development of cell culture-based vaccines is an attractive alternative approach to the production of inactivated vaccine production.

Live attenuated vaccine that can be administered by nasal spray is nearing availability. It has been shown to be as efficacious as inactivated vaccine [8]. Live influenza vaccines elicit systemic and local mucosal immune responses that include stimulating secretory immunoglobulin IgA in the respiratory tract, a portal for the virus. They also elicit cellular immunity, which might provide better protection than that given by inactivated vaccines [9]. Live attenuated vaccines will not be used until the H5N1 virus has become widespread among humans so as not to introduce new influenza viral HA and NA genes into human population.

These vector-based vaccines are egg-independent replication-incompetent human adenoviral vector-based H5 influenza vaccines and have been shown to successfully induce HA-specific humoral and cellular immune responses. These vaccines provide protective effects against homologous and antigenically distinct H5N1 strains in a mouse model [10].

Rapid and early diagnosis of influenza virus infection is important to aid surveillance of circulating strains and enable the early vaccination or prophylactic treatment of high-risk groups. Laboratory diagnosis of influenza is made by the detection of virus in respiratory secretions by serological methods.

Table 1. Serological criteria to meet CPMP/BWP/214/96 requirements by age group [12].

Test	HI	SRH	CPMP/BWP/214/96 criterion	
			Age group 18–60 years	Age group >61 years
Geometric mean ratio (pre- to post-vaccination)			>2.5	>2
Seroprotection	Titer ≥40	≥25 mm^2	>70% of subjects	>60% of subjects
Seroconversion or significant increase	Negative at pre-vacc and post-vacc titer ≥40	Negative at pre-vacc and post-vacc titer ≥25 mm^2	>40% of subjects	>30% of subjects

The influenza virus surface glycoprotein HA is a very important antigenic determinant for production of virus-neutralizing antibodies generated during infection or immunization. Serological assays commonly used to quantify antibodies specific for influenza viruses include hemagglutination inhibition (HAI), single radial hemolysis (SRH or single radial diffusion, SRD), virus microneutralization (MN), ELISA and Western blot; the most widely used assays are HAI and SRH.

Serological measures are used to characterize the amount of antibody before and after vaccination, and to compare the seroresponse in subjects with different treatment regimens or other characteristics (dose, age, etc.). Measures most frequently used are geometric mean titer (GMT), seroconversion, significant titer increase, and seroprotection rate. Considerable discrepancies were found for the use of serological measures in several studies [11].

Three European criteria need to be fulfilled (and at least one of the assessments should meet the indicated requirements) for the yearly vaccine registration in the European Union (CPMP/BWP/214/96) [12]. A tabular presentation of these criteria is provided in Table 1. As there are currently no criteria for the licensure of pandemic vaccines, the serological results were analyzed using the CPMP criteria required for the annual registration of interpandemic vaccines. According to guideline CPMP/VEG/4717/03 on dossier structure and content for Pandemic influenza vaccine marketing authorization application [13], it is anticipated that mock-up pandemic vaccines should at least be able to elicit sufficient immunological responses to meet all three of the current standards set for existing vaccines in adults or older adults defined in CPMP/BWP/214/96. All sera should be assayed for anti-HA antibody against the prototype strains by HAI or SRH tests. In the interpretation of HAI and SRH immunogenicity results, criteria established from the Committee for Medical Products for Human use (CHMP) are taken into consideration.

Strains with pandemic potential need to follow the Guidance on dossier structure and content for influenza vaccines derived from strains with a pandemic potential for use outside of the core dossier of marketing authorization (CHMP/VWP/263499/2006). This guideline addresses the content of marketing authorization applications for inactivated avian influenza vaccines produced in eggs or in cell cultures. The recommendations include the same three criteria as the seasonal vaccines (i.e., seroconversion rate, seroprotection, and significant increase in GMT) as defined in CHMP/BWP/214/96, with seroconversion rates being the most important. This guideline also is valid for vaccines containing or derived from influenza strains with a high pandemic potential from other animal (e.g., pig) or of non-H1/H3 human origin.

Each method used for antibody titration has different characteristics, and the validity index and specific use (seroepidemiology, serodiagnosis, response to vaccination, etc) have to be considered to select the most suitable one. Recently ELISA and MN tests have been developed thanks to the discovery of the HA structure. While the high quantity of data collected by conventional assays (HAI and SRH) has permitted a fairly good standardization, some problems are found with the routine application of MN and ELISA tests.

In vitro *assays to assess protective antibody levels (HAI, SRH, MN)*

HAI is easy to use and is the most often employed assay for serum influenza antibody titration; it was standardized in 1942 [14]. The HAI test is also the simplest technique for measurement of antibody to influenza virus, and, because it detects antibody to the hemagglutinin, is usually concordant with the MN test. HAI assay is not a functional test for measuring immunity to influenza, and sometimes suffers from several technical drawbacks. This assay may be influenced by the binding avidity of the virus and by the species of red blood cells (RBCs) used. Furthermore, antibody to NA can sterically block the access of the HA of the virus to RBCs receptors and thereby inhibit hemagglutination. The HAI test works thank to receptors for influenza viruses that are present on the RBCs of many animal species. In the presence of virus, cells that have these receptors are agglutinated in the hemagglutination reaction. Specific antibodies present in some sera incubated with virus can prevent this hemagglutination. This hemagglutination inhibition by antibodies allows quantification of these antibodies and the determination of the antigenic relatedness of influenza virus strains.

Because serum may contain nonspecific inhibitors to which virus may bind, treatment with receptor-destroying enzyme (RDE) is necessary before the test. For H1 and H3 strains, this test has the advantage of good sensitivity, but great care is necessary to achieve reproducible results. It does have the disadvantage of relatively low sensitivity for antibody to influenza

B virus [15]. It is possible to increase sensitivity by ether treatment of the virus, but this can also be a source of variability, and potentially reduces the strain specificity of the test [16, 17]. Another disadvantage is that sera must be treated to remove nonspecific inhibitors before being tested.

The HAI assay is a serological technique used to detect HA antibody in serum resulting from infection or vaccination with influenza virus, and HAI titers correlate with protection from influenza in humans [18]. This assay is suitable for screening a large number of samples; there is a good correlation with MN tests (see below) for seasonal H1 and H3 strains; only a biosafety level 2 is needed; and this test is EMEA approved.

Although this test is a standard technique for the measurement of antibody to influenza virus, its sensitivity is much too low to detect responses to avian viruses in mammalian sera, so that alternative serological tests are needed [19]. Studies have shown that the HAI assay sensitivity for avian influenza viruses can be improved through the use of subunit HA rather than intact virus [20].

The HAI antibody titer is expressed as the reciprocal of the highest serum dilution showing complete hemagglutination using four viral hemagglutination units. An HAI titer of 1:40 or higher is taken as seroprotective, and a fourfold increase in HAI titer in samples taken after and before vaccination is the minimum increase considered necessary for classification of seroconversion.

The HAI test has a number of advantages. It can be performed with a variety of erythrocyte species and the test is simple to perform and is highly reproducible. The test is strain specific.

This assay underestimates antibody for pandemic strains because the efficiency of binding of influenza virus is dependent on the specificity of the sialic acid cellular receptor. The receptor specificity of influenza viruses correlates with ability to agglutinate RBCs from different species. An important source of variation in the HAI test is the differing sensitivity of RBCs from individual animals of the same species.

Human viruses preferentially bind to oligosaccharides containing *N*-acetylneuraminic acid α2,6-galactose (NeuAc α2,6Gal), while avian and equine influenza strains bind to NeuAc α2,3Gal. Many animal species, including the horse and cow, have high amount of NeuAc α2,3Gal receptors but virtually no NeuAc α2,6Gal receptors in erythrocytes. Chicken RBCs have less NeuAc α2,6Gal and more NeuAc α2,3Gal, turkey RBCs have more NeuAc α2,6Gal than chicken RBCs [14]. Therefore, seasonal H1 and H3 influenza viruses preferentially agglutinate chicken, or turkey, but not horse or cow RBCs, whereas avian viruses agglutinate preferentially RBCs from horse or cow.

In accordance with these hypotheses, the sensitivity of the HAI assay is largely determined by the type of erythrocytes used, and the measurement of HAI titers against avian viruses has been significantly improved by use of horse erythrocytes. Turkey erythrocytes could be responsible for the relative

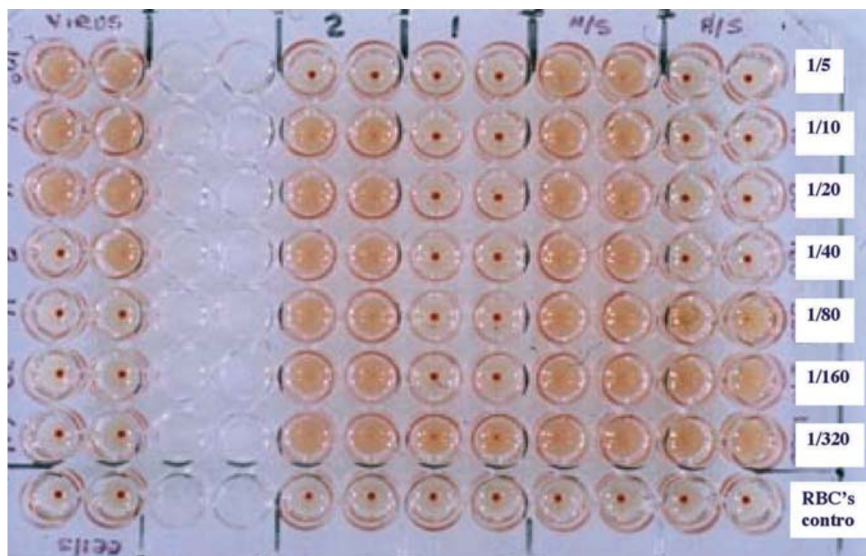

Figure 1. Basics of horse RBC HAI assay
Conditions are: V-bottom 96-well plates; RDE-treated sera; 1:5 starting dilution; serial twofold dilutions in final volume of 25 μl; virus: H5N1/Vietnam/1203 BPL-inactivated; 4 HAU in 25 μl; horse RBCs; collected in citrate dextrose acid (ACD) solution; washed and standardized to 1% v/v in PBS/0.5% BSA; added to assay in 50-μl volume; 1 h incubation time at room temperature to allow horse RBC to settle.

insensitivity of HAI for the detection of H5 antibody [21]. The main problem with horse HAI is a horse-to-horse variation, and specificity is reduced with increasing age of the erythrocytes (Fig. 1).

SRH was developed in 1975 [22]. It is routinely used for the detection of influenza-specific and rubella IgG antibodies. The test utilizes antibody diffusion in agar gel to measure the antibody content of test sera. Complement-mediated hemolysis induced by influenza antigen-antibody complexes produces easily discernible zones, the size of which are proportionate to concentrations of specific antibody in sera (Fig. 2) [23, 24]. Advantages of this assay are that sera do not need to be pretreated to inactivate nonspecific inhibitors, sera can be analyzed without dilution, only a preincubation of samples at 56 °C for 30 min is needed to inactivate complement, the test is easily standardized, and it may be more sensitive than HAI particularly for pandemic H5 strains. This test appears to be as sensitive as MN assay [25].

SRH is usually performed in PVC immunoplates, which are prepared using sheep RBCs for H1 or H3 antibody detection. The amount of live or inactivated whole influenza virus used to sensitize the RBCs is 2000 UE/ml in a 10% RBCs suspension, and 5 μl heated-inactivated serum is added to wells in SRH plates. After incubation for 18 h at 4 °C and 3 h at 37 °C, halos

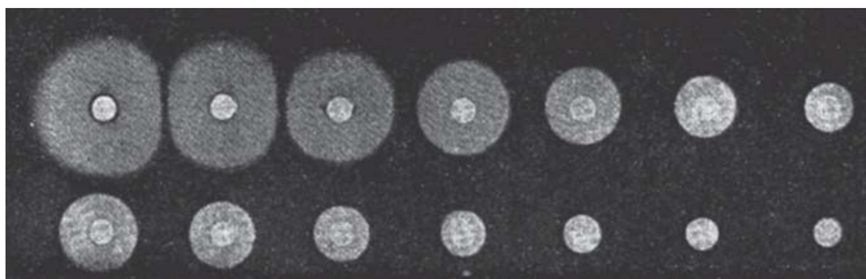

Figure 2. SRH reactions
SRH of human serum in agarose gel immunoplates containing guinea pig complement (final concentration 1:30) and chicken erythrocytes (1%) treated with A/Port Chalmers/1/73 virus. The clear areas represent zones of lysed erythrocytes produced by antibody to HA. The wells in the top row contain serial, twofold dilutions (1:1–1:64) of a potent human serum having an HAI titer of 1:2560 with A/PC/1/73 virus. The bottom row contains similar dilutions of a serum with an HAI titer of 1:256 [22].

of hemolysis are measured and areas are calculated. Areas of hemolysis equal or higher than 25 mm^2 are considered seroprotective. In the case of H5 strains, better results were obtained using turkey erythrocytes.

SRH is suitable for screening for large number of samples. This feature has made the test useful for rapid screening of antibodies against newly detected influenza variants, making it valuable for large-scale sero-epidemiological studies. It has good correlation with MN for pandemic H5 strains and is EMEA approved.

The SRH test works well with inactivated viruses so that serology of H5N1 can be safely analyzed at a biosafety level 2 containment. Although this test can detect H5N1 antibody, it cross-reacts with nonspecific antibody in human and rabbit sera. Therefore, preliminary screen for cross-reactivity, and confirmatory tests with an alternative technique are recommended.

This test allows smaller differences in antibody level to be detected than is possible by conventional HAI tests. It is a convenient test that can be performed with undiluted serum. Antibodies responses to natural infection and vaccination are readily detected by SRH tests.

Whether comparing HAI and SRH tests for seasonal strains (Fig. 3), a close correlation between the antibodies potencies measured in both test systems was observed. Serum samples with high HAI titers (1:1256–1:5120) gave zone diameter of 9–11 mm (hemolysis area 64–95 mm*2*). Of the 15 samples shown in Figure 3 that were negative in HAI tests (titer $<$1:10), 10 were also negative by SRH [22]. Completely different results were achieved when comparing HAI with SRH and MN for H5 strains (Fig. 4).

The MN assay (or virus neutralization assay, VN) requires only a stock of infectious virus as the antigen. The advantages of the MN assay are that it is more sensitive than HAI, more specific, and is suitable for automation

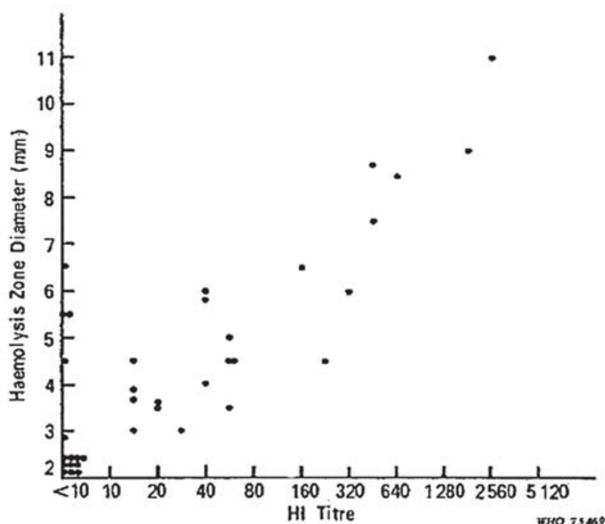

Figure 3. Correlation between HAI titer and SRH zone diameter for A/Port Chalmers/1/73 (H3N2) virus in 37 human serum samples [22].

[26]. This assay detects functional anti-HA antibody, which is highly specific for the subtype in question. Moreover, this assay can be developed quickly upon recognition of a novel virus and can be used even before suitable recombinant or purified viral proteins become available for use in other assays. In addition, MN will also measure neutralizing responses against other envelope glycoproteins, i.e., NA, in contrast to the HAI assay that only measures responses against the HA component. The MN test seems to have advantages when antibody levels are low, with negligible or negative titers in the HAI test [27, 28].

MN tests have not been used widely in serological studies because they are lengthy (an overnight test), thus this assay is not easy when screening a large number of samples [20]. Moreover, this assay needs live virus and thus, a high containment in case of pandemic strains. At the front line of an outbreak, especially in resource-limited regions, biosafety level 3 laboratory facilities or higher are not always available. Therefore, this assay is the gold standard for confirmation.

For the MN assay, there is no recognized correlate of protection; however, a fourfold increase in titer after vaccination has been used in the literature to assess immune responses to H5 viral antigens by MN [29, 30].

Based on the sensitivity and specificity of analysis described, the MN assay is now being used as part of a sero-epidemiological investigation of the 1997 outbreak in Hong-Kong to detect H5 virus in sera from thousands of subjects evaluated. Because avian viruses may also induce low levels of serum antibody, and may not have been detected in previous surveys that

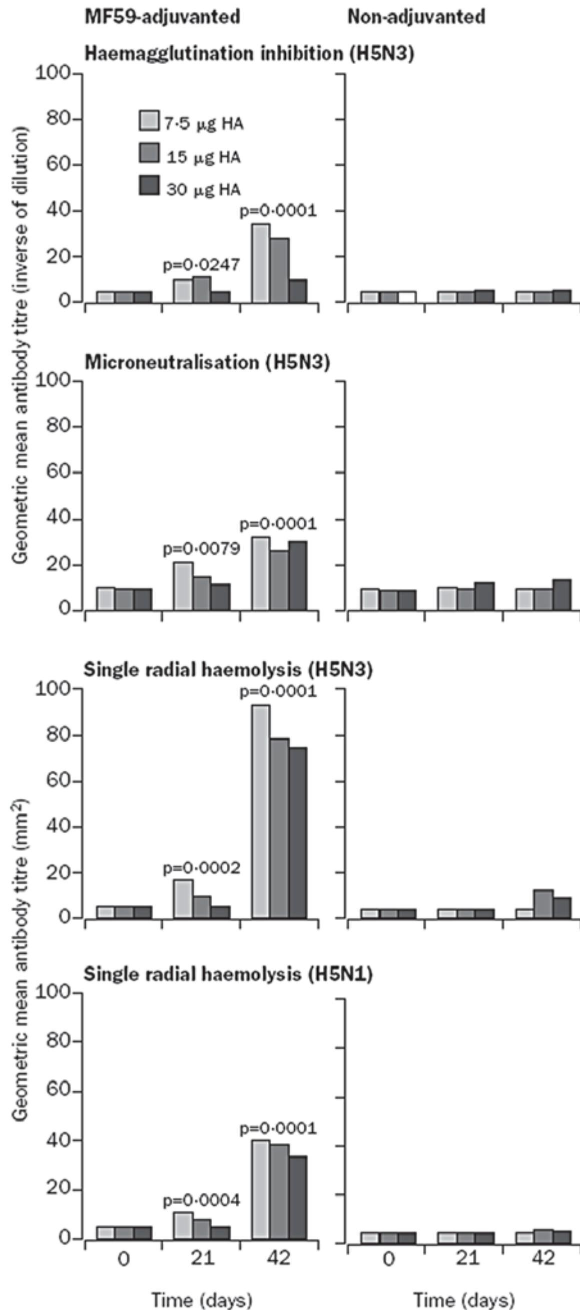

Figure 4. Geometric mean titers of antibody for MF59-adjuvanted and conventional surface-antigen H5N3 vaccine before and after 2 and 3 doses of vaccine, (A) HAI test using H5N3 antigen. (B) MN using H5N3 antigen. (C) SRH using H5N3 antigen. (D) SRH using H5N1 antigen [41].

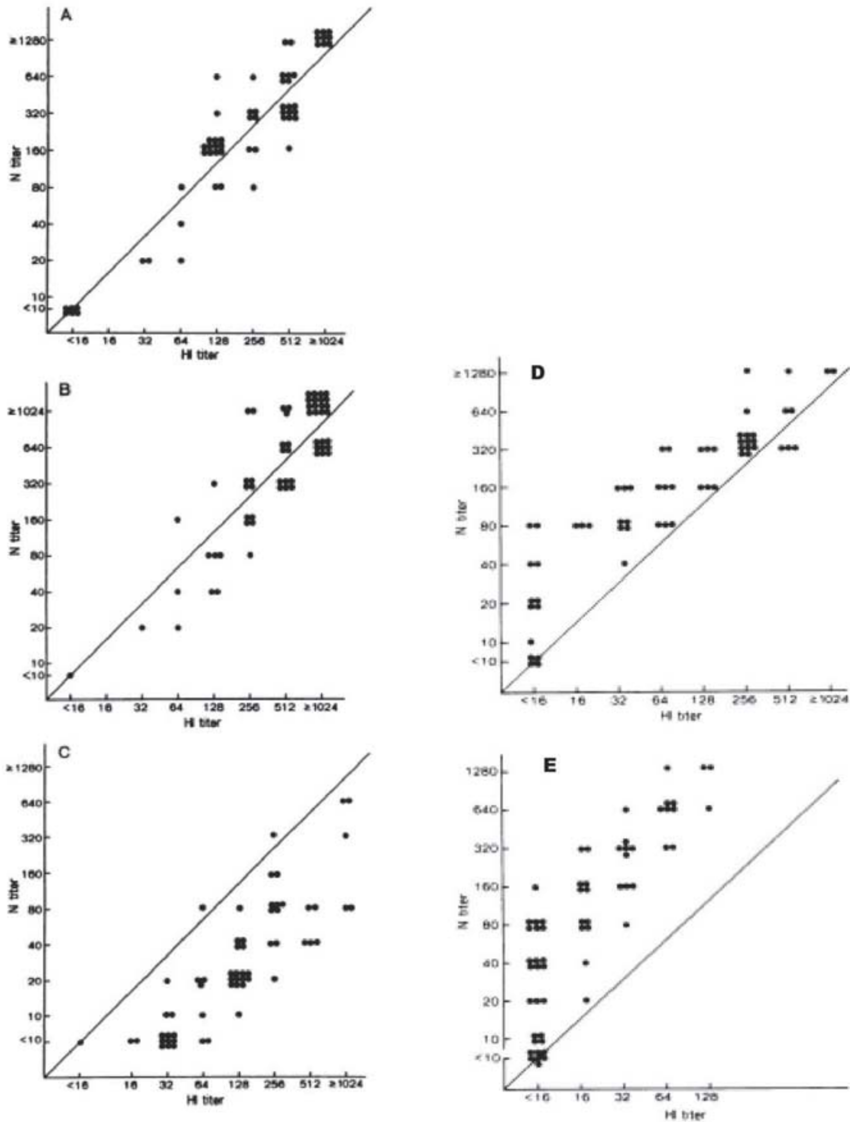

Figure 5. Relationship between HAI and neutralizing-antibody (MN) titers against A/Yamagata/120/86 (H1N1) (A), A/Fukuoka/C29/85 (H3N2) (B), A/Shisen/2/87 (H3N2) (C), B/Nagasaki/1/87 (D) and B/Osaka/152/88 (E).

The serum antibody was measured by MN and HAI tests. When titrated against vaccine strains (A, B, D) differences between the MN and HAI titers are small, when titrated against heterologous strains the differences are large. The possibility that noninfectious virus particles consume neutralizing antibodies, and thus result in lower actual titers, as suspected in the neutralization tests with heterologous strains, seems unlikely because the ratio between infectivity and the hemagglutinin titer of A/Shisen/2/87 was not lower than that of other strains. Therefore, these observations seem to indicate that the neutralization test is more specific than the HAI test. [42].

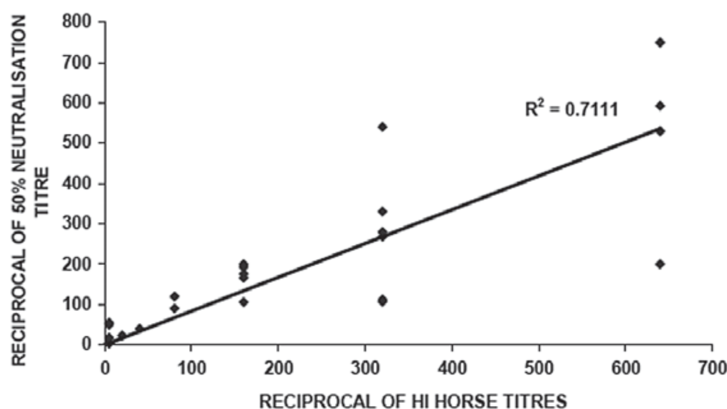

Figure 6. Correlation between MN and horse RBCs HAI titers using samples vaccinated with adjuvanted A/H5N1/Duck/Singapore/97 vaccine MF59 (n=48) (R: correlation coefficient) [33].

relied on the HAI assay [31], the MN assay may also be applied to serosurveys to detect evidence of human infection with avian influenza A viruses of other subtypes.

MN assays are usually done in 96-wells plates, where sera (heat-inactivated for 30 min at 56 °C) are tested through mixing with an equal volume of influenza virus at 2×10^3 $TCID_{50}$/ml. After 2-h incubation at 37 °C under 5% CO_2, 100 µl cells (1.3×10^5) is added to each well. After a second incubation at 37 °C for 18 h under 5% CO2, the cells are fixed in acetone for 10 min. The presence of viral protein is detected by ELISA using a monoclonal antibody to the influenza nucleoprotein. Alternatively, the presence of virus can be detected in the medium of culture using simple hemagglutination of animal RBCs [32].

Figure 5 shows the relationship between HAI and MN antibody titers against seasonal strains. The MN antibody titers are slightly higher than the HAI titers against the homologous strains. This figure also demonstrates that correlation is dependent on the strain analyzed. Less correlation between MN and HAI was also observed for detection of antibodies that may be present in sera from individuals receiving the H5N1 Vietnam vaccine (Fig. 6).

Other assays

An ELISA has been developed to detect antibodies to HA from novel influenza viruses. This assay is suitable for screening a large number of samples, with the preferable use of HA (with HA1 being purified only if this gives less cross-reactivity). Thus, this offers the possibility of automation, but the

Table 2. Sensitivities and specificities of serological assays for detection of antibodies to H5N1 virus.

Age group[a]	Parameter[b]	Values (%)				
		Individual serological tests[c]			Combination of tests[d]	
		N	E	W	N-W	E-W
Child	Sensitivity ($n=8$)	88	100	100	88	100
	Specificity ($n=24$)	100	92	93	100	100
Adult	Sensitivity ($n=85$)	80	80	80	80	80
	Specificity ($n=85$)	93	62[e,f]	85	96	84[e]

[a]Serum samples from individuals 1–14 years of age (Child) or from individuals 18–59 years of age (Adult).
[b]Sensitivity, number of H5N1 virus-infected patients testing positive for antibody divided by the total number of patients with confirmed H5N1 infections tested. Specificity, number of control age-matched sera tested minus the number of control sera testing positive for antibody divided by total number of control sera.
[c]Tests for determination of H5N1 virus positivity (N, microneutralization test; W, Western blotting; E, ELISA).
[d]Combination of tests. Microneutralization test with A/Hong Kong/156/97 virus followed by Western blot confirmation with rHA of A/Hong Kong/156/97 virus (N-W) and ELISA with rHA of A/Hong Kong/156/97 virus followed by Western blot confirmation with rHA of A/Hong Kong/156/97 virus (E-W).
[e]Number of samples, 50.
[f]Statistical analysis for positive association between test result and known status of samples was not significant (Fisher exact test; $p=0.067$). All other assays were significant ($p=0.003$) [20].

assay has no correlate of protection. If HA is produced using a baculovirus expression system, false positive reactivity can be found due to contaminating baculovirus/insect cell proteins. When combined with Western blotting (WB), ELISA shows improved specificity and retains improved sensitivity compared with the MN assay and WB combination [20, 33, 34].

WB is useful only for confirmation. This technique is too labor intensive to be considered a diagnostic test for screening several thousand sera, and is generally used as a secondary serological test to confirm other EMEA-accepted serological tests, i.e., MN assay or ELISA.

The sensitivities of WB and ELISA are generally higher than MN, and specificities of MN are higher than WB and ELISA particularly for pandemic strains (Tab. 2). To determine whether the MN assay and/or ELISA could be used to detect H5-specific antibody in single serum samples, the relative sensitivities and specificities of the assays were compared (Table 2). The test using the MN assay was notably superior to that of ELISA. When combined with WB, each test improved in specificity; however, maximum

sensitivity and specificity were still achieved by a combination of the MN assay and WB [20]. ELISA and WB test detected antibody of lower avidity and/or quantity than that required for detection by the MN assay.

The development of polymerase chain reaction (PCR) [35] has provided a highly specific and sensitive method for the detection of viral genomes. PCR uses two oppositely oriented primer flanking a specific DNA region, which, by repeated cycles of heat denaturation, annealing, and extension of the primers with Taq polymerase, allow the amplification of the specific target sequence within the virus genome. Studies have carried out reverse transcriptase (RT)-PCR to detect influenza virus RNA in clinical material and cell culture fluids. Although PCR is not as rapid as ELISA, which can be performed in a few hours, it is considerably more rapid than cell culture of virus. Furthermore, the sensitively of PCR has been demonstrated to be comparable to that of isolation of virus by cell culture [36].

There are two realistic options for rapid development of neutralization assays to make them more widely applicable: to use reverse genetics to engineer a safer, attenuated virus by deletion of the polybasic cleavage site in HA, as is done for the development of inactivated vaccines for pandemic influenza [37, 38], or the construction of viral pseudotypes bearing the influenza HA glycoproteins as surrogate viruses for use in neutralization assays. The first option has its inherent problems, i.e., the issue of possible reversion to the wild-type virus *via* genetic reassortment [39].

With the pseudotype system, however, only the HA from influenza is required, with no possibility of recombination or virus escape. These particles undergo abortive replication and do not give rise to replication-competent progeny. These pseudotypes encode reporter genes and bear foreign viral envelopes of interest [40]. The transfer of marker genes to target cells depends on the function of the envelope protein; therefore, the titer of neutralizing antibodies against the envelope can be measured by a reduction in marker gene transfer (Fig. 7).

Retroviral pseudotype-based assay facilitates the accurate determination of neutralizing antibody body responses to influenza H5N1 without the need to use replication-competent virus, and thus can be carried out at biosafety level 2. The pseudotype assay is significantly more sensitive than the turkey erythrocyte HAI, and at least as sensitive as the horse erythrocyte HAI and MN for the detection of antibodies to H5 HA (Fig. 8). The safety, specificity and sensitivity of the pseudotype assay suggest that it may have a role as an alternative or supplement to conventional assays.

Further development of β-Gal-based pseudotype assays will allow wider application as an ELISA-type assay in laboratories without specialized equipment. In addition, with minor modifications, these assays could be used to screen HA and NA inhibitors with high-throughput usage.

The application of pseudotypes in immunological assays (such as MN, ELISA, HAI) using sera from vaccinated volunteers has been demonstrated for H5N1/Vietnam/1194 strain. The pseudotype-based neutralization

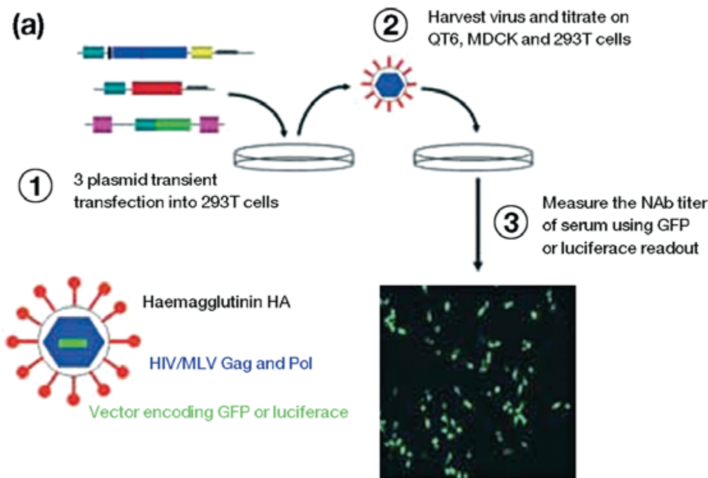

Figure 7. H5N1 HA retroviral pseudotypes. MLV (HA) and HIV (HA) pseudotype construction and neutralization assay for influenza A/H5N1 [39]. Confluent plates of 293T cells were prepared the day before transfection. Each plate was transfected with 1 μg gag/pol construct, 1.5 μg of GFP or Luc reporter construct and 1.5 μg HA construct using Fugene-6 transfection reagent. At 24h post-transfection, 1U of exogenous neuraminidase was added to induce the release of HA-pseudotyped particles from the surface of the producer cells. The supernatant was harvested 72h post-transfection and passed through 0.45-μm filters. MLV vector titers were measured on human 293T, QT6, MDCK cells and are presented as infectious units (IU) per milliliter. HIV vector titers were measured on 293T, QT6 and MDCK cells.

assay is promptly adaptable to a high-throughput format for the evaluation of vaccine efficacy. Furthermore, using plasmids encoding for any other HA sequence, a panel of pseudotypes expressing HA from different viral strains could be generated and used to evaluate cross-protective antibodies.

Conclusion

Traditional HAI method, performed using turkey RBCs, offers a simpler assay for detection of human antibody to some influenza strains particularly H1 and H3; this assay may be useful for large serosurveys as an initial screening tool. Detection of antibodies to avian influenza viruses in mammalian species, including humans, using HAI has generally failed, even in cases where infection was confirmed by virus isolation. HAI assay is less difficult and less time consuming to perform than MN or SRH assays.

SRH assay requires only a small amount of whole inactivated virus, thus there is no need to adapt the virus to rapid growth. This feature has made this test useful for rapid screening of antibodies against newly detected influenza variants. It can be valuable for large-scale sero-epidemiological studies of new influenza virus variants. SRH has a big disadvantage in detecting

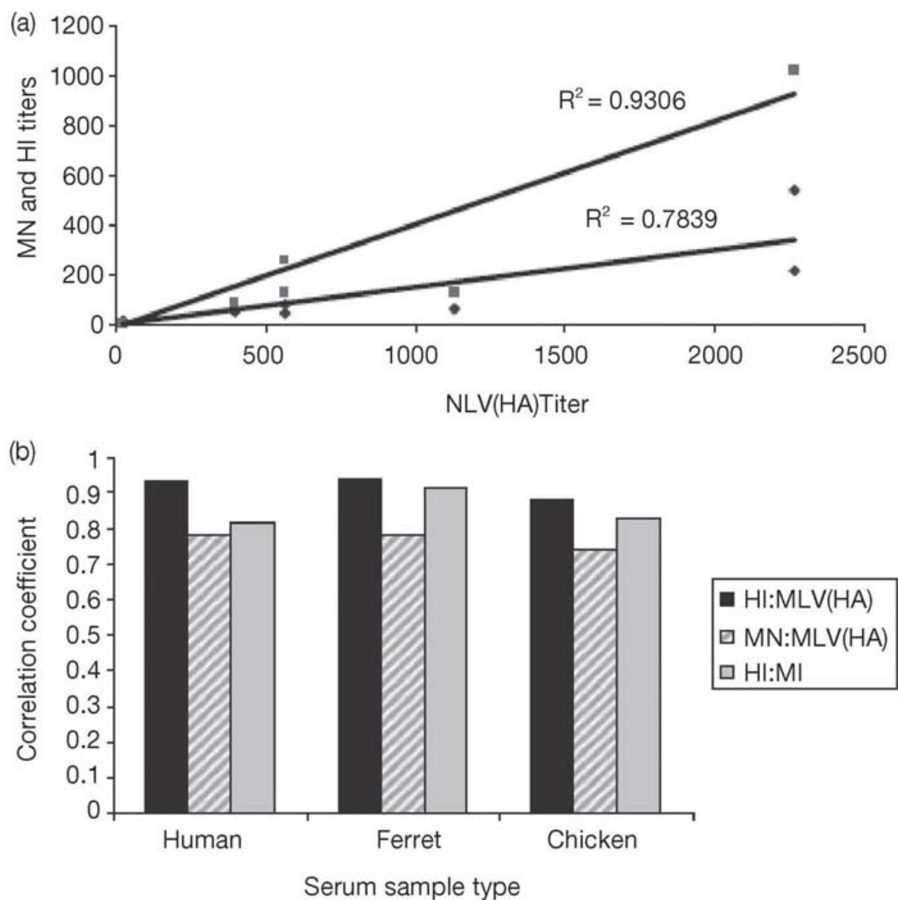

Figure 8. Comparison of MLV (HA) with HAI and MN for the measurement of antibodies to H5N1.
(a) Anti-H5 antibody responses in human sera. Scatterplot showing the correlation of antibody geometric mean titers (GMT) measured by horse HAI (squares) and MN (diamonds) with IC90 GMT measured by MLV (HA) pseudotype assay with a green fluorescent protein (GFP) reporter. HAI and MN assays performed against NIBRG-14 virus. Total number of sera assayed is 56 (6 positive for antibodies against H5, and 50 H5 negative control sera). Linear trend lines were fitted to the data by using Microsoft Excel 2003. (b) Correlation coefficients of HAI (horse):MLV (HA), MN:MLV (HA) and HAI (horse):MN for human, ferret and chicken sera. Data plotted are r^2 correlation coefficients determined using Microsoft Excel 2003 [39].

antibody to internal virus antigens in addition to those antibodies directed against surface glycoproteins, and may lack specificity for the detection of antibodies to HA. However, interpretation of results is complicated as the relationship between HAI titer and the hemolytic area obtained may not be easy to read.

MN assay is specific, more sensitive than the HAI assay, and suitable for automation. However, this assay requires time and live and titrated virus, thus a high containment area is needed when testing pandemic strains.

An ELISA test specific for HA antibodies requires highly purified antigen, which can be difficult to obtain in sufficient amount.

The pseudotype neutralization assay is as sensitive as horse erythrocytes HAI and MN for the detection of antibodies against H5N1. It is safer, and can be applied in a high-throughput format for human and animal surveillance and for the evaluation of vaccines. To achieve maximum sensitivity in serological assays, the selection of virus isolated from the same influenza outbreak, or the use of an antigenically equivalent strain is required for optimal antigenic match. Competent molecular virology laboratories could produce HA pseudotype virus within 2–3 weeks of the availability of viral RNA. Further studies are underway, making use of a panel of H5 retroviral pseudotypes with HA components derived from H5N1 viruses involved in the recent human and avian outbreaks.

Development of such assays will be an important further step in preparation of new influenza vaccines, not only for prepandemic and pandemic products, but also for cell-derived vaccines. Additional immunological assessments such as cell-mediated immunity and NA inhibition need to be explored to give more insight into the overall effects of vaccination.

References

1 Treanor J (2004) Influenza vaccine – Outmaneuvering antigenic shift and drift. *N Engl J Med* 350: 218–220

2 Webby RJ, Webster RG. (2003) Are we ready for pandemic influenza? *Science* 302: 1519–1522

3 Barker WH, Mullooly JP (1980) Impact of epidemic type A influenza in a defined adult population. *Am J Epidemiol* 112: 798–811

4 Barker WH (1986) Excess pneumonia and influenza-associated hospitalization during influenza epidemics in the United States, 1970–78. *Am J Public Health* 76: 761–765

5 Dorrell L, Hassan I, Marshall S, Chakraverty P, Ong E (1997) Clinical and serological responses to an inactivated influenza vaccine in adults with HIV infection, diabetes, obstructive airways disease, elderly adults and healthy volunteers. *Int J STD AIDS* 8: 776–779

6 Künzel W, Glathe H, Engelmann H, Van Hoecke C (1996) Kinetics of humoral antibody response to trivalent inactivated split influenza vaccine in subjects previously vaccinated or vaccinated for the first time. *Vaccine* 12: 1108–1110

7 Stephenson I, Nicholson KG (2001) Influenza: Vaccination and treatment. Eur Respir J 17: 1282–1293

8 Couch RB, Kasel JA, Glezen WP, Cate TR, Six HR, Taber LH, Frank AL, Greenberg SB, Zahradnik JM, Keitel WA (1986) Influenza: Its control in persons and populations. *J Infect Dis* 153: 431–440

9 Beyer WE, Palache AM, de Jong JC, Osterhaus AD (2002) Cold-adapted live influenza vaccine *versus* inactivated vaccine: Systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis. *Vaccine* 20: 1340–1353
10 Hoelscher MA, Garg S, Bangari DS, Belser JA, Lu X, Stephenson I, Bright RA, Katz JM, Mittal SK, Sambhara S (2006) Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet* 367: 475–481
11 Beyer WE, Palache AM, Lüchters G, Nauta J, Osterhaus AD (2004) Seroprotection rate, mean fold increase, seroconversion rate: Which parameter adequately expresses seroresponse to influenza vaccination? *Virus Res* 103: 125–132
12 EMEA (1996) Note for Guidance on harmonisation of requirements for influenza vaccines (CPMP/BWP/214/96)
13 EMEA (2003) Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application. (CPMP/VEG/4717/03)
14 Hirst GK (1941) The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science* 94: 22–23
15 Wright PF, Bryant JD, Karzon DT (1980) Comparison of influenza B/Hong Kong virus infections among infants, children, and young adults. *J Infect Dis* 141: 430–435
16 Monto AS, Maassab HF (1981) Ether treatment of type B influenza virus antigen for the hemagglutination inhibition test. *J Clin Microbiol* 13: 54–57
17 Kendal AP, Cate TR (1983) Increased sensitivity and reduced specificity of hemagglutination inhibition tests with ether-treated influenza B/Singapore/222/79. *J Clin Microbiol* 18: 930–934
18 Palmer DF, Coleman MT, Dowdle WR, Schild GC (1975) Advanced laboratory techniques for influenza diagnosis. *Immunology Series, Procedural Guide 6*: U.S. Department of Health, Education, and Welfare; Public Health Service, Center for Disease Control, 34
19 Hinshaw VS, Webster RG, Easterday BC, Bean WJ Jr (1981) Replication of avian influenza A viruses in mammals. *Infect Immun* 34: 354–361
20 Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, Fukuda K, Cox NJ, Katz JM (1999) Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* 37: 937–943
21 Stephenson I, Wood JM, Nicholson KG, Zambon MC (2003) Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol* 70: 391–398
22 Schild GC, Pereira MS, Chakraverty P (1975) Single-radial-hemolysis: A new method for the assay of antibody to influenza haemagglutinin. Applications for diagnosis and seroepidemiologic surveillance of influenza. *Bull World Health Organ* 52: 43–50
23 Mumford J, Wood J (1993) WHO/OIE meeting: Consultation on newly emerging strains of equine influenza. 18–19 May 1992, Animal Health Trust, Newmarket, Suffolk, UK. Vaccine 11: 1172–1175
24 Wood JM, Gaines-Das RE, Taylor J, Chakraverty P (1994) Comparison of

influenza serological techniques by international collaborative study. *Vaccine* 12: 167–174
25 Wood JM, Melzack D, Newman RW, Major DL, Zambon M, Nicholson KG, Podda A (2001) A single radial haemolysis assay for antibody to H5 haemagglutinin. *International Congress Series* 1219: 761–766
26 Gross PA, Barry DW, D'Esopo N (1976) Influenza immunization in chronic bronchitis: Local and systemic immune response. *Am Rev Respir Dis* 114: 305–313
27 Benne CA, Harmsen M, de Jong JC, Kraaijeveld CA (1994). Neutralization enzyme immunoassay for influenza virus. *J Clin Microbiol* 32: 987–990
28 Harmon MW, Rota PA, Walls HH, Kendal AP (1988) Antibody response in humans to influenza virus type B host cell-derived variants after vaccination with standard (egg-derived) vaccine or natural infection. *J Clin Microbiol* 26: 333–337
29 Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M (2006) Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* 354: 1343–1351
30 Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, Höschler K, Zambon MC (2006) Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: Phase I randomised trial. *Lancet* 367: 1657–1664
31 Profeta ML, Palladino G (1986) Serological evidence of human infections with avian influenza viruses. *Arch Virol* 90: 355–360
32 Frank AL, Puck J, Hughes BJ, Cate TR (1980) Microneutralization test for influenza A and B and parainfluenza 1 and 2 viruses that uses continuous cell lines and fresh serum enhancement. *J Clin Microbiol* 12: 426–432
33 Stephenson I, Wood JM, Nicholson KG, Charlett A, Zambon MC (2004) Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. *Virus Res* 103: 91–95
34 Doller G, Schuy W, Tjhen KY, Stekeler B, Gerth HJ (1992) Direct detection of influenza virus antigen in nasopharyngeal specimens by direct enzyme immunoassay in comparison with quantitating virus shedding. *J Clin Microbiol* 30: 866–869
35 Sakai RK, Gelfand DH, Stoffel S, Scharf FJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* 239: 487–491
36 Donofrio JC, Coonrod D., Davidson JN, Betts RF (1992) Detection of influenza A and B in respiratory secretions with the polymerase chain reaction. *PCR Method Appl* 1: 262–268
37 Wood JM, Robertson JS (2004) From lethal virus to life-saving vaccine: Developing inactivated vaccines for pandemic influenza. *Nat Rev Microbiol* 2: 842–847
38 Hoffmann E, Krauss S, Perez D, Webby R, Webster RG (2002) Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* 20: 3165–3170
39 Temperton NJ, Hoschler K, Major D, Nicolson C, Manvell R, Hien VM, Ha DQ, de Jong M, Zambon MC, Takeuchi Y, Weiss RA (2007) A sensitive retroviral

pseudotype assay for influenza H5N1-neutralizing antibodies. *Influenza* 1: 105–112

40 Sanders DA (2002) No false start for novel pseudotyped vectors. *Curr Opin Biotechnol* 13: 437–442

41 Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: A randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 357: 1937–1943

42 Okuno Y, Tanaka K, Baba K, Maeda A, Kunita N, Ueda S (1990) Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *J Clin Microbiol* 28: 1308–1313

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The role of animal models in influenza vaccine research

Catherine J. Luke and Kanta Subbarao

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

A major challenge for research on influenza vaccines is the selection of an appropriate animal model that accurately reflects the disease and the protective immune response to influenza infection in humans. Vaccines for seasonal influenza have been available for decades and there is a wealth of data available on the immune response to these vaccines in humans, with well-established correlates of protection for inactivated influenza virus vaccines. Many of the seminal studies on vaccines for epidemic influenza were conducted in human subjects. Studies in humans are performed less frequently now than they were in the past. Therefore, as the quest for improved influenza vaccines continues, it is important to consider the use of animal models for the evaluation of influenza vaccines, and a major challenge for research on influenza vaccines is the selection of an appropriate animal model that accurately reflects the disease and the protective immune response to influenza infection in humans.

The emergence of highly pathogenic H5N1 avian influenza (AI) viruses and the threat of a pandemic caused by AI viruses of this or another subtype has resulted in a resurgence of interest in influenza vaccine research. The development of vaccines for pandemic influenza presents a unique set of obstacles, not the least of which is that the demonstration of efficacy in humans is not possible. Since the correlates of protection from pandemic influenza are not known, we rely on extrapolation of lessons from seasonal influenza vaccines and on data from the evaluation of pandemic influenza vaccines in animal models to guide our decisions on vaccines for use in humans. The features and contributions of commonly used animal models for influenza vaccine research are discussed.

Influenza viruses

Influenza is a negative-sense, single-stranded RNA virus belonging to the family *Orthomyxoviridae*. *Orthomyxoviridae* consist of four genera: influenza A, influenza B, influenza C and Thogoto viruses. The proteins of influenza A viruses are encoded by genes on eight RNA segments. Influenza A viruses are widely distributed in nature and can infect a wide variety of birds

and mammals, including humans. Influenza A virus subtypes are classified on the basis of the antigenicity of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1, 2] into 16 HA subtypes and 9 NA subtypes, and all of these subtypes have been found to infect birds [2, 3]. Waterfowl and shorebirds are the natural reservoirs of AI viruses.

In their natural hosts, most AI infections are not associated with clinical disease, and the viruses are generally thought to be in evolutionary stasis [4]. In the human population, relatively few subtypes of influenza A viruses have caused sustained outbreaks of disease; viruses bearing H1, H2 and H3 HA and N1 and N2 NA genes have circulated in the human population during the 20th century. H1N1 viruses appeared in 1918 and circulated until 1957, when they were replaced by H2N2 viruses. These in turn were replaced in 1968 by H3N2 viruses, which continue to circulate at the present time. In 1977, H1N1 viruses reappeared and have continued to co-circulate with the H3N2 viruses. Influenza A and B viruses cause epidemics in humans each winter.

In addition to the seasonal influenza epidemics, the potential also exists for an influenza pandemic at any time. A pandemic occurs when an influenza strain with a novel HA subtype (with or without a novel NA subtype) appears and spreads in a susceptible human population. In the 20th century, influenza pandemics occurred in 1918, 1957 and 1968, and were associated with significant morbidity and mortality [5]. It is estimated that, in the United States alone, the next influenza pandemic could cause 89,000–207,000 deaths, and 314,000–734,000 hospitalizations, and tens of millions of outpatient visits and illnesses [6]. AI viruses in their natural reservoir in waterfowl and shorebirds are the source from which novel HA and NA subtypes are introduced into the human population. A novel AI HA and/or NA can be introduced into the human population by direct spread from either wild birds or domestic poultry, as was seen when an H5N1 AI virus infected humans in 1997 [7]. Alternatively, human and avian influenza viruses can reassort, generating virus that can efficiently spread in humans, as happened in the case of the 1957 H2N2 and 1968 H3N2 pandemic viruses [8].

Influenza A viruses also infect and cause disease in a wide variety of mammalian species, including swine, horses, ferrets, mink, dogs, seals and whales. The currently circulating highly pathogenic AI H5N1 viruses that emerged in Asia in 2003 can also infect and cause lethal infection in felids, including tigers, leopards and domestic cats [9, 10].

Although several animal species are infected with influenza A viruses naturally and experimentally, an ideal animal model for studying infection and immunity to human influenza has not been identified. Several animal species are permissive to infection with influenza A and B viruses to varying degrees and some exhibit clinical signs of illness and pathological changes in the respiratory tract that are similar to those seen in human influenza. In this chapter, we discuss the main features of the animal models used for

evaluation of influenza vaccines, their advantages and disadvantages, and their contribution to research on vaccines against influenza in humans. We also discuss the role of animal models in the development of vaccines against pandemic influenza. Veterinary vaccines for swine, equine, avian and canine influenza can be evaluated in their natural hosts and are not be discussed.

Influenza vaccines

Vaccines have been available for epidemic or 'seasonal' influenza since the 1940s. Inactivated influenza virus vaccines are largely the same now as they were when first developed. They are still generally produced in embryonated hen's eggs. There has been much recent investment in the development of cell-based influenza vaccines, of which at least two are licensed in Europe and several others are in development in Europe and the United States. In 2003, live attenuated influenza vaccines were licensed in the United States for annual use in healthy individuals between 5 and 49 years of age.

A serum hemagglutination inhibiting (HAI) antibody titer of 1:32 or 1:40 or greater is associated with protection from seasonal influenza [11–13], and this is used as a measure to predict the protective efficacy of seasonal inactivated influenza virus vaccines. The correlates of protection for live attenuated vaccines are less clear-cut. These vaccines elicit systemic and mucosal immune responses and mucosal antibody in the respiratory tract is believed to play a major role in protection afforded by these vaccines [14–16].

Antigenic drift describes the gradual change in antigenicity of an influenza virus that allows the virus to escape neutralization by antibodies induced by infection or immunization with previously circulating strains. Antigenic drift results from point mutations in and around antibody-combining sites in the HA and NA proteins. Influenza virus vaccines are unusual in that one or more of the components of the trivalent vaccine formulation may have to be changed annually to keep pace with antigenic drift of the virus but as long as the licensed manufacturing process is used, the change in composition of the vaccine is considered a strain change and it is not treated as a new vaccine. Approval of seasonal influenza vaccines for use in humans requires limited testing in animals, and an evaluation of immunogenicity in humans is required in Europe but not in the United States.

In recent years, a resurgence of interest in improvement of seasonal influenza vaccines, and the looming threat of a possible influenza pandemic have spurred efforts to develop vaccines that could thwart the spread of an emerging pandemic virus. Extensive pre-clinical characterization of these new vaccines in animals will be necessary. Many researchers are engaged in efforts to develop 'universal' influenza vaccines that will protect against both epidemic and pandemic strains by targeting the more conserved antigens of the virus, such as nucleoprotein (NP) or matrix protein (M), thus

eliminating the need for constant updating of the composition of the annual seasonal influenza vaccine. The immune responses to candidate universal vaccines are entirely different from those elicited by the currently licensed seasonal inactivated influenza virus vaccines, where protective immunity is based mainly on neutralizing antibodies produced against the HA protein. Animal models are needed in which different types of immune responses can be evaluated.

One of the major challenges in the development of pandemic influenza vaccines is that correlates of protection from AI viruses of pandemic potential are not known. Efficacy of these novel influenza viruses cannot be established in humans, so assessment of efficacy is based on information gleaned from challenge studies in animals.

Animal models for influenza

Despite the diversity of mammalian species infected by influenza viruses in nature, only a few species are amenable to study in the laboratory. Tables 1–3 and the following sections summarize the features of the most commonly used small animal models for the study of influenza, and their respective utilities in the evaluation of influenza vaccines are summarized in Table 4. Commonly used laboratory animal species may not be fully permissive for infection with wild-type, non-adapted isolates of influenza viruses, and can vary in susceptibility to infection by specific virus strains and subtypes. Other variables that can influence the outcome of infection are the use of anesthesia, route of virus administration and the volume of inoculum.

Rodent models

Rodent models of infectious diseases are attractive for a number of scientific and practical reasons. They are small and relatively inexpensive to purchase and house. Many inbred strains are available and a battery of immunological reagents are available for some species.

Mice

Mice have been used for influenza vaccine research from the earliest days of the study of influenza virus biology. Shortly after the first human influenza virus was isolated from ferrets in 1933 by Wilson Smith and colleagues at the National Institute for Medical Research in London [17], it was discovered that human influenza viruses would cause disease in mice only if they were first adapted to the species by serial passages in the lungs [18]. This was sub-

sequently found to be true of all human influenza virus isolates. One of the most commonly used human influenza viruses in mouse studies is influenza A/Puerto Rico/8/34 (PR8), an H1N1 virus with a complex passage history, including several passages in ferrets, and hundreds of passages in eggs and mice (C.B. Smith, CDC, Atlanta, GA, personal communication). This virus is well adapted to mice and causes a lethal infection. The need for adaptation through serial passage of human influenza viruses is one of the major drawbacks of using mice in influenza research, because many mutations can arise during adaptation to the murine host [19–22] that can alter their replication kinetics, and can result in the ability of the virus to escape innate immune responses [23].

Influenza viruses that cause disease and are lethal for mice provide a useful endpoint for vaccine efficacy studies. Depending on the strain of virus used, mice may become lethargic, anorexic, develop ruffled fur, and may also exhibit neurological symptoms of infection, in addition to weight loss, which is often the primary objective measure of the severity of infection. Body temperature is not a useful measurement in mice because hypothermia can occur following infection with mouse-adapted viruses. Irrespective of whether an influenza virus induces morbidity or mortality in mice, the level of replication of influenza viruses in the lungs is the most informative endpoint for efficacy studies in mice, since even a modest reduction in titer of infectious virus in the lungs can be associated with survival from lethal infection [24, 25]. Mice immunized with influenza viruses or vaccines develop serum HAI and neutralizing antibodies, the titers of which correlate with protection from subsequent challenge. Studies by Virelizier [26] demonstrated that antibody alone could protect against influenza infection in mice. Passive transfer of immune serum to naive mice reduced replication of virus in the lungs, and protected the recipient mice from lethal influenza pneumonitis but did not prevent tracheitis or replication of virus in the upper respiratory tract [27]. The observation that passively transferred serum antibodies can reduce pulmonary virus replication but not viral replication in the upper respiratory tract is not unique to influenza A. Similar observations have been reported in influenza C virus [28], respiratory syncytial virus (RSV) [29] and severe acute respiratory syndrome-associated coronavirus (SARS-CoV) infection [30]. When measuring the amount of virus in various tissues in cases where high levels of serum antibody are present, for example, when vaccines are administered with adjuvant, the presence of virus should be measured by quantitative molecular methods, to rule out the possibility of *ex vivo* neutralization by serum antibody during tissue preparation. Such *ex vivo* neutralization accounted for a reduction in detectable virus of up to 300-fold in the lungs of mice that had undergone passive transfer of immune serum against SARS-CoV [30]. The use of nasal and bronchiolar wash samples instead of tissue homogenates for viral quantitation was also employed as a solution to this issue [28].

Table 1. The use of the mouse model for the evaluation of vaccines against influenza.

Influenza virus subtypes tested	Findings	Refs.
1. Human influenza H1N1, H3N2, H2N2	- Human influenza virus isolates require adaptation to cause illness (lethality) in mice. - Infection under anesthesia results in viral pneumonia. - Clinical signs include ruffled fur, hunching, labored breathing, unsteady gait, hypothermia and weight loss. - Inflammation is observed in the respiratory tract	[18, 31, 32, 155]
2. Reconstructed 1918 H1N1 pandemic virus	- Causes illness in mice and replicates efficiently in the respiratory tract without prior adaptation. - Up to 13% loss of body weight is observed. - Lethal to mice with an MDT of 4.5 days. - No extrapulmonary spread observed. - Necrotizing bronchitis and bronchiolitis, and moderate to severe peribronchial and alveolar edema present.	[131]
3. HPAI H5N1	- Most isolates cause severe illness and death without prior adaptation. - Replicate efficiently in the respiratory tract without prior adaptation. - Cause significant weight loss. - Most isolates are lethal in mice with a MDT of 6–8 days. - Some isolates are detected in extrapulmonary sites including the brain. - Variable virulence in mice is observed with isolates from Hong Kong from 1997, and 2003–2004, and viruses isolated from Europe and South America.	[100–104, 126, 133]
4. H7	- HP and LP isolates replicate efficiently in respiratory tract of mice without prior adaptation, with some viruses causing weight loss and death. - Extrapulmonary spread to the brain and spleen observed following intranasal infection with some isolates. - Histopathological observations following intranasal infections with human isolates include necrosis and inflammation throughout the respiratory tract, but no lesions in the brain, heart, spleen, liver or kidneys. - Histopathological lesions are observed following intranasal infection with HP avian isolates.	[114–117]
5. H9N2	- Replicate efficiently in lungs of mice without prior adaptation. - Conflicting reports of lethality in mice. - Adaptation by passage in mouse lungs results in increased virulence. - Replication in brain reported following intranasal infection with non-adapted and mouse-adapted viruses.	[121, 125–130, 156, 157]
6. H6N1	- A/teal/W312/HK/97 (H6N1) replicates efficiently without prior adaptation and is lethal for mice when administered at high titers. - Significant weight loss (average 24%) is observed in infected mice. - Adaptation to mice results in increased virulence and spread to brain.	[125]

MDT,mean time to death; HPAI, highly pathogenic avian influenza; HP, highly pathogenic; LP, low pathogenicity

Table 2. The use of the ferret model for the evaluation of vaccines against influenza.

Influenza virus subtypes tested	Findings	Refs.
1. Human influenza H1N1, H3N2, H2N2 viruses	- Efficient replication of non-adapted isolates in respiratory tract. - Isolated report of the presence of an H3N2 human influenza virus in the brain. - Signs of illness include fever, sneezing, rhinorrhea, and weight loss. - Mild inflammatory changes are observed upon histopathological examination of lungs of infected animals.	[17, 132, 158, 159]
2. Reconstructed 1918 H1N1 pandemic virus	- Replication to high titers in respiratory tract. - Severe disease observed including lethargy, anorexia, severe weight loss and high fever. - Infection is lethal in 2/3 of inoculated animals; death occurs by day 11. - Virus is not detected in brain or heart. - Necrotizing bronchiolitis, and moderate to severe alveolitis with edema observed upon histopathological examination.	[141]
3. HPAI H5N1	- Efficient replication in respiratory tract and evidence of extrapulmonary spread to brain, spleen and intestines. - Most isolates cause severe disease, including fever, rhinitis, sneezing, severe lethargy, hind limb paresis and diarrhea. - Many isolates cause lethal infection in ferrets. - Histopathological observations include inflammatory changes in the lungs (bronchioloitis, bronchitis, interstitial pneumonia) and inflammation in the brain.	[104, 132, 134]
4. AI subtypes H1N1, H2N1, H6N2, H2N2, H2N3, H3N2, H10N7, H3N6, H7N7, seal H7N7 isolate	- Efficient replication in the upper respiratory tract. - No signs of illness with any of these isolates.	[140]

AI, avian influenza

The level of anesthesia can influence the outcome of influenza infection in mice. Mice infected under anesthesia develop pneumonia, while infection is limited to the upper respiratory tract when awake mice are infected [31, 32]. Volume of inoculum administered intranasally also influences the extent to which virus is distributed in the respiratory tract [32]. Immunologically, the lack of a functional Mx gene in standard laboratory strains of mice presents a disadvantage in using this model for studies in which the innate immune response to infection is important [33, 34]. However, the ready availability of mice, their relatively low cost, and the variety of genetic backgrounds and targeted genetic defects, and the immunological reagents available still make the mouse an attractive and heavily utilized animal model for studies of influenza.

Table 3. The use of the hamster model for the evaluation of vaccines against influenza.

Influenza virus subtypes tested	Findings	Refs.
1. Human influenza H3N2	- Non-adapted isolates replicate in the upper and lower respiratory tract. - No clinical signs of infection are observed.	[35–37, 146]
2. HPAI H5N1	- Non-adapted A/HK/483/97 (H5N1) resulted in lethal infection with deaths of all inoculated animals by day 6 post inoculation. - Virus is detectable in the lungs and brain.	[146]
3. H9N2	- Non-adapted A/HK/1073/99 (H9N2) replicates to high titers in the lungs but is not detected in the brain. - Infection is not lethal.	[146]
4. H9N5	- Non-adapted A/dk/HK/702/79 (H9N5) replicates efficiently in the lungs. - Infection is not lethal.	[146]

Hamsters

Influenza virus infection of hamsters with non-adapted human influenza viruses does not result in clinical disease, but the virus replicates to high titers in the nasal turbinates and lungs following intranasal infection [35–37]. As with mice, the hamster represents a readily available small animal model that can be used for pre-clinical evaluation of candidate vaccines, but it has not been used as extensively as mice have been for studies of inactivated influenza virus vaccines. The body temperature of Golden Syrian hamsters is about 39 °C, while that of mice is 37 °C. Thus, hamsters have been used for the evaluation of live attenuated temperature-sensitive vaccines with shut-off temperatures of ≥38 °C [38].

Guinea pigs

Guinea pigs can be infected with non-adapted human influenza viruses, although the amount of virus needed to infect guinea pigs is about ten times more than the amount of virus needed to infect hamsters or ferrets [39]. Infection of guinea pigs with A/England/42/72 (H3N2) did not result in febrile illness or other clinical signs of influenza infection. Virus was isolated from nasal washes of animals infected with influenza A/England/42/72 (H3N2), A/Hong Kong/1/1968 (H3N2) or A/FM/1/47 (H1N1), but titers shed in the nasal secretions were not as high as those observed following experimental infection of ferrets. Infection of guinea pigs with influenza A/HK/1/68 (H3N2) virus resulted in pneumonia, which developed slowly and was reversible. This model was used to study the

Table 4. Comparison of the utility of commonly used animal models in the evaluation of influenza vaccines.

Species	Utility in vaccine evaluation
Mouse	- Determination of level of replication of live attenuated vaccine candidates in comparison to wild-type viruses. - Evaluation of antibody responses to vaccination by HAI assay, Nt Ab assay, ELISA. - Evaluation of cellular immune responses to vaccination. - Evaluation of vaccine efficacy and effects of adjuvants. - General safety test for manufactured candidate vaccines.
Ferret	- Determination of level of replication of live attenuated vaccine candidates in comparison to wild-type viruses. - Evaluation of antibody responses to vaccination by HAI assay, Nt Ab assay, ELISA. - Limited evaluation of cellular immune responses to vaccination. - Evaluation of vaccine efficacy and effects of adjuvants. - Toxicology studies.
Hamster	- Determination of level of replication of temperature-sensitive live attenuated vaccine candidates. - Evaluation of vaccine immunogenicity by HAI assay, Nt Ab assay and ELISA. - Evaluation of vaccine efficacy.

HAI: hemagglutination inhibition; NtAb: neutralizing antibody; ELISA: enzyme-linked immunosorbent assay.

effects of environmental pollutants or drugs on the respiratory tract [40]. Lowen and colleagues [41] reported that Hartley strain guinea pigs are highly susceptible to non-adapted influenza A/Panama/2007/99 (H3N2) virus. Intranasal infection resulted in virus replication in the nose and lungs, with higher titers of virus recovered from the lungs. Virus was recovered from the upper respiratory tract for up to 9 days post inoculation, whereas shedding declined to undetectable levels in the lungs by day 5. Virus replication was not associated with any effects on body temperature or weight of the animals, and no other clinical signs of illness were observed.

Rats

Common laboratory strains of rat are described as 'semi-permissive' for influenza infection, and infant rats are of some utility in the evaluation of live attenuated influenza vaccines, but they have not been used extensively to study influenza infection [42–44].

The cotton rat (*Sigmodon hispidus*) has been used in the laboratory as a model for several infectious diseases (reviewed in [45]). In particular, the cotton rat model was used extensively for the development of therapeutic

antibody treatments for RSV and has provided much useful information for vaccine development against this pathogen. Sadowski and co-workers reported that intranasal administration of human influenza virus to lightly anesthetized, outbred young adult cotton rats resulted in virus replication in the respiratory tract, production of pulmonary lesions and a strong immune response [46]. In recent years, there has been some renewed interest in the cotton rat as a laboratory animal model for human influenza virus infection. Species-specific reagents that permit more detailed analysis of viral pathogenesis and immune responses in this species have been developed [45] and inbred cotton rats are now available. The advantages of this model include the fact that cotton rats can be infected by non-adapted human influenza viruses, inbred animals are available, the virus replicates in the upper and lower respiratory tract, some clinical parameters can be measured, and virus infection results in histopathological changes in the lungs that are similar to those seen in the natural infection of humans [47]. To date only a limited number of human influenza viruses have been evaluated in cotton rats.

Ferrets

Ferrets are exquisitely susceptible to infection with human influenza viruses. The initial isolation of a human influenza virus by Smith and colleagues involved ferrets [17]. The ferret model of influenza has remained the same since this fortuitous discovery, and, in the opinion of many researchers, the ferret remains the ideal small animal model for influenza research. Ferrets can be infected with non-adapted human influenza virus isolates. Influenza virus infection in ferrets is primarily an upper respiratory tract infection, and infected ferrets exhibit clinical signs of infection similar to those seen in human influenza including fever, rhinitis and sneezing. The disadvantages of the ferret as a model for studying influenza vaccines include expense, special housing requirements, a limited number of suppliers, the difficulty in obtaining animals that are seronegative for influenza virus, their exquisite sensitivity to other respiratory pathogens and ease of acquiring infection from their handlers, and the lack of species-specific reagents, although this last point does not present an obstacle for the evaluation of HAI and neutralizing antibody responses. In addition, the high body temperature of ferrets (average temperature of 38.8 °C) may limit their utility in the evaluation temperature-sensitive live attenuated influenza vaccines.

Non-human primates

Non-human primates have not been used extensively for influenza vaccine research. From a practical standpoint, these animals are expensive and they

have not proven to be the best model for the study of vaccines for influenza. Old and New World species of monkeys have been evaluated as models of human influenza infection. It was determined early in the days of the study of influenza virus biology that non-human primate species were not as susceptible to human influenza viruses as their human relatives. Burnet reported in 1941 [48] that clinical signs of infection were only apparent in cynomolgus macaques when they were infected *via* the intratracheal route as opposed to intranasally. Interestingly, mortality was observed in animals inoculated with the 'W.S. Egg' strain, but the details of the derivation of this strain beyond the original isolation from Wilson Smith are not clear. Burnet reported that pathological changes consistent with those seen in human influenza infection were observed in the lungs of infected monkeys. The observation that intratracheal infection of monkeys might be required to achieve clinical signs of infection was supported by studies conducted by Saslaw and colleagues [49] in Rhesus macaques. Intratracheal infection of Rhesus macaques with a lung filtrate from mice infected with mouse-adapted A/PR/8/34 (H1N1) resulted in clinical signs of illness on day 2 post infection (p.i.), that resolved by day 4 p.i., whereas no signs of illness were apparent in monkeys inoculated with the same virus preparation intranasally, although both groups of animals showed hematological and serological evidence of infection.

Cynomolgus macaques were explored as a model for evaluation of the immunogenicity and efficacy of an immunostimulating complex (ISCOM) influenza vaccine by Rimmelzwaan and colleagues [50]. Cynomolgus macaques inoculated intratracheally with the human influenza A/Netherlands/18/94 (H3N2) virus did not develop clinical signs of illness but virus was recovered from lung lavage, nasal swabs and pharyngeal swab samples. Histopathological examinations were not performed.

Pigtailed macaques (*Macaca nemestrina*) were infected with a recombinant human influenza A/Texas/91 (H1N1) virus following virus administration *via* the trachea, tonsils and conjunctiva [51]. The animals exhibited clinical signs of infection, including loss of appetite, weight loss, nasal discharge and moderate fever, and histopathological observations that were consistent with progressive pneumonia. Virus was recovered from lung tissue at day 4 but not at day 7 p.i.

New World monkeys – including squirrel and cebus monkeys – have been evaluated as models for influenza vaccine studies. Murphy et al. [52] demonstrated that adult squirrel monkeys could be infected with intratracheally administered human influenza viruses. Mild illness that manifested as afebrile coryza was seen and, although radiographic evidence of pneumonia was not observed, the animals shed virus from the respiratory tract. Further studies evaluated the ability of AI viruses to replicate and cause illness in this species [53]. Different viruses caused varying degrees of clinical illness; some influenza viruses were completely attenuated in squirrel monkeys, while others replicated efficiently and caused clinical signs of similar sever-

ity to that seen in human H3N2 influenza infection. Squirrel monkeys were employed to evaluate the level of attenuation of avian/human influenza virus reassortants in a study comparing the replication of reassortants with findings in chimpanzees and human volunteers [54]; the findings in squirrel monkeys were not predictive of the level of attenuation of the reassortant viruses in humans.

Cebus apella and *Cebus albifrons* monkeys were evaluated as models for influenza infection by Grizzard et al. [55]. The monkeys were inoculated either intranasally or intratracheally with two human influenza A viruses: A/Victoria/75 (H3N2) and A/New Jersey/76 (H1N1). All animals that received the A/Victoria/75 (H3N2) strain developed clinical signs of illness, and had evidence of infection by either virus shedding or serology. Radiographic evidence of pulmonary disease was only seen in animals inoculated intratracheally with A/Victoria/75 (H3N2). Eight of ten animals inoculated intratracheally with the A/New Jersey/76 (H1N1) virus had mild upper respiratory tract illness, but only one of ten animals shed virus. However, all of these animals seroconverted. Histopathological evidence of inflammation in the lungs and trachea was seen in animals inoculated intratracheally with either strain, although the lesions in the animals that received A/Victoria/75 (H3N2) were more severe.

Chimpanzees are considered to be a valuable animal model to study infections of humans because of their close evolutionary relationship with the human species. However, the use of chimpanzees as animal models in research is logistically difficult. They are extremely expensive animals that require long-term care and stringent isolation since they are susceptible to several human pathogens. Chimpanzees have been used for some studies with influenza [54, 56, 57]. Influenza A and B viruses replicated to high titer in seronegative chimpanzees, but viral replication was not associated with illness. Advantages of studying influenza in this species include the fact that chimpanzees have the same body temperature as humans, the lower respiratory tract can be repeatedly sampled safely, they display permissiveness for vectored vaccines similar to humans (for example, vaccinia-based vaccines) and they are evolutionarily close to humans and this may mean that similar host-range restrictions for replication of viruses may be present, which could facilitate selection of live attenuated candidate vaccines for testing in humans.

There is renewed interest in the use of non-human primates for evaluation of vaccines for pandemic influenza (see *Vaccines for pandemic influenza* below).

Animal models in influenza vaccine research

The three general areas of vaccine research and development in which animal models are utilized are for the evaluation of vaccine safety, immunoge-

nicity and efficacy. The following sections describe the use of animal models in each of these aspects of the pre-clinical evaluation of influenza vaccines.

Safety

Early in the days of clinical testing of live attenuated vaccines against seasonal influenza, it was recognized that an animal model that could predict the attenuation of these vaccines would allow progression to immunogenicity and efficacy testing to occur more rapidly. Ideally, systematic comparisons of the behavior of attenuated virus vaccine candidates in animal models and in humans are needed to achieve this end. Researchers began to address this question in the late 1970s and early 1980s, and the infant rat was extensively investigated as a model to predict the restriction of replication of live attenuated influenza vaccines in humans [42–44]. In general, attenuation in the infant rat model correlated with attenuation in humans, although there were exceptions. Other species evaluated for this purpose include mice, hamsters, ferrets and chimpanzees.

Although vaccine safety can only be fully assessed when a vaccine is administered to human subjects, regulatory authorities usually recommend standard tests for pre-clinical evaluation of the safety of new vaccine candidates. The primary safety concern for inactivated influenza virus vaccines is reactogenicity, and for live attenuated influenza vaccines, it is the level of attenuation and genetic stability. Standard toxicology tests on new vaccine candidates are often performed in rabbits, although current WHO guidelines for nonclinical evaluation of vaccines recommend that toxicology studies be performed in an animal species that most closely reflects the immune response to the vaccine in humans, or is 'sensitive to the biological effects of the vaccine', using the dose and route of administration to be studied in clinical trials [58]. The design and results of such studies should be reviewed with special attention to experimental details such as the route of administration, volume and quantity of virus in the inoculum, and whether or not anesthesia was used, particularly for live attenuated vaccines, because each of these factors can influence the outcome. Toxicity following administration of very high doses of live influenza virus to animals *via* a variety of routes has been reported in the literature. For example, administration of 10^9 EID_{50} of influenza virus administered intranasally resulted in complete pulmonary consolidation and death in mice, and this pathology occurred despite restricted replication of virus in lung tissue [59]. Henle and Henle [60] reported inflammation in the gut, damage to the liver and spleen, and death in mice given high doses of influenza virus intraperitoneally. Similar findings were observed in rats, rabbits and guinea pigs. Lung inflammation was observed in ferrets administered high titer live attenuated influenza viruses intranasally [60a] and systemic signs of illness were reported in human volunteers who received attenuated influenza viruses at doses that exceeded 10^7 $TCID_{50}$

[61–63]. In these studies, signs of clinical illness, including fever and other systemic signs, appeared within 48 h of administration of the virus, which is more rapid in general than the appearance of symptoms associated with productive influenza virus infection. The systemic symptoms did not correlate with the titer of virus shed in respiratory secretions, or with the occurrence of respiratory symptoms. The occurrence of systemic illness in humans following administration of high doses of influenza virus in the absence of high levels of virus replication may be explained by the innate immune response to an abortive infection of epithelial cells.

The current procedures for marketing approval of vaccines for seasonal influenza do not involve extensive safety testing in animals. In the US, a standard general safety test, which is designed to detect extraneous toxic components in the vaccine preparation, is usually performed with the final drug product in mice and guinea pigs [64]. This test is performed for both inactivated and live attenuated virus vaccines. For inactivated influenza virus vaccines, vaccine can be administered *via* either the subcutaneous or intraperitoneal routes for the guinea pig test, whereas only the intraperitoneal route can be used for other types of vaccine. The vaccine formulation must also be certified to be free of endotoxin.

New vaccine candidates or novel preparations (including vaccines prepared by currently licensed methodologies that are now formulated with adjuvant), require extensive pre-clinical safety testing. In addition to tests such as repeat dose toxicology testing and general safety testing, some tests would be appropriate for the specific type of vaccine, e.g., demonstration of attenuation of live attenuated vaccines compared to the wild-type parent virus in more than one animal species [16, 65, 66], and biodistribution studies for plasmid-based vaccines [67–71].

Ferrets have been used to assess the attenuation of cold-adapted live attenuated vaccines for influenza [72]. These studies showed that cold-adapted 6-2 reassortant vaccine viruses generated from human influenza viruses failed to replicate in the lower respiratory tract of ferrets. Since ferrets are a good model for influenza infection in humans, they can also be used in toxicological studies of influenza vaccines.

The attenuation phenotype of several live attenuated influenza vaccine candidates was evaluated using the hamster model [35, 37]. For the small number of temperature-sensitive, cold-adapted reassortant influenza viruses tested in hamsters and later in humans, there was generally a correlation between the level of replication in hamsters and humans. However, in studies with AI/human influenza virus reassortants, the findings in hamsters did not accurately predict the level of attenuation of the viruses for humans [73]. Such data are important because they demonstrate that the genetic determinants for attenuation of influenza viruses are different in different species.

Non-human primate species have not been used extensively in studies of the safety of influenza vaccines. Chimpanzees were used in several studies to evaluate the level of attenuation and safety of candidate live attenuated

vaccines [73]. Regulatory authorities in Europe require neurovirulence testing of live attenuated influenza vaccines and inactivated vaccines that are to be administered intranasally [74]. Since influenza viruses are not central nervous system pathogens in humans, the wisdom of such requirements, which were designed to determine the safety of live attenuated vaccines for truly neurotropic viruses such as poliovirus, can be questioned. The neonatal rat was recently proposed as a model in which to study neurovirulence of intranasally administered influenza vaccines [75], and a few influenza strains were evaluated in this model. Some viruses replicated in the brain following intranasal administration, but pronounced lesions or dramatic behavioral changes were not demonstrated in infected animals.

Immunogenicity

The vast majority of studies conducted in animals in influenza vaccine research are those that evaluate the immune response to candidate vaccines. Although it is clear that the immune responses to vaccines in animals are not often identical to and may not be directly predictive of those seen in humans, the first step in the proof-of-principle of a new vaccine is to establish the immunogenicity of a vaccine candidate in animals before proceeding to clinical evaluation. The immune responses measured in the animal model should be relevant to the desired response in humans. Such studies may provide useful information regarding regimen and routes of vaccination to guide the design of clinical trials.

Strain-specific immunity directed against the HA

It is well established that the primary correlate of protection for inactivated whole-virus or subunit influenza vaccines administered parenterally is serum antibody directed against the HA protein. Most studies that are conducted to evaluate immune responses to influenza vaccines are conducted in mice and ferrets. Measurement of antibody responses in animal models is very straightforward, since HAI and neutralizing antibody assays do not require species-specific reagents. Limited studies have been conducted to evaluate the guinea pig as a model to study immunity to influenza virus. Phair and colleagues [39] demonstrated that infection of guinea pigs with unadapted human influenza viruses resulted in resistance to challenge with homologous virus, and that passive transfer of hyperimmune serum to naive guinea pigs also conferred protection against infection. However, the levels of HAI antibody detected in serum following infection were lower than those observed in ferrets or hamsters, and infected guinea pigs did not produce detectable levels of local antibody in nasal secretions. In addition, high levels of nonspecific inhibitors of hemagglutination were present in guinea

pig sera, making measurement of specific HAI antibodies problematic [39]. Phair et al. did, however, demonstrate that guinea pigs exhibited a delayed-type hypersensitivity response to influenza infection that resembled that seen in humans, although this response did not appear to be involved in resistance to infection.

Humoral immune responses to the HA of human influenza viruses and vaccines have been studied extensively in ferrets. Early studies determined that naive ferrets were not protected against influenza infection by vaccination with killed virus [76]. These observations were confirmed in later studies using formalin-inactivated vaccines [77]. However, killed vaccine administered with adjuvant to naive ferrets provided partial protection against infection [78]. Thus, immune responses in the ferret to vaccination with inactivated virus vaccines against human influenza viruses do not appear to be identical to those seen in humans, since humans do not generally require adjuvant to achieve protective levels of HAI antibodies. In contrast to the findings with inactivated influenza viruses, immunization with live influenza virus resulted in protection against subsequent challenge [77]. An explanation for this difference may be that in ferrets, influenza infection is primarily an upper respiratory tract infection, and adjuvant is required to elicit higher levels of serum antibody needed to restrict replication of virus in the upper respiratory tract. Several studies have demonstrated that higher levels of serum antibody are required to provide protection against respiratory viruses in the nose of animals than in the lungs [27–29].

Heterosubtypic immunity

In recent years, and particularly since the emergence of the highly pathogenic H5N1 viruses in Asia in 2003, and the challenges in developing H5N1 vaccines, there has been a resurgence of interest in heterosubtypic immunity – the ability of an immune response elicited by a particular influenza A virus to protect against an influenza A virus of a different subtype. Heterosubtypic immunity against influenza has been demonstrated in a number of studies in mice but the precise mechanism of this immunity is not clear [79–82]. Previously, it was thought that this phenomenon was mediated by cellular immune responses, but recent studies suggest that antibody is the primary mechanism of heterosubtypic immunity [82] and that the diversity of the antibody repertoire is important [83].

Heterosubtypic immunity has also been observed in ferrets [84, 85], although there was some debate as to the length of time that such immunity persists. McLaren and Potter [84] reported that it did not persist beyond 10 weeks after vaccination, but in another study, protection against infection with a heterosubtypic virus was observed 18 months following immunization [86]. In both cases, heterosubtypic immunity did not prevent infection but limited virus replication following challenge.

The utility of the cotton rat model to address the question of heterosubtypic immunity was explored [87]. The endpoints in this study were respiratory rate, virus replication in lungs and nasal tissues, and pulmonary histopathology. A statistically significant reduction in respiratory rate was seen following challenge with A/Wuhan/359/95 (H3N2) in cotton rats that had been immunized with either the homologous virus or with a virus of a different subtype, A/PR/8/34 (H1N1), 4 weeks earlier, compared to non-immunized animals. This reduction in respiratory rate correlated with a statistically significant reduction in virus titers in the lungs and nasal tissues in immunized animals. Cotton rats that were immunized with the heterosubtypic A/PR/8/34 (H1N1) virus had the same extent of alveolitis, interstitial pneumonia and airway debris as non-immune, infected animals, and, like the cotton rats that were immunized with homologous virus, they had more severe early peribronchiolitis than was observed in primary infection. This peribronchiolitis could be indicative of a memory response in the heterosubtypic immune animals. However, heterosubtypic immune cotton rats had less bronchiolar epithelial damage than those animals immunized with homologous virus.

The role of heterosubtypic immunity through prior exposure or vaccination in man, although inferred from retrospective analysis of data from influenza pandemics [88], is extremely complex and cannot be readily determined. Studies in young infants and children in which the effect of pre-existing immunity on replication and immunogenicity of heterosubtypic attenuated influenza viruses suggested that heterosubtypic immunity in humans is weak [89].

Immune responses to other influenza proteins

An approach that is being explored in the development of novel vaccines for influenza is that of universal influenza vaccines that target the conserved proteins of the virus – NP, M1 and M2. A number of modalities such as NP and M DNA vaccines [90–92], baculovirus-expressed recombinant M2 protein [93], M2 peptides [94] and recombinant M2 protein incorporated into hepatitis B core antigen [95–97] have been tested in mice, and prevent death, but not illness, following challenge with heterologous virus. In the case of candidate universal vaccines for influenza, new animal models and assays are needed in which antibody and cellular responses to viral antigens other than the HA and NA can be measured. Since the immune responses to these conserved antigens are not well characterized in humans, at present it is not clear whether these responses are accurately reflected in animal models. Undoubtedly more information will be obtained in this area in the future as candidate universal vaccines are evaluated in clinical trials.

There has also been recent interest in the role of immune responses to the NA component of seasonal vaccines in protection against related subtypes of influenza, including potential pandemic strains [98]. Antibodies to

the NA protein can modulate the severity of influenza illness [99] but the NA content of inactivated influenza virus vaccines is not standardized.

Efficacy

Animal models are also used to evaluate the efficacy of new candidate influenza vaccines. The most commonly used animal models for such studies are mice and ferrets. In mice and ferrets it has been established that antibody against the HA can prevent infection or ameliorate disease following challenge with influenza virus. Reduction in virus titer in the lower respiratory tract following challenge correlates with protection, so quantitative virology is the most relevant measure of vaccine efficacy for vaccines designed to generate antibody responses to the HA. Additional endpoints such as morbidity, mortality and pathological findings may provide supporting evidence of protection from infection and disease. Although demonstration of vaccine efficacy in an animal model is not an absolute requirement in pre-clinical evaluation of a vaccine candidate from a regulatory standpoint, it provides evidence that immune responses to the vaccine are biologically relevant.

Vaccines for pandemic influenza

The direct transmission of HPAI H5N1, H7N7 and low pathogenicity AI (LPAI) H9N2 viruses from birds to humans, associated in many cases with severe morbidity and mortality, has raised concerns about the emergence of a new pandemic virus and has prompted efforts to develop vaccines against AI viruses of pandemic potential. Evaluation and characterization of a suitable animal model for these other influenza virus subtypes is a critical step in the development of such vaccines.

Animal models

In the following section we describe the features of the animal models that have been developed to study AI viruses, and their contributions to the evaluation of pandemic vaccines.

Mice

Mice have been used in pre-clinical studies of inactivated and live attenuated pandemic influenza virus vaccines. Most reports in the literature that describe the characterization of replication, pathogenicity and the immune

response of AI viruses in mice focus on viruses of the H5, H7 and H9 subtypes.

H5N1 viruses and vaccines

Several studies demonstrated that the H5N1 viruses isolated from human cases in Hong Kong in 1997 cause disease and death in mice without prior adaptation [100–102]. The Hong Kong H5N1 viruses isolated from humans in 1997 varied in their ability to cause disease and death in BALB/c mice and generally fell into two distinct groups – those that were highly virulent, and those of low virulence for mice – and one virus (A/HK/156/97) was of intermediate virulence in two of the studies [101, 102], but Gao et al. [100] found this isolate to be one of the most highly virulent in this model. The 50% lethal dose of H5N1 viruses that were highly virulent for mice were 10–1000 times lower than those of low virulence, they replicated to titers up to 1000 times higher in the lungs of mice early in the course of infection, and they replicated in extrapulmonary sites including the brain. Viral antigen was observed by immunohistochemistry in the lungs of mice infected with A/HK/483/97 (H5N1), a highly virulent strain, and A/HK/486/97 (H5N1), a less virulent strain, and was associated with necrotic bronchi. Viral antigen was also observed in both glial cells and neurons in the brain of mice infected with the highly virulent influenza A/HK/483/97 (H5N1) virus, a finding also reported by Gao et al. [100]. In addition, Gao et al. reported the presence of viral antigen in the cardiac myofibers in mice infected with the highly virulent influenza A/HK/483/97 (H5N1) virus. The ability of the H5N1 viruses to replicate and cause disease and death in mice did not correlate with their ability to kill chickens [102], and the relevance of replication of these viruses in extrapulmonary sites in mice to the human disease is not clear, although a general correlation between the level of virulence in mice and the severity and outcome of disease in humans was observed with 11 of 15 viruses evaluated [101]. Dybing and colleagues [103] reported that infection of mice with highly pathogenic H5 AI viruses that were isolated from Scotland [influenza A/ck/Scotland/59 (H5N1)], Italy [influenza A/ck/Italy/1485-330/97 (H5N2)], Queretero [influenza A/ck/Queretero/7653-20/95 (H5N1)] and England [influenza A/tk/England/91 (H5N1)], caused little or no disease in BALB/c mice. HPAI H5N1 influenza viruses isolated from humans in Asia in 2004 caused weight loss, ruffled fur, listlessness and pronounced leukopenia, and were lethal in mice without prior adaptation, and replicated outside the respiratory tract [104]. In the same study, HPAI H5N1 viruses isolated from birds, and a single human isolate, were less virulent for mice.

Lu et al. [102] used the BALB/c mouse model to evaluate the immunogenicity and efficacy of a vaccine for H5N1 influenza based on an antigenically related non-pathogenic AI virus, A/duck/Singapore-Q/F119-3/97

(H5N3). They found that two doses of inactivated vaccine were required to elicit HAI antibody responses of a magnitude that would be protective in human influenza in the majority of vaccinated animals, and that the addition of alum adjuvant resulted in higher levels of HAI antibody and a greater seroconversion rate. These findings generally agreed with the observations made in humans when a similar vaccine was tested in clinical studies: two doses of vaccine were necessary to achieve acceptable levels of antibody, and the addition of adjuvant, in this case MF59 (instead of alum used in the studies in mice), increased the magnitude of the antibody response as well as the seroconversion rate [105–107]. Efficacy of this vaccine in mice was determined by measurement of the level of virus replication in the lungs and protection against lethal challenge with an H5N1 isolate that was highly virulent in mice.

The efficacy of several different H5N1 virus vaccines have been evaluated in mice and in all cases, the vaccines were immunogenic and protective in mice (reviewed in [108]). When tested in Phase I studies in humans, inactivated H5N1 virus vaccines were found to be suboptimally immunogenic, requiring high doses [109, 110] to elicit neutralizing and HAI antibody responses. Administration of whole virion vaccines and inactivated virus vaccines with adjuvant increased immunogenicity in mice, and in humans [109, 111]. It is unclear whether data obtained in mice with pandemic influenza vaccines are predictive of vaccine immunogenicity in humans since pre-clinical data for the specific vaccine formulations that have been tested in humans to date have not been reported.

Cold-adapted live attenuated vaccine candidates against H5N1 AI viruses have been developed, and were found to be immunogenic and to confer protection against challenge with homologous and heterologous wild-type viruses in mice [65, 112, 113]. Some of these vaccines are currently in clinical trials, and so the predictive value of the information gained from studies in mice cannot be fully assessed at this time.

H7 viruses and vaccines

Representative low pathogenicity and highly pathogenic H7 AI viruses from both the Eurasian and North American lineages replicated in mice without prior adaptation [114]. Highly pathogenic H7 viruses demonstrated extrapulmonary spread to the spleen and brain, as has been observed with HPAI H5N1 isolates, although H7 viruses were detected in the brain earlier in infection (day 1 p.i. for H7 and day 4 for H5). de Wit et al. [115] reported that intranasal infection of mice with the non-adapted HPAI A/Netherlands/219/2003 H7N7 virus, that was isolated from a fatal human case, resulted in severe illness indicated by weight loss, lethargy, ruffled fur, and lethality. The rate of loss in body weight was similar over a range of doses of virus between 3×10^3 and 3×10^6 EID_{50}. The virus was detected

in the spleen, liver, kidneys and brain, as well as in the lungs of mice. This model was used for the evaluation of the immunogenicity and efficacy of candidate H7 influenza vaccines [115]. A single dose of an ISCOM vaccine and two doses of a subunit vaccine failed to protect mice against lethal infection with the A/NL/219/2003 (H7N7) virus, with one exception. Mice vaccinated with two doses of 1 μg or 5 μg ISCOM vaccine exhibited a small temporary loss in body weight but otherwise appeared healthy after challenge. Vaccination with two doses of the ISCOM vaccine resulted in at least a 1000-fold reduction in virus replication in the lungs, and near-complete reduction of extrapulmonary replication of challenge virus. However, in all vaccinated mice, virus was still present in the lungs at high titers.

Munster et al. [116] reported that the human HPAI H7N7 viruses A/NL/219/2003 and A/NL/33/2003 both caused lethal infection in mice when administered intranasally at a high dose (dose not specified). At a dose of 5×10^2 $TCID_{50}$, influenza A/NL/219/2003 virus, which was isolated from a fatal human case, resulted in loss of body weight, ruffled fur, lethargy, and respiratory problems from day 2 p.i., and infected mice were euthanized on day 5 p.i., whereas mice infected intranasally with 5×10^2 $TCID_{50}$ of influenza A/NL/33/2003 virus, isolated from a human with conjunctivitis in the same outbreak, no signs of illness or loss in body weight were observed up to day 7 p.i. The influenza A/NL/219/2003 virus replicated to a titer that was more than 1000-fold higher in the lungs of infected mice than influenza A/NL/33/2003 virus, and was isolated from the brain, spleen, liver and kidney of all infected animals. Influenza A/NL/33/2003 virus was isolated from the brain of only one out of three mice, and was not detected from the other organs examined. Histopathological findings for all mice infected with influenza A/NL/219/2003 virus included necrosis and inflammation throughout the respiratory tract that was pronounced in the trachea, and became progressively milder in the bronchi, bronchioles and alveoli, In contrast, lesions in the respiratory tract were only observed in one out of four mice infected with influenza A/NL/33/2003 virus, and were characterized as mild to moderate cell necrosis, with neutrophil infiltrates in the trachea, bronchi and bronchioles. Lesions were not observed upon histopathological examination of brain, heart, spleen, liver or kidneys of mice infected with either virus. Viral antigen expression was limited to the respiratory tract tissues in mice infected with either virus, but was more abundant in mice infected with influenza A/NL/219/2003 virus. Rigoni and colleagues [117] reported that HPAI H7N1 viruses isolated from chickens and ostriches could infect and replicate in mice without adaptation, and were associated with disease signs of varying severity. Bronchitis, tracheitis, alveolitis and brain lesions were observed in mice infected with three HPAI H7N1 influenza viruses. However, the influenza A/ostrich/2332/00 virus caused more severe lesions and spread more rapidly in the lungs and brain than the other two viruses (influenza A/ostrich/984/00 and influenza A/ck/5093/99) [117].

Low pathogenicity H7 viruses replicated to high titers in the upper and lower respiratory tract of mice, but were not lethal, even at high doses. Immunogenicity of these viruses was also evaluated in mice [114].

H9 viruses and vaccines

Human infections with H9N2 AI viruses were first reported in 1999 [118, 119], and, although the illness in the infected individuals was relatively mild, there is still concern over the pandemic potential of H9 viruses because viruses of this subtype are highly prevalent in birds [120–124]. The pathogenicity of human and avian H9 influenza viruses in mice has been studied by several laboratories, with a view to the establishment of an animal model that can be used to study strategies for prevention, including vaccines and antiviral drugs. Some H9 influenza viruses replicate in the respiratory tract of mice without prior adaptation [121, 125–127], but serial passage of the A/quail/Hong Kong/G1/97 (H9N2) virus in mice resulted in an increase in virulence and in extrapulmonary spread and lethality of this virus in intranasally infected mice [125, 126]. Data from different laboratories using the same H9N2 virus to infect mice are not consistent. Some of the factors that can influence the outcome of infection are anesthesia, dose, volume and route of virus administration and passage history. It is difficult to compare studies when complete information is not provided. For example, in studies reported by Lu et al. [127], the human influenza A/Hong Kong/1073/99 (H9N2) virus replicated efficiently in the lungs of mice but failed to cause death or signs of disease, significant weight loss or to spread to extrapulmonary sites. However, Leneva et al. [125] reported that infection of mice with this virus resulted in 40% mortality and significant weight loss in the surviving mice. In these discordant studies, mice were anesthetized with CO_2 [127] or with metofane [125], were infected by the same route using virus that had been propagated in embryonated eggs, at approximately the same dose (10^6 EID_{50}), but inoculum volumes used were not stated in either study, so it is not clear why this virus was lethal in one study and not in the other. Similarly, lethal challenge of mice with the human influenza A/Hong Kong/1073/99 (H9N2) virus was reported as part of a study to determine the efficacy of an M2 liposome vaccine [128], although this virus did not cause disease or lethality in the hands of other investigators [127, 129, 130]. All laboratories delivered virus intranasally to anesthetized mice. However, in the study reported by Ernst et al. [128], mice were anesthetized intraperitoneally with ketamine/xylazine, whereas in the other two studies, inhalational anesthesia was used, which may result in a lighter state of anesthesia.

The mouse model was used to evaluate the level of attenuation and the protective efficacy of a cold-adapted live attenuated H9N2 vaccine candidate bearing the HA and NA from the influenza A/ckHK/G9/97 (H9N2) virus and the internal protein genes from the influenza A/Ann Arbor/6/60

cold-adapted virus [129]. The H9N2 live attenuated vaccine was restricted in replication and protected mice from challenge with homologous and heterologous wild-type H9N2 influenza viruses. This vaccine is being evaluated for safety and immunogenicity in clinical trials.

1918 H1N1 pandemic virus

Like the highly pathogenic H5N1 AI viruses, the fully reconstructed recombinant 1918 H1N1 pandemic influenza virus was highly lethal in mice without prior adaptation [131]. The mean time to death in mice infected intranasally was 4.5 days. However, in contrast to the highly pathogenic H5N1 influenza viruses, this virus was not detected in extrapulmonary tissues. Histopathological findings included necrotizing bronchitis and bronchiolitis, moderate to severe alveolitis and severe peribronchial and alveolar edema.

The mouse model appears to be potentially useful for the evaluation of pandemic influenza vaccines. Most AI viruses studied in mice to date can replicate without adaptation, although the outcome of infection with some AI viruses is clearly different depending not only on the particular virus being studied, but also on the laboratory in which the studies were conducted. It is important that AI viruses continue to be evaluated in mice, using standardized inoculation procedures and doses, with measurement of the same endpoints, so that the utility of this model can be maximized for the evaluation of pandemic influenza vaccines.

Ferrets

H5N1 viruses and vaccines

The ability of a limited number of AI subtypes to replicate and cause disease in ferrets has been investigated and, not surprisingly, the behavior of H5 subtype viruses has been studied in the most detail. Zitzow and colleagues [132] demonstrated that two H5N1 influenza viruses isolated from human cases of infection in Hong Kong in 1997 were capable of replication not only in the respiratory tract, but also in the brain, spleen and intestines of ferrets. Virus replication was associated with clinical signs of disease such as severe lethargy, sneezing, rhinitis, hind limb paresis and, in some cases, diarrhea, and some H5N1 viruses were lethal to ferrets. However, the hierarchy of severity of disease seen with the different H5N1 1997 isolates in infection of mice was not observed in ferrets: influenza A/HK/483/97 and A/HK/486/97 were equally virulent after intranasal infection of ferrets, whereas the A/HK/483/97 virus was more virulent in mice than the A/HK/486/97 virus was in several studies [100–102, 133]. As with mice, the significance for humans of the disease signs and extrapulmonary replication of H5N1 viruses in ferrets is not clear, par-

ticularly since in the same study, Zitzow et al. reported isolation of a human H3N2 influenza virus from the brain of ferrets following intranasal infection. Similar studies were conducted using human and avian H5N1 viruses isolated in 2004–2005 [104, 134]. Govorkova et al. [134] evaluated four human H5N1 influenza isolates and nine avian H5N1 isolates from Asia from 2004. A wide spectrum of infectivity, severity of disease and lethality was observed in ferrets inoculated with these viruses. The H5N1 viruses isolated from humans, and two of the avian isolates, caused severe disease in ferrets, with some lethality. However, it is difficult to draw general conclusions regarding the behavior of these viruses in this model because small numbers of animals were used (only two animals per group for all but one of the viruses tested), and there was variability in infectivity. For example, although the influenza A/Vietnam/3046/2004 virus caused severe disease in two out of two ferrets inoculated, it was lethal in only one animal, and virus was only recovered from the nasal washes. In contrast, the influenza A/Vietnam/3062/2004 virus, which was also lethal in one out of two ferrets inoculated, was recovered from the lungs, brain, spleen and intestine of these animals. Similarly, Maines et al. [104] evaluated H5N1 isolates from Asia from 2004 in the ferret model. Although different viruses were used in this study compared to that conducted by Govorkova et al. (with the exception of A/Vietnam/1203/2004), similar findings were reported: the human isolates caused severe disease, with some lethality, in ferrets. Again, small numbers of animals were used (three per group for most of the isolates tested) and some variability in infectivity and severity of disease was observed. In the study conducted by Zitzow et al., gross pathological changes observed in ferrets infected with highly virulent HPAI H5N1 viruses included focal areas of redness in the lungs, consolidation of the lungs and rare discoloration of the liver, petechiae on the liver and lesions on the intestines and kidneys [132]. Maines et al. [104] reported the presence of hemorrhage in the adipose tissue surrounding the liver, kidney and bladder in two thirds of infected ferrets. Histopathological findings in the lungs of infected ferrets included acute bronchiolitis, bronchopneumonia, interstitial pneumonia, with suppurative exudates in the bronchi, bronchioles and adjacent alveolar spaces, with prominent epithelial necrosis and marked intraalveolar edema, by day 3 p.i., and bronchitis, bronchiolitis and pneumonia observed on days 6–7 p.i. [104, 132, 134]. Inflammatory changes were also evident in the brain of ferrets infected with highly virulent HPAI H5N1 viruses, from days 5–6 p.i., including glial nodules, perivascular infiltration of lymphocytes and polymorphonuclear leukocytes in the brain parenchyma, neuronophagia and lymphocytic infiltrates in the choroid plexus [132, 134]. Viral antigen was observed by immunohistochemistry in neurons in the same areas of the brain as the inflammation [104]. Govorkova et al. [134] reported histopathological changes in the liver, including diffuse vacuolization of the hepatocellular cytoplasm, mononuclear infiltrates, periportal hemorrhage, and hepatocellular necrosis. Generally, the viruses isolated from avian species caused less severe disease than those isolated from humans.

The number of ferrets inoculated with each virus was small and ferrets are an outbred species so the significance of variability in data such as virus replication and clinical illness are difficult to interpret. Until the scientific community has more experience with the behavior of AI viruses in animal models, it would be prudent to compare new isolates with well-characterized strains and to study these pathogens in more than one model.

The ferret model has been used to evaluate the efficacy of several experimental inactivated [135, 136] and live attenuated [65, 112, 113] vaccines against H5N1 influenza. Inactivated H5N1 vaccines were immunogenic and protective in the ferret model [135, 136]. However, inactivated H5N1 vaccines that were tested in clinical trials were suboptimally immunogenic [109, 110]. The attenuation of cold-adapted live attenuated H5N1 vaccines was demonstrated in ferrets. These vaccine candidates were also immunogenic and protective against challenge with homologous and heterologous H5N1 wild-type viruses in ferrets [65]. Whether the observations of attenuation and cross-reactive immune responses in ferrets are borne out in clinical studies in humans remains to be seen. Clinical evaluation of the safety and immunogenicity of these vaccines is currently underway.

Protection from lethal H5N1 infection and level of replication of the challenge virus in the lungs and other tissues are the endpoints used for evaluation of efficacy in this model. Van Riel et al. [137] demonstrated that the pattern of attachment of H5N1 influenza human isolates in the respiratory tract of ferrets was similar to that seen in the human respiratory tract, whereby virus attached predominantly to type II pneumocytes, alveolar macrophages and nonciliated cuboidal epithelial cells of the terminal bronchioles in the lower respiratory tract, and became progressively rarer more proximally in the respiratory tract, towards the trachea. This pattern of H5N1 virus attachment predominantly to the lower respiratory tract is thought to be related to the distribution of α-2,3 sialic acid receptors [138]. However, other investigators found that H5N1 influenza viruses were able to infect *ex vivo* cultures of the human upper respiratory tract, i.e., nasopharyngeal, adenoid and tonsillar tissues, despite a lack of α-2,3 sialic acid receptors in these tissues [139]. The tropism of H5N1 influenza viruses in the respiratory tract of humans and other species remains equivocal and further studies in which a number of different isolates are evaluated in larger numbers of animals are needed.

Other AI subtypes

There are only isolated reports in the literature that describe the replication and clinical signs resulting from infection of ferrets with other AI subtypes. Hinshaw et al. [140] demonstrated that AI viruses of the H2, H3, H6, H7 and H10 subtypes, as well as an H7N7 virus isolated from a seal, replicated in the upper respiratory tract of ferrets, but elicited low or undetectable

levels of antibody. None of these AI isolates tested caused signs of disease in infected ferrets.

Joseph et al. [114] evaluated the immunogenicity of H7 AI viruses in ferrets and demonstrated that the pattern of antigenic relatedness of the viruses studied was similar to that observed in mice.

1918 H1N1 pandemic virus

The reconstructed 1918 H1N1 influenza virus replicated to high titers in the upper respiratory tract of ferrets following intranasal inoculation [141]. All inoculated ferrets exhibited severe signs of disease that included lethargy, anorexia, sneezing, rhinorrhea, severe weight loss and high fever from day 2 p.i., and two out of three animals succumbed to infection by day 11. Unlike the highly pathogenic H5N1 viruses in ferrets, viral replication was not detected in tissues outside the respiratory tract. Necrotizing bronchiolitis, moderate to severe alveolitis and edema were observed in the lungs of infected ferrets on day 3 p.i. The presence of viral antigen in the upper and lower portions of the bronchi, bronchial and bronchiolar epithelium and hyperplasic epithelium within the alveoli was observed.

Cats

There are few reports in the literature on influenza infection in cats. In studies conducted by Paniker and Nair in the 1970s [142, 143], intranasal infection of anesthetized cats with influenza A/Hong/Kong/1968 (H3N2) virus freshly isolated from human cases or laboratory- and egg-adapted isolates did not result in clinical signs of influenza but virus was recovered from pharyngeal secretions, and infection induced HAI antibodies and was transmitted to contact animals. Infected cats did not display clinical signs of influenza. Hinshaw and colleagues [140] later demonstrated that intranasally administered H7N7 and H7N3 AI viruses replicated in the upper respiratory tract of cats without clinical signs of disease, and the cats developed HAI antibodies after infection.

H5N1 AI viruses

There was little interest in influenza infection and immunity in cats until the recent re-emergence of highly pathogenic avian H5N1 viruses in Asia, when it was reported that a number of big cats, namely tigers and leopards, in zoos in Thailand, became infected with HPAI H5N1 viruses, apparently after they were fed infected chicken carcasses [9]. Infection in many of these felids was fatal, and later anecdotal reports of H5N1 infection in domestic

cats in areas where there were outbreaks of H5N1 infection in avian populations contributed to a surge in interest in H5N1 influenza in cats. The pattern of attachment of a human H5N1 influenza virus to respiratory tract tissues of a cat was similar to that seen with human tissue [137].

Experimental infection of European short haired cats with an H5N1 virus isolated from a human in Vietnam in 2004 resulted in clinical disease, virus replication in respiratory and extra-pulmonary tissues, and pathological changes consistent with H5N1 infections in humans [10, 144]. Clinical signs, including significant elevation in body temperature, decreased activity, conjunctivitis and labored breathing were seen in experimentally infected cats that were infected intratracheally or by feeding on infected chicks [10]. Similar disease symptoms were observed in sentinel cats that became infected from being housed with cats that had been infected intratracheally. Illness in contact cats became apparent about 3 days later than in the cats infected *via* the intratracheal route. Peak viral titers in throat swabs of intratracheally infected cats were ~ $10^{4.5}$ $TCID_{50}$/ml, whereas the peak titers observed in nasal swabs ranged from $10^{2.5}$ to $10^{5.0}$ $TCID_{50}$/ml [144]. Virus was also recovered from rectal swabs of cats infected by feeding on infected chicks, but the titers of virus in these samples varied widely. In addition, cats infected through feeding had lesions in the intestines. In animals infected intratracheally or by feeding, virus was also recovered from extra-pulmonary tissues, most often from the brain, liver, kidney and heart. Infected sentinel cats did not have detectable virus in tissues outside the respiratory tract; however, pathological changes were observed in the adrenal glands in one of the two sentinel cats infected in this manner. These studies demonstrated that HPAI H5N1 viruses are capable of extrapulmonary spread in cats, and can cause severe disease and even death in animals infected intratracheally or by feeding on infected bird carcasses. The observations also raise the possibility that HPAI H5N1 influenza in cats may be spread from the gastrointestinal tract.

Karaca et al. [145] reported studies on the immunogenicity of a fowlpox-based H5 vaccine in cats. HAI antibodies were detected in serum of cats following a single subcutaneous dose of vaccine, and a significant boost in antibody titers was observed following a second vaccination.

It remains to be seen whether cats will be used extensively in the evaluation of pandemic influenza vaccines.

Hamsters

H9 viruses and vaccines

Saito and colleagues conducted a study to evaluate the replication and pathogenicity of influenza viruses of various subtypes in Syrian hamsters [146]. The influenza A/HK/1073/99 (H9N2) virus replicated to high titers in

the lungs, but was not lethal to hamsters and was not detected in the brain. The HPAI H5N1 influenza A/HK/483/97 virus that was highly virulent in mice was also lethal in hamsters, with all animals succumbing to infection by day 6 p.i., and, as in mice, virus was recovered from the brain of infected hamsters. Avian H9N2 and H9N5 isolates replicated in the lungs of hamsters, but to lower titers than human isolates. The human H9N2 virus elicited low levels of neutralizing antibody in infected hamsters, whereas the avian H9N2 isolate did not elicit detectable neutralizing antibody. The behavior of this limited number of AI isolates in the Syrian hamster model suggest that these viruses may be similar to that observed in mice, and further evaluation of this model for evaluating the efficacy of pandemic influenza vaccines is warranted.

Non-human primates

There is renewed interest in the use of non-human primates for immunogenicity studies for pandemic vaccines, based on the presumption that immune responses in these animals, having a closer evolutionary relationship to humans, may be more predictive of the responses in humans than smaller animals like mice and ferrets. To date, there are few data available on the serological responses in non-human primates to AI virus vaccines.

H5N1 AI viruses

The use of cynomolgus macaques as a model for influenza virus infection in humans was revisited following the emergence of the highly pathogenic H5N1 AI viruses in 1997 [147]. The initial human H5N1 influenza isolate, A/Hong Kong/156/1997, isolated from a fatal case of influenza in a child [7], was inoculated at multiple sites, including the trachea, tonsils and conjunctiva. Three of four animals developed fever within 2 days, and one showed signs of anorexia and acute respiratory distress. High titers of virus were recovered from lungs on day 4 p.i., and virus was also isolated from the trachea, tracheobronchial lymph nodes and heart. Virus was not recovered from these tissues on day 7 p.i. Virus was also recovered from bronchioalveolar lavage from two out of two animals on days 3 and 5 p.i.; from pharyngeal swabs from two animals on day 5 p.i., and from nasal swabs from one animal on days 3 and 7. Viral RNA was detected in the brains of two animals by RT-PCR on day 4 p.i., and in the spleen of all four animals tested on day 7 p.i. Pathological changes in the lungs of infected animals included pulmonary consolidation, necrotizing broncho-interstitial pneumonia and flooding of alveoli with edema fluid, fibrin, erythrocytes, cell debris, macrophages and neutrophils and inflammatory changes were seen in multiple organs [148].

Infection of Rhesus macaques with avian H5N1 isolates reported by Chen et al. [149] indicated that results of intranasal inoculation varied depending on the influenza virus isolate used. Clinical signs of infection, including elevation in body temperature, anorexia and increased respiratory rate were observed in macaques inoculated with the following H5N1 viruses: A/bar-headed goose/Qinghai/1/2005, A/great cormorant/Qinghai/3/2005 and A/duck/Guangxi/35/2001. Pathological changes were seen in the lungs of all infected animals, but were more pronounced in the monkeys inoculated with the duck isolate. However, the only virus to be re-isolated from infected animals was A/duck/Guangxi/35/2001, and this virus was isolated from respiratory tract secretions and tissues and also from spleen, liver and heart.

1918 H1N1 pandemic virus

Cynomolgus macaques were evaluated as a model for the reconstructed 1918 H1N1 pandemic influenza virus [150]. Monkeys were infected by multiple routes – intratracheally, orally, on the tonsils and conjunctiva – based on the earlier studies with HPAI H5N1 influenza viruses in this species [147]. Animals infected with the reconstructed 1918 virus had severe clinical illness, high levels of virus replication in the respiratory tract and severe pathological changes in the lungs, compared to control animals infected with a recombinant human H1N1 influenza virus, A/Kawasaki/173/01 [150].

There may be a place for non-human primates in the evaluation of pandemic influenza vaccines, but the currently available data are not sufficient to support the use of these models for immunogenicity or efficacy studies. Further studies are needed to characterize AI infection and the immune responses to AI viruses and vaccines in these species.

Correlates of protection from AI viruses and regulatory concerns

Despite the fact that the correlates of protection from AI virus infections in humans are not known, the criteria for licensing pandemic influenza vaccines are based on the previous human experience with vaccines against seasonal influenza. In Europe and the United States, regulatory authorities have published guidance for vaccine manufacturers that attempt to balance the need for expedited approval of pandemic influenza vaccines with the requirements of demonstration of safety and immunogenicity of candidate vaccines.

In the United States, for example, a guidance for vaccine manufacturers was published in 2007 [151], which states that licensure of both inactivated and live attenuated vaccines for pandemic influenza should be based on the percent of subjects achieving an HAI antibody titer of 1:40 or greater, and upon the rate of seroconversion, which is defined as a fourfold or greater rise

in post-vaccination HAI antibody titer. Efficacy studies in animal models, although not an absolute requirement, may at least provide evidence that biologically relevant immune responses are elicited by candidate vaccines.

This guidance is intended to allow for the rapid marketing approval of pandemic influenza vaccines that are produced using manufacturing processes that are already validated for seasonal influenza vaccines, so that the licensure of the pandemic vaccine is essentially a strain change. Such approval requires much more limited testing of the candidate vaccines in animal models. In the European Union, manufacturers are required to submit information on the production and pre-clinical testing of a 'mock-up' pandemic vaccine. In the event of a pandemic, a vaccine made in the same way as the mock-up vaccine, but based on the nascent pandemic virus, will be produced and will be subject to limited pre-clinical characterization, including immunogenicity studies in animals on at least one batch of the product [152]. Efficacy studies of the actual pandemic vaccine formulation in animals are not required. However, extensive pre-clinical testing of the vaccine candidate is required for new vaccine modalities and formulations, including formulation of approved vaccines with adjuvants.

In the United States, a regulatory mechanism was introduced under what is commonly referred to as the 'animal rule' [153] for marketing approval of vaccines for which efficacy studies in healthy human volunteers are either unethical or not feasible. This regulation stipulates that, in cases where efficacy of vaccines in humans cannot be definitively determined, marketing approval for a vaccine may be granted based on 'adequate and well-controlled animal studies' providing that the basis for vaccine efficacy is reasonably well understood, and that the animal responds to the vaccine in a manner that is predictive for humans. Studies in more than one animal species would typically be required, unless a single animal model is available that faithfully predicts efficacy in humans. It is unclear at this time whether this rule will eventually be applied to vaccines for pandemic influenza. In any event, it is critical that the predictive value of the available animal models for immunogenicity and efficacy of pandemic influenza vaccines be determined systematically using the same vaccine formulations that are progressing into clinical studies.

Conclusion

Although several animal species support the replication of human and AI viruses, a survey of the literature leads to the conclusion that there is no single ideal animal model for the evaluation of influenza vaccines. Some animal models are more suitable than others to predict the attenuation of live virus vaccines, or more closely reflect the human immune response to vaccines. Animal models certainly play a crucial role in the evaluation of influenza vaccines, but the limitations of the models must be taken into account

when decisions are made regarding which vaccine candidates should move forward into clinical trials.

The evaluation of vaccines for pandemic influenza presents additional challenges in that the correlates of protection from AI viruses are not known, and so there may be a greater need for reliance on data from animal studies for these vaccines. It is critical that the behavior of AI viruses with pandemic potential be characterized in a range of animal models. Even from limited observations it is clear that replication of AI viruses and their ability to cause disease in animals depends on the host species, and is subtype and even strain specific. Therefore, pre-clinical safety, immunogenicity and efficacy data from animal studies must be carefully considered in the evaluation of pandemic influenza vaccines.

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References

1 Palese P, Shaw ML (2007) Orthomyxoviridae: The viruses and their replication. In: DM Knipe, PM Howley, DE Griffin, RA Lamb, MA Martin, B Roizman, SE Straus (eds): *Field Virology* (5th ed), Lippincott Williams and Wilkins, Philadelphia, 1647–1689

2 Wright PF, Neumann G, Kawaoka Y (2007) Orthomyxoviruses. In: DM Knipe, PM Howley, DE Griffin, RA Lamb, MA Martin, B Roizman, SE Straus (eds): *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, 1691–1740

3 Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 79: 2814–2822

4 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of Influenza A viruses. *Microbiol Rev* 56: 152–179

5 Cox NJ, Subbarao K (2000) Global epidemiology of influenza: Past and present. *Annu Rev Med* 51: 407–421

6 Meltzer MI, Cox NJ, Fukuda K (1999) The economic impact of pandemic influenza in the United States: Priorities for intervention. *Emerg Infect Dis* 5: 659–671

7 Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J et al (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279: 393–396

8 Kawaoka Y, Krauss S, Webster RG (1989) Avian-to-human transmission of the

PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol* 63: 4603–4608
9 Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theamboonier A, Tantilertcharoen R et al (2004) Avian influenza H5N1 in tigers and leopards. *Emerg Infect Dis* 10: 2189–2191
10 Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, Osterhaus A (2004) Avian H5N1 influenza in cats. *Science* 306: 241
11 Couch RB, Kasel JA (1983) Immunity to influenza in man. *Annu Rev Microbiol* 37: 529–549
12 Hobson D, Curry RL, Beare AS, Ward-Gardner A (1972) The role of serum hemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg* 70: 767–777
13 Treanor J, Wright PF (2003) Immune correlates of protection against influenza in the human challenge model. In: F Brown, LR Haaheim, GC Schild (eds): *Laboratory Correlates of Immunity to Influenza – A Reassessment*. Karger, Basel, 97–104
14 Clements ML, Murphy BR (1986) Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. *J Clin Microbiol* 23: 66–72
15 Gorse GJ, O'Connor TZ, Newman FK, Mandava MD, Mendelman PM, Wittes J, Peduzzi PN (2004) Immunity to influenza in older adults with chronic obstructive pulmonary disease. *J Infect Dis* 190: 11–19
16 Murphy BR, Coelingh K (2002) Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. *Viral Immunol* 15: 295–323
17 Smith W, Andrewes CH, Laidlaw PP (1933) A virus obtained from influenza patients. *Lancet* II: 66–68
18 Andrewes CH, Laidlaw PP, Smith W (1934) The susceptibility of mice to the viruses of human and swine influenza. *Lancet* II: 859–862
19 Brown EG (1990) Increased virulence of a mouse-adapted variant of Influenza A/FM/1/47 virus is controlled by mutations in genome segments 4, 5, 7 and 8. *J Virol* 64: 4523–4533
20 Brown EG, Liu H, Chang Kit L, Baird S, Nesrallah M (2001) Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: Identification of functional themes. *Proc Natl Acad Sci USA* 98: 6883–6888
21 Smeenk CA, Brown EG (1994) The Influenza virus variant A/FM/1/47–MA possesses single amino acid replacements in the hemagglutinin, controlling virulence, and in the matrix protein, controlling virulence as well as growth. *J Virol* 68: 530–534
22 Smeenk CA, Wright KE, Burns BF, Thaker AJ, Brown EG (1996) Mutations in the hemagglutinin and matrix genes of a virulent influenza virus variant, A/FM/1/47–MA, control different stages in pathogenesis. *Virus Res* 44: 79–95
23 Grimm D, Staeheli P, Hufbauer M, Koerner I, Martinez-Sobrido L, Solorzano A, Garcia-Sastre A, Haller O, Kochs G (2007) Replication fitness determines

high virulence of influenza A virus in mice carrying functional Mx resistance gene. *Proc Natl Acad Sci USA* 104: 6806–6811
24 Epstein SL, Lo CY, Misplon JA, Lawson CM, Hendrickson BA, Max EE, Subbarao K (1997) Mechanisms of heterosubtypic immunity to lethal influenza A virus infection in fully immunocompetent, T cell-depleted, β2-microglobulin-deficient, and J chain-deficient mice. *J Immunol* 158: 1222–1230
25 Tumpey TM, Szretter KJ, Van Hoeven N, Katz JM, Kochs G, Haller O, Garcia-Sastre A, Staeheli P (2007) The *Mx*1 gene protects mice against pandemic 1918 and highly lethal human H5N1 influenza viruses. *J Virol* 81: 10818–10821
26 Virelizier J (1975) Host defenses against influenza virus: The role of anti-hemagglutinin antibody. *J Immunol* 115: 434–439
27 Ramphal R, Cogliano RC, Shands JWJ, Small PAJ (1979) Serum antibody prevents lethal murine influenza pneumonitis but not tracheitis. *Infect Immun* 25: 992–997
28 Takiguchi K, Sugawara K, Hongo S, Nishimura H, Kitame F, Nakamura K (1992) Protective effect of serum antibody on respiratory infection of influenza C virus in rats. *Arch Virol* 122: 1–11
29 Prince GA, Horswood RL, Chanock RM (1985) Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. *J Virol* 55: 517–520
30 Subbarao K, McAuliffe J, Vogel L, Fahle G, Fischer S, Tatti K, Packard M, Shieh WJ, Zaki S, Murphy B (2004) Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J Virol* 78: 3572–3577
31 Iida T, Bang FB (1963) Infection of the upper respiratory tract of mice with influenza virus. *Am J Hyg* 77: 169–176
32 Yetter RA, Lehrer S, Ramphal R, Small PAJ (1980) Outcome of influenza infection: Effect of site of initial infection and heterotypic immunity. *Infect Immun* 29: 654–662
33 Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O (1988) Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol* 8: 4518–4523
34 Staeheli P, Haller O, Boll W, Lindenmann J, Weissmann C (1986) Mx protein: Constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. *Cell* 44: 147–158
35 Abou-Donia H, Jennings R, Potter CW (1980) Growth of influenza A viruses in hamsters. *Arch Virol* 65: 99–107
36 Heath AW, Addison C, Ali M, Teale D, Potter CW (1983) *In vivo* and *in vitro* hamster models in the assessment of virulence of recombinant influenza viruses. *Antiviral Res* 3: 241–252
37 Murphy BR, Wood FT, Massicot JG, Chanock RM (1978) Temperature-sensitive mutants of influenza virus. XVI. Transfer of the two *ts* lesions present in the Udorn/72-*ts*-1A2 donor virus to the Victoris/3/75 wild-type virus. *Virology* 88: 244–251
38 Subbarao EK, Kawaoka Y, Murphy BR (1993) Rescue of an influenza A virus wild-type PB2 gene and a mutant derivative bearing a site-specific temperature-sensitive and attenuating mutation. *J Virol* 67: 7223–7228

39 Phair JP, Kauffman CA, Jennings R, Potter CW (1979) Influenza virus infection of the guinea pig: Immune response and resistance. *Med Microbiol Immunol* 165: 241–254
40 Azoulay-Dupuis E, Lambre CR, Soler P, Moreau J, Thibon M (1984) Lung alterations in guinea-pigs infected with influenza virus. *J Comp Pathol* 94: 273–283
41 Lowen AC, Mubareka S, Tumpey TM, Garcia-Sastre A, Palese P (2006) The guinea pig as a transmission model for human influenza viruses. *Proc Natl Acad Sci USA* 103: 9988–9992
42 Ali M, Maassab HF, Jennings R, Potter CW (1982) Infant rat model of attenuation for recombinant influenza viruses prepared from cold-adapted attenuated A/Ann/Arbor/6/60. *Infect Immun* 38: 610–619
43 Mahmud MIA, Jennings R, Potter CW (1979) The infant rat as a model for assessment of the attenuation of human influenza viruses. *J Med Microbiol* 12: 43–54
44 Teh C, Jennings R, Potter CW (1980) Influenza virus infection of newborn rats: Virulence of recombinant strains prepared from influenza virus strain A/Okuda/57. *J Med Microbiol* 13: 297–306
45 Niewiesk S, Prince G (2002) Diversifying animal models: The use of hispid cotton rats (*Sigmodon hispidus*) in infectious diseases. *Lab Anim* 36: 357–372
46 Sadowski W, Wilczynski J, Semkow R, Tulimowska M, Krus S, Kantoch M (1987) [The cotton rat (*Sigmodon hispidus*) as an experimental model for studying viruses in respiratory tract infections. II. Influenza viruses types A and B]. *Med Dosw Mikrobiol* 39: 43–55
47 Ottolini MG, Blanco JC, Eichelberger MC, Porter DD, Pletneva L, Richardson JY, Prince GA (2005) The cotton rat provides a useful small-animal model for the study of influenza virus pathogenesis. *J Gen Virol* 86: 2823–2830
48 Burnet FM (1941) Influenza virus "A" infections of cynomolgus monkeys. *Aust J Exp Biol Med Sci* 19: 281–290
49 Saslaw S, Wilson HE, Doan CA, Woolpert OC, Schwab JL (1946) Reactions of monkeys to experimentally induced influenza virus A infection. An analysis of the relative roles of humoral and cellular immunity under conditions of optimal or deficient nutrition. *J Exp Med* 84: 113–125
50 Rimmelzwaan GF, Baars M, van Beek R, Van Amerongen G, Lovgren-Bengtsson K, Claas ECJ, Osterhaus ADME (1997) Induction of protective immunity against influenza virus in a macaque model: Comparison of conventional and ISCOM vaccines. *J Gen Virol* 78: 757–765
51 Baskin CR, Garcia-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villan E, Katze MG (2004) Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). *J Virol* 78: 10420–10432
52 Murphy BR, Lewis Sly D, Hosier NT, London WT, Chanock RM (1980) Evaluation of three strains of influenza A virus in humans and in owl, cebus and squirrel monkeys. *Infect Immun* 28: 688–691
53 Murphy BR, Hinshaw VS, Lewis Sly D, London WT, Hosier NT, Wood FT, Webster RG, Chanock RM (1982) Virulence of avian influenza A viruses for squirrel monkeys. *Infect Immun* 37: 1119–1126

54 Snyder MH, Clements ML, Herrington D, London WT, Tierney EL, Murphy BR (1986) Comparison by studies in squirrel monkeys, chimpanzees, and adult humans of avian-human Influenza A virus reassortants derived from different avian influenza virus donors. *J Clin Microbiol* 24: 467–469

55 Grizzard MB, London WT, Sly DL, Murphy BR, James WD, Parnell WP, Chanock RM (1978) Experimental production of respiratory tract disease in cebus monkeys after intratracheal or intranasal infection with influenza A/Victoria/3/75 or influenza A/New Jersey/76 virus. *Infect Immun* 21: 201–205

56 Murphy BR, Hall SL, Crowe J, Collins PL, Subbarao EK, Connors M, London WT, Chanock RM (1992) The use of chimpanzees in respiratory virus research. In: J Erwin, JC Landon (eds): *Chimpanzee Conservation and Public Health: Environments for the Future.* Diagnon/Bioqual, Rockville

57 Snyder MH, London WT, Tierney EL, Maassab HF, Murphy BR (1986) Restricted replication of a cold-adapted reassortant influenza A virus in the lower respiratory tract of chimpanzees. *J Infect Dis* 154: 370–371

58 WHO (2005) *WHO Guidelines on nonclinical evaluation of vaccines.* Annex 1, World Health Organization

59 Sugg JY (1949) An Influenza virus pneumonia of mice that is nontransferable by serial passage. *J Bacteriol* 57: 399–403

60 Henle W, Henle G (1946) Studies on the toxicity of influenza viruses. II. The effect of intra-abdominal and intravenous injection of influenza viruses. *J Exp Med* 84: 639–661

60a Jin H, Manetz S, Leininger J, Luke C, Subbarao K, Murphy B, Kemble G, Coelingh KL (2007) Toxicological evaluation of live attenuated cold-adapted H5N1 vaccines in ferrets. *Vaccine* 25: 8664–8672

61 Betts RF, Douglas GRJ, Maassab HF, DeBorde DC, Clements ML, Murphy BR (1988) Analysis of virus and host factors in a study of A/Peking/2/79 (H3N2) cold-adapted vaccine recombinant in which vaccine-associated illness occurred in normal volunteers. *J Med Virol* 26: 175–183

62 Murphy BR, Holley HP, Berquist EJ, Levine MM, Spring SB, Maassab HF, Kendal AP, Chanock RM (1979) Cold-adapted variants of influenza A virus: Evaluation in adult seronegative volunteers of A/Scotland/840/74 and A/Victoria/3/75 cold-adapted recombinants derived from the cold-adapted A/Ann Arbor/6/60 strain. *Infect Immun* 23: 253–259

63 Okuno Y, Nakamura K, Yamamura T, Takahashi M, Toyoshima K, Kunita N, Sugai T, Fujita T (1960) Studies on attenuation of influenza virus. *Proc Jpn Acad* 36: 299–303

64 Center for Biologics Evaluation and Research (CBER), 21 CFR PART 610 General Biological Products Standards (Food and Drug Administration)

65 Suguitan AL Jr, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K (2006) Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med* 3: e360

66 WHO (2003) *Production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses*. Interim biosafety risk assessment. World Health Organization

67 Gonin P, Gaillard C (2002) Gene transfer vector biodistribution: Pivotal safety studies in clinical gene therapy development. *Gene Ther* 11: S98–S108
68 Leamy VL, Martin T, Mahajan R, Vilalta A, Rusalov D, Hartikka J, Bozoukova V, Hall KD, Morrow J, Rolland AP et al (2006) Comparison of rabbit and mouse models for persistence analysis of plasmid-based vaccines. *Hum Vaccines* 2: 113–118
69 Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, Harper LB, Schock HB, Zhang H, Faris JE et al (2002) Plasmid DNA vaccines: Assay for integration into host genomic DNA. *Dev Biol* 104: 33–43
70 Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, Griffiths TG, Niu Z, Denisova L, Follmer TT et al (2000) Plasmid DNA vaccines: Tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirology* 43: 273–281
71 Winegar RA, Monforte JA, Suing KD, O'Loughlin KG, Rudd CJ, Macgregor JT (1996) Determination of tissue distribution of an intramuscular plasmid vaccine using PCR and in situ DNA hybridization. *Hum Gene Ther* 7: 2185–2194
72 Maassab HF, Kendal AP, Abrams GD, Monto AS (1982) Evaluation of a cold-recombinant influenza virus vaccine in ferrets. *J Infect Dis* 146: 780–790
73 Murphy BR, Sly DL, Tierney EL, Hosier NT, Massicot JG, London WT, Chanock RM, Webster RG, Hinshaw VS (1982) Reassortant virus derived from avian and human influenza A viruses is attenuated and immunogenic in monkeys. *Science* 218: 1330–1332
74 EMEA (2003) *Points to Consider on the Development of Live Attenuated Influenza Vaccines.* European Agency for the Evaluation of Medicinal Products, CPMP/BWP/2289/01
75 Rubin SA, Liu D, Pletnikov M, McCullers JA, Ye Z, Levandowski RA, Johannessen J, Carbone KM (2004) Wild-type and attenuated influenza virus infection of the neonatal rat brain. *J Neurovirol* 10: 305–314
76 Smith W, Andrewes CH, Laidlaw PP (1935) Influenza: Experiments on the immunization of ferrets and mice. *Br J Exp Pathol* 16: 291–302
77 Potter CW, Oxford JS, Shore SL, McLaren C, Stuart-Harris CH (1972) Immunity to influenza in ferrets. I. Response to live and killed virus. *Br J Exp Pathol* 53: 153–167
78 Potter CW, Shore SL, McLaren C, Stuart-Harris CH (1972) Immunity to influenza in ferrets. 2. Influence of adjuvants on immunization. *Br J Exp Pathol* 53: 168–179
79 Benton KA, Misplon JA, Lo CY, Brutkiewicz RR, Prasad SA, Epstein SL (2001) Heterosubtypic immunity to Influenza A virus in mice lacking IgA, all Ig, NKT cells or gd T cells. *J Immunol* 166: 7437–7445
80 Epstein SL, Lo CY, Misplon JA, Bennink JR (1998) Mechanism of protective immunity against influenza virus infection in mice without antibodies. *J Immunol* 160: 322–327
81 Epstein SL, Lo CY, Misplon JA, Lawson CM, Hendrickson BA, Max EE, Subbarao K (1997) Mechanisms of heterosubtypic immunity to lethal influenza A virus infection in fully immunocompetent, T cell-depleted, β2-microglobulin-deficient, and J chain-deficient mice. *J Immunol* 158: 1222–1230
82 Nguyen HH, van Ginkel FW, Vu HL, McGhee JR, Mestecky J (2001)

Heterosubtypic immunity to influenza A virus infection requires B cells but not $CD8^+$ cytotoxic T lymphocytes. *J Infect Dis* 183: 368–376

83 Nguyen HH, Zemlin M, Ivanov II, Andrasi J, Zemlin C, Vu HL, Schelonka R, Schroeder HWJ, Mestecky J (2007) Heterosubtypic immunity to influenza A virus infection requires a properly diversified antibody repertoire. *J Virol* 81: 9331–9338

84 McLaren C, Potter CW (1974) Immunity to influenza in ferrets. VII. Effect of previous infection with heterotypic and heterologous influenza viruses on the response of ferrets to inactivated influenza virus vaccines. *J Hyg* 72: 91–100

85 McLaren C, Potter CW, Jennings R (1974) Immunity to influenza in ferrets. X. Intranasal immunization of ferrets with inactivated influenza A virus vaccines. *Infect Immun* 9: 985–990

86 Yetter RA, Barber WH, Small PAJ (1980) Heterotypic immunity to influenza in ferrets. *Infect Immun* 29: 650–653

87 Straight TM, Ottolini MG, Prince GA, Eichelberger MC (2006) Evidence of a cross-protective immune response to influenza A in the cotton rat model. *Vaccine* 24: 6264–6271

88 Epstein SL (2006) Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957. *J Infect Dis* 193: 49–53

89 Steinhoff MC, Fries LF, Karron RA, Clements ML, Murphy BR (1993) Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. *J Clin Microbiol* 31: 836–838

90 Epstein SL, Tumpey TM, Misplon JA, Lo CY, Cooper LA, Subbarao K, Renshaw M, Sambhara S, Katz JM (2002) DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. *Emerg Infect Dis* 8: 796–801

91 Okuda K, Ihata A, Watabe S, Okada E, Yamakawa T, Hamajima K, Yang J, Ishii N, Nakazawa M, Okuda K et al (2001) Protective immunity against influenza A virus induced by immunization with DNA plasmid containing influenza M gene. *Vaccine* 19: 3681–3691

92 Ulmer J, Donnelly J, Parker S, Rhodes G, Felgner P, Dwarki V, Gromkowski S, Deck R, DeWitt C, Friedman A et al (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259: 1745–1749

93 Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ (1995) Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine* 13: 1399–1402

94 Fan J, Liang X, Horton M, Perry HC, Citron MP, Heidecker GJ, Fu TM, Joyce J, Przysiecki CT, Keller PM et al (2004) Preclinical study of influenza virus A M2 peptide conjugate in mice, ferrets, and rhesus monkeys. *Vaccine* 22: 2993–3003

95 DeFilette M, Friers W, Martens W, Birkett A, Ramne A, Lowenadler B, Lycke N, Jou WM, Saelens X (2006) Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine* 24: 6597–6601

96 DeFilette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, Resch S, Friers W (2005) Universal influenza A vaccine: Optimization of M2-based constructs. *Virology* 337: 149–161

97 Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Friers W (1999) A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med* 5: 1157–1163
98 Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, Webby RJ (2007) Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. *PLoS Med* 4: e59
99 Murphy BR, Kasel JA, Chanock RM (1972) Association of serum anti-neuraminidase antibody with resistance to influenza in man. *N Engl J Med* 286: 1329–1332
100 Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y (1999) Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J Virol* 73: 3184–3189
101 Katz JM, Lu X, Tumpey TM, Smith CB, Shaw MW, Subbarao K (2000) Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *J Virol* 74: 10807–10810
102 Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM (1999) A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J Virol* 73: 5903–5911
103 Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML (2000) Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *J Virol* 74: 1443–1450
104 Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P et al (2005) Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J Virol* 79: 11788–11800
105 Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: A randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 357: 1937–1943
106 Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, Ypma E, Zambon M (2003) Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine* 21: 1687–1693
107 Stephenson I, Nicholson KG, Gluck R, Mischler R, Newman RW, Palache, AM, Verlander NQ, Warburton F, Wood JM, Zambon MC (2003) Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: Phase I randomised trial. *Lancet* 362: 1959–1966
108 Subbarao K, Luke CJ (2007) H5N1 viruses and vaccines. *PLoS Pathog* 3: e40
109 Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, Hoschler K, Zambon MC (2006) Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: Phase I randomised trial. *Lancet* 367: 1657–1664
110 Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M (2006) Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* 354: 1343–1351

111 Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, Gao Q, Zhang Z, Liu Y, Wang Z et al (2006) Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: A Phase I randomised controlled trial. *Lancet* 368: 991–997
112 Desheva JA, Lu XH, Rekstin AR, Rudenko LG, Swayne DE, Cox NJ, Katz JM, Klimov AI (2006) Characterization of an influenza A H5N2 reassortant as a candidate for live-attenuated and inactivated vaccines against highly pathogenic H5N1 viruses with pandemic potential. *Vaccine* 24: 6859–6866
113 Lu X, Edwards LE, Desheva JA, Nguyen DC, Rekstin AR, Stephenson I, Szretter KJ, Cox NJ, Rudenko LG, Klimov A, Katz JM (2006) Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. *Vaccine* 24: 6588–6593
114 Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K (2007) Evaluation of replication and pathogenicity of avian influenza A H7 subtype viruses in a mouse model. *J Virol* 81: 10558–10566
115 de Wit E, Munster V, Spronken MIJ, Bestebroer TM, Baas C, Beyer WEP, Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM (2005) Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. *J Virol* 79: 12401–12407
116 Munster VJ, de Wit E, van Riel D, Beyer WEP, Rimmelzwaan GF, Osterhaus ADME, Kuiken T, Fouchier RAM (2007) The molecular basis of the pathogenicity of the Dutch highly pathogenic human influenza A H7N7 viruses. *J Infect Dis* 196: 258–265
117 Rigoni M, Shinya K, Toffan A, Milani A, Bettini F, Kawaoka Y, Cattoli G, Capua I (2007) Pneumo- and neurotropism of avian origin Italian highly pathogenic avian influenza H7N1 isolates in experimentally infected mice. *Virology* 364: 28–35
118 Guo Y, Li J, Cheng X (1999) [Discovery of men infected by avian influenza A (H9N2) virus]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 13: 105–108
119 Peiris M, Yuen KY, Leung CW, Chan KH, Ip PLS, Lai RWM, Orr WK, Shortridge KF (1999) Human infection with influenza H9N2. *Lancet* 354: 916–917
120 Guan Y, Shortridge KF, Krauss S, Webster RG (1999) Molecular characterization of H9N2 influenza viruses: Were they the donors of the “internal” genes of H5N1 viruses in Hong Kong ? *Proc Natl Acad Sci USA* 96: 9363–9367
121 Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, Norwood M, Shortridge KF, Webster RG, Guan Y (2000) Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology* 267: 279–288
122 Shortridge KF (1999) Poultry and the influenza H5N1 outbreak in Hong Kong, 1997: Abridged chronology and virus isolation. *Vaccine* 17 (Suppl 1): S26–29
123 Xu KM, Li KS, Smith GJ, Li JW, Tai H, Zhang JX, Webster RG, Peiris JS, Chen H, Guan Y (2007) Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000–2005. *J Virol* 81: 2635–2645
124 Xu KM, Smith GJ, Bahl J, Duan L, Tai H, Vijaykrishna D, Wang J, Zhang JX, Li

KS, Fan XH et al (2007) The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. *J Virol* 81: 10389–10401
125 Leneva IA, Goloubeva O, Fenton RJ, Tisdale M, Webster RG (2001) Efficacy of zanamivir against avian influenza A viruses that possess genes encoding H5N1 internal proteins and are pathogenic in mammals. *Antimicrob Agents Chemother* 45: 1216–1224
126 Leneva IA, Roberts N, Govorkova EA, Goloubeva OG, Webster RG (2000) The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses. *Antiviral Res* 48: 101–115
127 Lu X, Renshaw M, Tumpey TM, Kelly GD, Hu-Primmer J, Katz JM (2001) Immunity to influenza A H9N2 viruses induced by infection and vaccination. *J Virol* 75: 4896–4901
128 Ernst WA, Kim HJ, Tumpey TM, Jansen AD, Tai W, Cramer DV, Adler-Moore JP, Fujii G (2006) Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. *Vaccine* 24: 5158–5168
129 Chen H, Matsuoka Y, Swayne D, Chen Q, Cox NJ, Murphy BR, Subbarao K (2003) Generation and characterization of a cold-adapted influenza A H9N2 reassortant as a live pandemic influenza virus vaccine candidate. *Vaccine* 21: 4430–4436
130 Chen H, Subbarao K, Swayne D, Chen Q, Lu X, Katz J, Cox N, Matsuoka Y (2003) Generation and evaluation of a high-growth reassortant H9N2 influenza A virus as a pandemic vaccine candidate. *Vaccine* 21: 1974–1979
131 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A (2005) Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310: 77–80
132 Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM (2002) Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *J Virol* 76: 4420–4429
133 Katz JM, Lu X, Frace AM, Morken T, Zaki SR, Tumpey TM (2000) Pathogenesis of and immunity to avian influenza A H5 viruses. *Biomed Pharmacother* 54: 178–187
134 Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT et al (2005) Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J Virol* 79: 2191–2198
135 Lipatov AS, Hoffmann E, Salomon R, Yen HL, Webster RG (2006) Cross-protectiveness and immunogenicity of influenza A/Duck/Singapore/3/97(H5) vaccines against infection with A/Vietnam/1203/04(H5N1) virus in ferrets. *J Infect Dis* 194: 1040–1043
136 Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, McCain-Moss LR, Peiris JS, Rehg JE, Tuomanen EI, Webster RG (2004) Responsiveness to a pandemic alert: Use of reverse genetics for rapid development of influenza vaccines. *Lancet* 363: 1099–1103
137 van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus ADME, Kuiken T (2006) H5N1 virus attachment to lower respiratory tract. *Science* 312: 399

138 Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y (2006) Influenza virus receptors in the human airway. *Nature* 440: 435–436
139 Nicholls JM, Chan MCW, Chan WY, Wong HK, Cheung CY, Kwong DLW, Wong MP, Chui WH, Poon LLM, Tsao SW et al (2007) Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. *Nat Med* 13: 147–149
140 Hinshaw VS, Webster RG, Easterday BC, Bean WJ (1981) Replication of avian influenza A viruses in mammals. *Infect Immun* 34: 354–361
141 Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A (2007) A two-amino acid change in the hemagglutinin of the 1918 Influenza virus abolishes transmission. *Science* 315: 655–659
142 Paniker CKJ, Nair CMG (1970) Infection with A2 Hong Kong influenza virus in domestic cats. *Bull World Health Organ* 43: 859–862
143 Paniker CKJ, Nair CMG (1972) Experimental infection of animals with influenza-virus types A and B. *Bull World Health Organ* 47: 461–463
144 Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, Osterhaus AD, Kuiken T (2006) Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. *Am J Pathol* 168: 176–183
145 Karaca K, Swayne DE, Grosenbaugh D, Bublot M, Robles A, Spackman E, Nordgren R (2005) Immunogenicity of fowlpox virus expressing the avian influenza virus H5 gene (TROVAC AIV-H5) in cats. *Clin Diagn Lab Immunol* 12: 1340–1342
146 Saito T, Lim W, Suzuki T, Suzuki Y, Kida H, Nishimura SI, Tashiro M (2002) Characterization of a human H9N2 influenza virus isolated in Hong Kong. *Vaccine* 20: 125–133
147 Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD (2001) Pathogenesis of influenza A (H5N1) virus infection in a primate model. *J Virol* 75: 6687–6691
148 Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD (2003) Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (Macaca fascicularis). *Vet Pathol* 40: 304–310
149 Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, Qi Q, Tian G, Fan S, Zhao H et al (2006) Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. *J Virol* 80: 5976–5983
150 Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Hyun Kim J, Halfmann P, Hatta M et al (2007) Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445: 319–323
151 Center for Biologics Evaluation and Research (CBER) (2007) *Guidance for Industry. Clinical data needed to support the licensure of pandemic influenza vaccines.* Food and Drug Administration
152 European Agency for the Evaluation of Medicinal Products (EMEA) (2004) *Guideline on Dossier Structure and Content for Pandemic Influenza Vaccine Marketing Authorization Application*
153 Federal Register 21 CFR Parts 314 & 610 (2002) *New Drug and Biological*

Drug Products; Evidence Needed to Demonstrate Effectiveness of New Drugs When Human Efficacy Studies are Not Ethical or Feasible
154 Iida T, Bang FB (1963) Infection of the upper respiratory tract of mice with influenza A virus. *Am J Hyg* 77: 169–176
155 Loosli CG (1948) The pathogenesis and pathology of experimental air-borne influenza virus A infections in mice. *J Infect Dis* 84: 153–168
156 O'Neill E, Krauss SL, Riberdy JM, Webster RG, Woodland DL (2000) Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice. *J Gen Virol* 81: 2689–2696
157 Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G (2005) Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. *Vaccine* 23: 5751–5759
158 Smith H, Sweet C (1988) Lessons from human influenza from pathogenicity studies with ferrets. *Rev Infect Dis* 10: 56–75
159 Smith W, Stuart-Harris CH (1936) Influenza infection of man from the ferret. *Lancet* 2: 121–123

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Live attenuated influenza vaccine

Harry Greenberg[1] and George Kemble[2]

[1]Departments of Medicine and Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA and Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA; [2]MedImmune Vaccines, Mountain View, CA, USA

Abstract

Development of the live, attenuated influenza vaccine, FluMist®, has spanned several decades. The vaccine contains three vaccine strains, two attenuated influenza A strains and one attenuated influenza B strain; these vaccine strains are genetic reassortants each harboring two gene segments from the wild type virus conferring the appropriate antigens (e.g. A/H3N2, A/H1N1 or B) and the remaining 6 gene segments of the live, attenuated influenza A or influenza B donor virus. Both donor viruses have complex genetic signatures that control the key biological traits of the resulting genetic reassortants, including temperature-sensitivity in vitro and attenuation in an animal model, and the overall attenuation of the vaccine. Studies in humans have demonstrated that the attenuated vaccine strains can elicit humoral antibodies as well as cellular immunity; both responses are generally more readily detectable in children than adults. A number of different clinical studies in children and adults have shown that this vaccine can reduce the burden of influenza illness in vaccinated subjects, including seasons in which the circulating wild type strain was antigenically drifted from the antigens included in the vaccine. These attributes, and others, of the live vaccine make it a potentially useful platform to generate an effective vaccine to combat a future influenza pandemic.

Introduction

One key principle in determining whether a vaccination strategy will likely be an effective means of controlling disease is the observation that natural infection by a pathogen results in the protection of the individual from subsequent illness with the same or highly related pathogens. Natural infection with wild-type influenza virus elicits a highly protective and long-lasting immune response that protects the individual from suffering influenza illness following re-exposure to the same, or similar, strain of influenza. Historical analyses have shown that individuals infected with an A/H1N1 strain in the early 1950s were protected from illness when the same virus circulated nearly 25 years later [1]. Despite this long-lasting and

highly effective immunity, adults are susceptible to influenza-like illness on a regular basis. This apparent paradox is not due to waning or ineffective immune responses, but rather to the fact that the influenza virus continually evolves in the human population by undergoing genetic changes in all its genes including those encoding the major antigens on the virion surface, which are targets of protective immunity. These changes in the two surface proteins, the hemagglutinin (HA) and neuraminidase (NA) glycoproteins, lead to antigenic drift. At some point, the newly evolved drifted influenza strain differs sufficiently from its progenitor so that the immunity built up to the progenitor in the human population is no longer capable of efficiently reacting with the new influenza strain, and the drifted variant is now poised to cause a new epidemic wave of disease. Immune responses to influenza can be measured in many different compartments including IgG and IgA in the serum, secretory IgA in the nasal secretions, and T, B and NK cells in the periphery as well as various lymphoid tissues, especially those in the respiratory tree. Functional antibodies that neutralize the virus or prevent it from binding its cognate receptor, frequently designated hemagglutination inhibiting (HAI) antibodies, can be found in the serum and occasionally in nasal secretions; the cellular immune responses and additional antibody responses target a variety of regions on the viral HA, NA and other proteins encoded by the virus particularly M, NP and NS. Several of the immune responses to the virus have been correlated with protection from disease especially the quantity of HAI or neutralizing antibodies in the serum and potentially, the titer of serum antibody to NA as well. Despite the presence of these multiple measures of immunological memory and effector functions and availability of substantial information correlating some of these measures with protection, the fundamental role each has in preventing illness following re-exposure to influenza remains to be elucidated. Due to the complexity of the immune response to influenza infection and the lack of a detailed mechanistic understanding of the specific components of the response that provide protection, designing an optimally effective vaccine that targets only a limited subset of viral peptides or antigens has been difficult. A vaccine strategy that effectively mimics the immune response elicited by natural infection would be expected to provide an effective, cross-reactive, and long-lasting immunity.

Background

Inactivated influenza vaccines were first put into use over 50 years ago for the military. In the late 1960s, the process of cold-adaptation was applied to influenza virus for the purpose of generating a live attenuated vaccine with the hope that such a vaccine would generate broader and higher levels of immunity. In 2003, following several decades of clinical study and development, a live attenuated influenza vaccine (LAIV) called FluMist® was

licensed in the United States for the active immunization against influenza-like illness in healthy children and adults, 5–49 years of age. FluMist is currently manufactured in specific pathogen-free embryonated chicken eggs. The vaccine is a trivalent blend of three LAIV vaccine strains, A/H1N1, A/H3N2 and B, each recommended annually by the U.S. Public Health Service to antigenically match the strains expected to circulate in the upcoming influenza season. The material is blended such that the dose of each strain is approximately 7 $\log_{10}$ of infectious particles and is filled into sprayer devices that produce a mist upon actuation. The original licensed vaccine formulation was stored frozen until immediately prior to use, while the current vaccine is stored at refrigerator temperature, 2–8 °C. The first two decades of LAIV clinical studies, many sponsored by the NIH, were performed using monovalent and bivalent formulations of the vaccine delivered by nasal drop rather than spray and have been extensively reviewed previously [2]. This chapter describes the key studies during the development and characterization of the trivalent formulation of FluMist.

Development of cold-adapted influenza vaccine strains

Live attenuated vaccines that are delivered by the same route of entry as the wild-type pathogen are expected to induce an immune response that is similar to the natural pathogen without eliciting the typical signs or symptoms associated with illness. This approach does not require a predetermined knowledge of the identity or structure of the crucial protective antigens nor a defined mechanistic understanding of the immune effector functions that mediate protection. In the 1960s, John Maassab at the University of Michigan set out to attenuate influenza virus for vaccine use through a process designated cold-adaptation. Forcing the virus to replicate efficiently at lower than normal temperatures was predicted to result in changes to its genetic makeup making it less fit to replicate at normal and elevated body temperatures, thereby attenuating the strain. The A/Ann Arbor/6/60 (H2N2) strain was isolated from a patient and serially passaged at reduced temperatures in both eggs and chicken cells along with biologically cloning the progeny at several intervals [3]. Biological characterization demonstrated that the resulting virus was cold adapted (*ca*), as defined by its ability to replicate to titers at 25 °C that were within 2 $\log_{10}$ of titers obtained at 33 °C, and temperature sensitive (*ts*), as defined by replication of the virus at 39 °C that was debilitated by at least 2 $\log_{10}$ compared to its replication at 33 °C [4]. These newly acquired properties of the cold-adapted progeny, designated *ca* A/Ann Arbor/6/60, distinguished it from its parent as well as most wild-type influenza strains. The spectrum of temperatures at which the *ca* virus replicated well was lower than the wild-type viruses that caused disease. Of note, further characterization of *ca* A/Ann Arbor/6/60 in the highly susceptible ferret model demonstrated that it was attenuated

compared to wild-type influenza viruses. In contrast to the parental wild-type A/Ann Arbor/6/60 strain, the *ca* virus was unable to replicate in the lung tissues of ferrets or elicit signs of influenza-like illness [5]. Following the success of adaptation of influenza A, Massaab and his colleagues later isolated and cold-adapted an influenza B virus in a similar manner. This virus, designated *ca* B/Ann Arbor/1/66, had similar *ca*, *ts* and attenuated (*att*) properties as its influenza A counterpart [6]. This virus was even more restricted at higher temperatures than *ca* A/Ann Arbor/6/60, in that it was significantly restricted in replication at temperatures as low as 37 °C. These two strains provide the genetic background of all FluMist® vaccine strains, imparting their *ca*, *ts* and *att* properties to the vaccine.

The influenza virus genome is comprised of eight different RNAs or gene segments. Individual monovalent LAIV strains are derived by combining the gene segments encoding the two surface glycoproteins, HA and NA, of a contemporary field isolate of influenza with the remaining six internal gene segments of the appropriate *ca* master donor virus (MDV), either *ca* A/Ann Arbor/6/60 or *ca* B/Ann Arbor/1/66. The resulting 6:2 genetic reassortant combines the attenuation inherent to the MDVs with the antigens needed to elicit a neutralizing immune response that should prevent disease caused by currently circulating strains of influenza.

Basic properties of the vaccine

Genetic basis of biological properties of the vaccine

Sequence analysis and comparison of the genomes of *ca* A/Ann Arbor/6/60 and *ca* B/Ann Arbor/1/66 to their respective parental strains confirmed that a number of changes had accumulated during passage. Which of these changes were key to controlling the newly acquired biological properties was not immediately evident by merely examining the sequences. The first studies to determine the genetic basis of the *ca*, *ts*, and *att* phenotypes were performed by creating reassortant viruses *via* co-infection of a cell with two biologically distinct strains, the MDV and typically a wild-type field isolate. The gene segments of these two viruses would reassort and the resulting progeny could be isolated from each other and independently characterized for retention, loss or modification of the specific phenotype and their genetic composition. Because there was little control over which segments would reassort, distinct strains were used to facilitate selection and screening for the desired progeny. These studies helped identify the contribution of the PB1 gene segment to the *ts* phenotype of *ca* A/Ann Arbor/6/60. Sometimes, however, the results were misleading in that the loss of a biological property was not due to a specific mutation or set of mutations but rather to an incompatibility of gene segments from two diverse parental influenza strains, also known as the constellation effect. One of the best-documented

constellation effects was observed with the MDV-A M gene segment. A recombinant wild-type virus harboring the M gene segment of MDV-A was attenuated in animals leading to the conclusion that specific mutations in this gene segment were responsible for the *att* phenotype [7]. However, later work showed that a similar recombinant harboring the M gene segment from the parental wild-type A/Ann Abor/6/60 strain was also attenuated [8]. The biological result was not due to the one nucleotide difference in the M gene segments between *ca* and wild-type pair; rather the phenotype was due to the inability of the Ann Arbor M gene segment to interact optimally with the other gene segments of the divergent field isolate.

The introduction of reverse genetics enabled biological traits to be associated with specific nucleotides without having to account for potential problems caused by constellation effects. Using reverse genetics recombinant viruses are derived directly from cDNA clones of the viral gene segments, allowing recombinant viruses with only one or a defined set of changes to be produced. No selection system or extensive screening procedure is required to obtain the desired recombinant virus; the genome of the recombinant virus accurately reflects the genetic content of the cDNAs used to produce it. To map and study the impact of the genetic changes between the wild-type and *ca* virus pairs two derivatives of A/Ann Arbor/6/60 and B/Ann Arbor/1/66 were produced, one contained the nucleotides present in the MDV, the other encoded either eight or nine amino acid changes in the internal gene segments, respectively, representing the wild-type progenitors. The changes resulting in the wild-type progenitor sequence were expected to result in non-*ts* and non-*att* properties, these properties were confirmed following biological characterization. Recombinant viruses were then derived by making individual or grouped changes in the wild-type or MDV version of the strain and evaluating the resulting phenotype. The culmination of these studies demonstrated that five nucleotide positions distributed between the PB1, PB2 and NP gene segments of A/Ann Arbor/6/60 controlled both the *ts* and *att* properties [9]. Studies with B/Ann Arbor/1/66 revealed that three positions (two in PA and one in NP) controlled the *ts* phenotype, an additional two nucleotides in M controlled the *att* phenotype and another subset of three changes in PA and PB2 were responsible for the *ca* phenotype [10, 11]. The robustness of these genetic signatures was demonstrated by placing only the minimal set of changes into divergent influenza strains and demonstrating the accompanying transfer of the biological traits. For example, the five changes responsible for controlling *ts* and *att* of MDV-A were introduced into A/PR8/34 (H1N1) and the resulting recombinant virus acquired both the *ts* and *att* phenotypes [12]. Similar studies with *ca* B/Ann Arbor/1/66 and B/Yamanashi/166/98 were conducted with similar results [11]. The fundamental mechanism resulting in the expression of these phenotypes is a result of changes in multiple different viral proteins and most likely the impact of these changes is exhibited at multiple points of the replica-

tion cycle of the virus. The basic biochemical nature of these phenotypes is unclear and continues to be investigated.

Genetic stability of the vaccine in manufacturing

Influenza virus, like other RNA viruses, has an RNA-dependent RNA polymerase that lacks a proofreading function. Picking and sequencing individual plaque isolates demonstrated an observed mutation frequency of one change per 10 000 nucleotides resulting in a rate of 1.5×10^{-5} mutations per replication cycle [13]. Because of this inherent capacity of influenza virus gene segments to change, the genetic stability of the vaccine was characterized both within the context of the manufacturing process as well as following intranasal administration. In general, manufacturing of the bulk vaccine only requires the seed material to be passaged once or twice in embryonated eggs. To evaluate the stability of the genetic elements during manufacture, the genomic sequences of bulk vaccine and its progenitor seed materials were analyzed, represented by over nine different strains distributed among nine independent seed materials and over 50 bulk vaccine stocks. Comparisons of the genomic sequences of these materials demonstrated that in all cases the bulk vaccine was identical to the seed material. These data demonstrated that the vaccine's genetic composition is stable and unchanged within the parameters used to manufacture the vaccine at large scale [14].

Genetic stability of the virus in the respiratory tract and transmission

Following intranasal administration, the vaccine virus infects and replicates in epithelial cells of the upper respiratory tract resulting in an immune response. Characterizing the genetic stability of the vaccine in humans is an important element of understanding the properties of the vaccine. Over the course of multiple decades studying monovalent, bivalent and trivalent formulations of this vaccine in clinical studies, no revertants of the vaccine have been identified [2]. To evaluate the stability of the vaccine following replication in the upper respiratory tract of humans, a prospective shedding and transmissibility study was designed. Young children were selected due to the relatively longer duration and greater level of shedding of the vaccine following administration. Therefore, this population was expected to represent the most permissive setting for detecting revertants if they were to arise. In the study of genetic stability, 98 children 9–36 months of age were vaccinated with FluMist and nasal swab samples were taken at frequent and regular intervals. Of the children in the study, 86% shed at least one of the three strains in the vaccine with peak titers ranging from 1 to 8 days post vaccination and the last isolate shed 21 days post vaccination. The *ca* and

ts phenotypes were preserved in all the shed viruses tested (135 of 250 isolates were tested) [15]. Of the isolates, 54 were chosen at random and their genomes sequenced in their entirety and compared to the sequences of the strains used to vaccinate the children. These analyses revealed that some genetic changes had occurred in a majority of shed isolates and in some cases the mutations were shared by multiple isolates [16]. These changes could have arisen during replication in the upper respiratory tract or could have preexisted in the vaccine material at a level not detected by sequence analysis of the bulk material. To address the latter hypothesis, samples of the vaccine material were obtained, amplified by RT-PCR and individual clones were sequenced. Interestingly, in most cases, the change(s) evident in the isolate shed from the child were representative of changes that preexisted in the bulk vaccine material. Despite the presence of these mutations, all isolates invariably retained their characteristic biological properties, confirming the exquisite genetic stability that had been previously described in observational studies.

A corollary concern associated with genetic stability and vaccine shedding is the potential for person-to-person transmission of the virus. Shedding of the vaccine from an individual is a necessary predecessor for transmission to an unimmunized contact; however, shedding is not necessarily sufficient for transmission to occur. The study of the genetic stability of the vaccine in children was also designed to assess the probability of transmission of the vaccine virus. Young children in a daycare setting were expected to increase the likelihood of detecting a transmission event should it occur due to the relatively high level of shed virus in respiratory secretions, relatively high level of susceptibility to vaccine take and the general absence of hygienic practices among young children that generally inhibit transmission of viruses in adults. In addition to the 98 children vaccinated with FluMist, 97 children received placebo. These children were intermixed and placed in cohorts that played together in a daycare environment for at least 4 h every day for 3 or more days each week. Nasal swabs were obtained at regular and frequent intervals from each child and the presence of vaccine virus was assessed. Vaccine virus was recovered from 80% of the vaccinated children and in only one confirmed case from a placebo recipient [15]. The influenza B vaccine virus recovered from the placebo recipient was shed on only 1 day and matched the genetic signature of an influenza B vaccine virus shed by a vaccinated member of the same playgroup several days earlier. The transmitted vaccine isolate was shown to retrain its characteristic *ca* and *ts* properties and exhibited the attenuation phenotype in ferrets; additionally, the placebo child had no signs or symptoms distinguishable from other children in the study. These results were applied to the Reed-Frost model that indicated a 0.58% probability of vaccine transmission occurring from a single contact of a vaccinated young child with an unvaccinated young child [15]. The likelihood of transmission from a vaccinated adult would be expected to be substantially lower, since adults shed virus less frequently and in lower

amounts than children. In a study designed to characterize the shedding of FluMist in adults, vaccine was recovered from nasal swabs of only 50% of individuals 3 days after vaccination and by 10 days after vaccination only 5% of individuals had vaccine detectable in their nasal swab [17]. This low probability of transmission combined with the vaccine's genetic stability give additional confidence in the use of FluMist in children.

Basis of the immune response

Infection with wild-type influenza virus leaves the individual with a strong immunological memory that will prevent the same or antigenically similar variant from causing disease again in the same individual for decades. This immunological memory can be detected in many different compartments including local mucosal immunity, serum antibody and T cells. The immune response to vaccination with LAIV has been studied in multiple different settings and the immune response is qualitatively similar but quantitatively less than that elicited by natural infection; immunity can be documented by mucosal IgA, serum HAI and neutralizing antibodies and cellular responses. This observation is not surprising given that the vaccine stimulates immunity by replication in the upper respiratory tract similar to that of the wild-type virus. Despite finding evidence for vaccine-induced immunity at both local and systemic compartments, the specific functional role of any particular immune response and validated correlates of protection from influenza disease in vaccinated individuals remains unproven.

LAIV elicits a robust immune response in young children, particularly those that are seronegative for influenza at the time of vaccination [18–20]. Seroconversion rates, measured by the presence of hemagglutination inhibition antibody in the serum, are frequently 80–90% or more after two doses of vaccine. Rates typically are lower for children that have preexisting antibody at the time of vaccination. This is not surprising given that the vaccine must replicate at the mucosal surfaces to be effective. The presence of antibody at the time of immunization may both limit the extent of replication of the vaccine as well as mask the boosting of the immune response using relatively crude measures of immunogenicity such as HAI antibody in the serum. Other immune responses to LAIV have been documented in children including secretory IgA in nasal secretions. Cellular immune responses have been evaluated in older children receiving FluMist. Following immunization of children aged 5–9 years, blood was collected at 10 and 28 days post vaccination and stimulated with the A/H3N2 strain *ex vivo*. Both the CD4 and CD8 influenza-specific T cells were increased in these children compared to their pre-vaccination values; additionally, these increases were greater than those observed for TIV-immunized children in the same study [21]. In this same population, antibody-secreting B cells were also detected in the periphery within 7–10 days post vaccination [22].

Immunological markers in adults have been more difficult to measure. Most adults have had multiple encounters with wild-type influenza and influenza vaccination during their lifetime and have readily measurable levels of influenza antibody in their serum. In contrast to studies in young children, vaccination of adults with FluMist infrequently produces a measurable increase in serum HAI antibody titers. Recent studies on T cell immunity following vaccination had similar results. There were no demonstrable increases in CD4, CD8 or NK activity when cells were stimulated *ex vivo* either 10 or 28 days following vaccination [21]. This may again be due to the a higher level of influenza immunity prior to vaccination in adults. This same study demonstrated that the levels of pre-vaccination influenza specific CD4 and CD8 cells increased with age of the subjects and that adults had significantly higher baseline quantities than children. In contrast to the T cell and HAI responses, adults were generally shown to have increased influenza-specific antibody-secreting B cells in the blood 7–10 days post LAIV vaccination. While only 16% of the adults had a serological response measured by a fourfold or greater increase of HAI antibody following immunization, approximately 80% of the subjects had a measurable increase in the number of influenza-specific IgG-secreting antibody-secreting cells in the periphery [22]. These data clearly demonstrated that LAIV elicited a readily detectable B cell response in most adults, which is consistent with the clinical experience that FluMist is highly efficacious in an adult population [23, 24]. The current immunological markers typically used to assess the function of influenza vaccines, such as HAI in the serum, may not be sufficiently sensitive nor monitor the appropriate compartment to detect a functional immune response to LAIV.

Vaccine studies often rely on correlate markers to demonstrate that the vaccine will perform as expected under the conditions being studied. A robust correlate of protection is an immunological marker that when present coincides with protection from disease upon subsequent exposure to the wild-type virus and the lack of which correlates with susceptibility to illness. Due to high rates of efficacy demonstrated for LAIV combined with the difficulty in using traditional serum-based influenza assays to measure an immune response in adults, these markers have been difficult to identify for LAIV. In children, particularly young children who are immunologically naive to influenza, vaccination with LAIV elicits a robust immune response that can be detected in multiple compartments. One study evaluated potential correlates of protection by inoculating children with either vaccine or placebo, followed by a relatively long interval at which point samples were taken to measure humoral immune responses, and then these children were given another dose of a monovalent vaccine strain. By correlating the level of immune response using a variety of immunological assay systems with shedding of the challenge vaccine virus, the utility of the various assays for predicting protective immunity was assessed [18]. First, the presence of serum HAI antibody strongly correlated with the absence of shed challenge

virus. These data demonstrated that at least in children, a positive correlation existed for this marker. However, when the two groups of children who were seronegative at the time of challenge were compared, there was a significant increase in the number of children who shed vaccine in the placebo group compared to the LAIV-vaccinated group. These data led to the conclusion that the absence of serum HAI did not correlate completely with susceptibility. The same trends were observed when secretory nasal IgA was used as the marker. The observation that the presence of these markers correlates with protection helps identify potentially useful immunological measures; however, the observation that absence does not correlate with susceptibility argues that other important immune mechanisms may be overlooked when only serum HAI or secretory IgA are evaluated. Studies have demonstrated that LAIV elicits both humoral and cellular immune responses and those responses can be found at both mucosal and peripheral sites. However, the functional immune mediators that govern protection from disease have not yet been elucidated, may be multi-factorial and may differ among populations as well. Further study will be needed to identify practical correlates for vaccine efficacy as well as detailed immunological profiling to understand the functional components of an effective immune response and how it controls disease.

Performance of the vaccine in clinical studies

Vaccines derived from *ca* A/Ann Arbor/6/60 and *ca* B/Ann Arbor/1/66 have been extensively characterized in clinical studies. Prior to the mid 1990s, monovalent and bivalent forms of these vaccines were evaluated in over 15,000 subjects in a number of different clinical studies, many of which were sponsored by the NIH [2]. More recently, studies focused on both the frozen and refrigerator stable trivalent formulations of FluMist, have been conducted in a wide range of settings in individuals from 6 months to over 80 years of age.

The efficacy of the trivalent form of the vaccine has been evaluated in a number of settings in different age cohorts throughout the world. Table 1 lists the study populations, control groups and overall efficacy against culture-confirmed influenza-like illness for studies in children. The vaccine has reproducibly been shown to prevent influenza-like illness (ILI) caused by all three influenza types including, A/H1N1, A/H3N2 and B. In the majority of well-powered placebo-controlled studies, vaccine efficacy ranged from 73% to 93% in children with respect to culture-confirmed influenza illness when compared to placebo [25–29]. Interestingly, four studies were conducted in which FluMist was compared to TIV-vaccinated subjects. In the largest of these studies, which included over 8000 children and approximately 491 isolates from children who had modified CDC-ILI. FluMist was shown to reduce the burden of illness by nearly 55% compared to TIV. Of note, all

Table 1. LAIV efficacy studies for protection against laboratory confirmed influenza-like illness in children (adapted from [33])

Study (author, year)	No.	Ages (months)	Control	Efficacy (%)	Circulating strains	Ref.
Belshe 1996–1997	1602	15–71	Placebo	93	Matched H3N2 Matched B	[27]
Belshe 1997–1998	1358	26–85	Placebo	86	Mismatched H3N2	[26]
Tam 2000–2001	3174	12–35	Placebo	73	Matched H3N2 Matched H1N1 Matched B	[28]
Tam 2001–2002	1265	24–47	Placebo	84	Matched H3N2 Match ed H1N1	[28]
Vesikari 2000–2001	1784	6–35	Placebo	85	Matched H3N2 Matched H1N1 Matched B	[29]
Vesikari 2001–2002	1094	18–47	Placebo	89	Matched H3N2 Matched H1N1 Matched B	[29]
			Reduction compared to TIV (%)			
Ashkenazi 2002–2003	2187	6–71 with recurrent respiratory illness	TIV	53	Matched H1N1 Matched B	[30]
Fleming 2002–2003	2229	6–17 years with asthma	TIV	35	Matched B	[31]
Belshe 2004–2005	8352	6–59	TIV	55	Mismatched H3N2 Matched H1N1 Matched / Mismatched B	[25]

the A/H3N2 strains circulating in this study were antigenically mismatched to the two vaccines and the children vaccinated with FluMist had 79% fewer cases of modified CDC-ILI compared to the TIV group [25]. In two other studies, one conducted in children with recurrent respiratory illness and the other in older children with asthma, FluMist was also shown to be more efficacious than TIV [30, 31]. The fourth trial, conducted in adults, contained three cohorts: placebo, TIV vaccinated or FluMist vaccinated. While only 32 isolates were available for analysis, FluMist was shown to be efficacious compared to placebo but not more efficacious than TIV [32]. The results of this study compared to the three studies in children may reflect the interaction of FluMist with the immune system of the adult host or may simply reflect a normal variation due to a relatively smaller sample size.

The safety of FluMist has been evaluated in over 49,000 subjects in 48 completed studies [33]. In controlled studies, the most common adverse events in children were runny nose or nasal congestion, low-grade fever, decreased activity and decreased appetite. In the youngest children, who received two doses of vaccine, no significant differences were observed following the second dose. In adults, the most common adverse events are runny nose/nasal congestion, cough and sore throat. The reactogenicity of the vaccine is consistent with replication of a live attenuated virus in the nasal epithelium of the subject. In a large safety database study performed in Northern California Kaiser Hospital system, a 3.5-fold increase in asthma events were noted within 42 days of vaccination in the pre-specified age stratum of 18–35 months [34]. The observation was further investigated in the large efficacy study of FluMist and TIV in young children. In this latter study in the age stratum less than 24 months of age (6–23 months), there were 3.2% of children in the FluMist group who had medically attended wheezing events within 42 days of vaccination compared to 2.0% in the TIV group. There was no significant difference in rates after 42 days or in the children 24 months of age or older [25]. In a study of older children with asthma, the reactogenicity and safety of FluMist was similar to children receiving TIV, and in placebo-controlled studies in children 9 years of age older with moderate to severe asthma, the vaccine was safe and generally well tolerated [31, 35]. These observations of increased wheezing in the youngest age groups require further investigation to understand the relationship between these events and vaccination with LAIV.

LAIV protects against antigenically different strains

Influenza virus continually evolves; changes in the HA molecule give rise to variants that are capable of escaping from the preexisting immunity in the population. Predicting which of these variant drifted strains will give rise to the next epidemic wave of seasonal influenza is an annual challenge addressed by the global public health authorities. In general, matching the vaccine antigen to upcoming season's influenza strain should result in the best opportunity to produce effective influenza vaccines; however, occasionally strains chosen for inclusion in the vaccine do not match well with the epidemic virus. Immunity elicited by FluMist may provide for a larger margin of error for antigenic matching than occurs after inactivated vaccine administration. FluMist has been shown to provide protection against significantly antigenically drifted variants in several clinical settings. In 1997–1998, children were immunized with a trivalent blend of FluMist containing the A/Wuhan/359/95 (H3N2) strain. The virus that circulated in the community that year was designated A/Sydney/05/97 (H3N2) and was antigenically quite distinct from the H3N2 antigen contained in the vaccine. Despite this

level of mismatch, the vaccine conferred efficacy greater than 85% against the A/Sydney/05/97 H3N2 virus [26]. That same season, FluMist was also shown to be effective in adults by monitoring febrile upper respiratory tract infections and other associated medical utilizations [23]. In the head to head study of FluMist and TIV in children, FluMist reduced modified CDC-ILI caused by an antigenically drifted A/H3N2 strain by 79% compared to TIV [25]. The cross-protective nature of FluMist immunity is reflected in the serum antibody response to the vaccine [19, 20]. Young children vaccinated with FluMist develop a robust immune response to vaccination that can be measured by a rise in serum HAI and neutralizing antibody titers. Children immunized with LAIV containing the A/Panama/2007/99 strain developed high levels of HAI and neutralizing antibody following one dose; in contrast only a minority of children receiving one dose of TIV with the same antigen responded to the vaccine. Notably, antibodies from children vaccinated with LAIV had significant reactivity to the drift variant that circulated through the community that year, the A/Fujian-like (H3N2), whereas the children receiving TIV had little to no reactivity to this strain [20].

Field studies of LAIV

Influenza vaccination has been shown to have indirect benefits to others in the community who are not vaccinated. In Japan, the implementation of mandatory vaccination of school-aged children with inactivated influenza vaccine resulted in a significant drop in rate of pneumonia and influenza (P&I) mortality in the elderly. The rate of P&I mortality remained low for the duration of mandatory vaccination program and returned to higher baseline levels within 2 years after the program was abolished, demonstrating the powerful impact of reducing the burden of illness in young children on the community at large [36, 37]. Two large field studies of the vaccine have been reported in which the impact of vaccination on both the vaccinated population and the nonvaccinated population were studied. In a large open-label study in the Temple-Bolton area of Texas, LAIV was administered to several thousand children and the rates of medically attended acute respiratory illness were measured and compared to a similar control community. LAIV vaccination significantly reduced illness in the vaccinated individuals in the intervention community even in years in which a drift strain circulated through the community [38, 39]. Recently, a school-based program was conducted in which 40% of the children in the intervention schools were vaccinated. The rates of absenteeism were lower in the vaccinated subjects compared to their unvaccinated schoolmates or children in control schools that were not actively recruited for vaccination [40]. Notably, other indicators of vaccine effectiveness were noted including a reduction in illness in older siblings in the same household as the vaccinated subjects.

LAIV and pandemic preparedness

Application of an effective and widely available vaccine is the only likely solution to prevent significant morbidity and mortality in the next pandemic. LAIV has many attributes that make it a good candidate for an effective pandemic vaccine. Several LAIV vaccine candidates have been constructed that express H5N1 HA and NA. The HA of highly pathogenic wild-type H5N1 strains, as well as highly pathogenic strains of other subtypes, have a stretch of basic amino acids between the HA1 and HA2 domains that contributes to the virulence of the strain. The LAIV H5N1 vaccine candidates were constructed by first deleting this multiple basic amino acid motif prior to rescuing the vaccine strain. The resulting vaccine candidates have been shown to exhibit the characteristic *ca*, and *ts in vitro* phenotypes of all LAIV strains. In addition, these candidates are highly attenuated in chickens, mice and ferrets yet produce immune responses that protect mice and ferrets from challenge with antigenically similar as well as antigenically drifted variants [41]. Several aspects of a pandemic are likely to be different than a typical seasonal epidemic and these unique features will alter the normal course of actions taken by public health authorities as well as vaccine manufacturers. First, the pandemic is likely to spread quickly and on a global scale. An effective vaccine will need to be administered to a large portion of the world's population. Currently, the annual worldwide distribution of influenza vaccines is only adequate for 300 million doses, far short of the 6 billion people who will need the vaccine. In addition, the nature of the pandemic antigen will be atypical; it will be comprised of an HA that has not circulated previously and vaccine seed strains will need to be quickly assembled. LAIV has clinically demonstrated its capacity for preventing disease caused by antigenically drifted strains. This attribute of LAIV may allow prebanked or stockpiled vaccines that are not antigenically identical to the circulating vaccine strain to be made well in advance of the pandemic and be effective during the early stages of a pandemic. A second essential feature of an effective pandemic strategy is rapid and large-scale production capacities. The dose of 7 $\log_{10}$ infectious particles of LAIV is a small antigenic mass. One dose of LAIV represents less than approximately 1% of an inactivated (15 μg) dose of antigen. This efficiency translates into the potential to rapidly produce large quantities of bulk LAIV compared to inactivated vaccine. The capacity to produce large amounts of LAIV rapidly combined with its cross-protective nature, ease of administration and high degree of efficacy in immunologically naive populations make this a promising candidate for pandemic preparedness.

Conclusion

The utilization of this novel vaccine technology continues to be refined and improved. Recent studies in children should enable greater use of this vaccine in this highly susceptible population. The current manufacturing methods used to make LAIV are based on production technologies that are over 50 years old, more modern production methods including manufacturing in cell culture substrates, are being developed. In addition, the generation of the 6:2 reassortant viruses used to initiate seed strain is being refined and integrated with the use of reverse genetics technology. Finally, the attributes that make this vaccine effective in young children is being further explored and developed to apply to pandemic solutions.

References

1 Wright PE, Neumann G, Kawaoka Y (2007) Orthomyxovirus. In: D Knipe, P Howley (eds): *Fields Virology*, 5th edn. Lippincott, Philadelphia
2 Murphy BR, Coelingh K (2002) Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. *Viral Immunol* 15: 295–323
3 Maassab HF (1967) Adaptation and growth characteristics of influenza virus at 25 °C. *Nature* 213: 612–614
4 Maassab HF (1968) Plaque formation of influenza virus at 25 °C. *Nature* 219: 645–646
5 Maassab HF, Francis T Jr, Davenport FM, Hennessy AV, Minuse E, Anderson G (1969) Laboratory and clinical characteristics of attenuated strains of influenza virus. *Bull World Health Organ* 41: 589–594
6 Maassab HF (1970) Developments of variants of influenza virus. In: RD Barry, BWJ Mahy (eds): *The Biology of Large RNA Viruses*. Academic Press, London, 542–566
7 Snyder MH, Betts RF, DeBorde D, Tierney EL, Clements ML, Herrington D, Sears SD, Dolin R, Maassab HF, Murphy BR (1988) Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. *J Virol* 62: 488–495
8 Subbarao EK, Perkins M, Treanor JJ, Murphy BR (1992) The attenuation phenotype conferred by the M gene of the influenza A/Ann Arbor/6/60 cold-adapted virus (H2N2) on the A/Korea/82 (H3N2) reassortant virus results from a gene constellation effect. *Virus Res* 25: 37–50
9 Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H (2003) Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology* 306: 18–24
10 Chen Z, Aspelund A, Kemble G, Jin He (2006) Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist). *Virology* 345: 416–423

11 Hoffmann E, Mahmood K, Chen Z, Yang CF, Spaete J, Greenberg HB, Herlocher ML, Jin H, Kemble G (2005) Multiple gene segments control the temperature sensitivity and attenuation phenotypes of *ca* B/Ann Arbor/1/66. *J Virol* 79: 11014–11021
12 Jin H, Zhou H, Lu B, Kemble G (2004) Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. *J Virol* 78: 995–998
13 Parvin JD, Moscona A, Pan WT, Leider JM, Palese P (1986) Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. *J Virol* 59: 377–383
14 Buonagurio DA (2006) Genetic stability of live, cold-adapted influenza virus components of the FluMist/CAIV-T vaccine throughout the manufacturing process. *Vaccine* 24: 2151–2160
15 Vesikari T, Karvonen A, Korhonen T, Edelman K, Vainionpää R, Salmi A, Saville MK, Cho I, Razmpour A, Rappaport R et al (2006) A randomized, double-blind study of the safety, transmissibility and phenotypic and genotypic stability of cold-adapted influenza virus vaccine. *Pediatr Infect Dis J* 25: 590–595
16 Buonagurio DA, O'Neill RE, Shutyak L, D'Arco GA, Bechert TM, Kazachkov Y, Wang HP, DeStefano J, Coelingh KL, August M et al (2006) Genetic and phenotypic stability of cold-adapted influenza viruses in a trivalent vaccine administered to children in a day care setting. *Virology* 347: 296–306
17 Talbot TR, Crocker DD, Peters J, Doersam JK, Ikizler MR, Sannella E, Wright PE, Edwards KM (2005) Duration of virus shedding after trivalent intranasal live attenuated influenza vaccination in adults. *Infect Control Hosp Epidemiol* 26: 494–500
18 Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K, Treanor J, Zangwill K, Hayden FG, Bernstein DI et al (2000) Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. *J Infect Dis* 181: 1133–1137
19 Lee MS, Mahmood K, Adhikary L, August MJ, Cordova J, Cho I, Kemble G, Reisinger K, Walker RE, Mendelman PM (2004) Measuring antibody responses to a live attenuated influenza vaccine in children. *Pediatr Infect Dis J* 23: 852–856
20 Mendelman PM, Rappaport R, Cho I, Block S, Gruber W, August M, Dawson D, Cordova J, Kemble G, Mahmood K et al (2004) Live attenuated influenza vaccine induces cross-reactive antibody responses in children against an a/Fujian/411/2002-like H3N2 antigenic variant strain. *Pediatr Infect Dis J* 23: 1053–1055
21 He X.S, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, Dekker CL, Greenberg HB, Arvin AM (2006) Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *J Virol* 80: 11756–11766
22 Sasaki S, Jaimes MC, Holmes TH, Dekker CL, Mahmood K, Kemble GW, Arvin AM, Greenberg HB (2007) Comparison of the influenza virus-specific

effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines. *J Virol* 81: 215–228

23 Nichol KL, Mendelman PM, Mallon KP, Jackson LA, Gorse GJ, Belshe RB, Glezen WP, Wittes J (1999) Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: A randomized controlled trial. *JAMA* 282: 137–144

24 Treanor JJ, Kotloff K, Betts RF, Belshe R, Newman F, Iacuzio D, Wittes J, Bryant M (1999) Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses. *Vaccine* 18: 899–906

25 Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, Kemble G, Connor EM, CAIV-T Comparative Efficacy Study Group (2007) Live attenuated *versus* inactivated influenza vaccine in infants and young children. *N Engl J Med* 356: 685–696

26 Belshe RB, Gruber WC, Mendelman PM, Cho I, Reisinger K, Block SL, Wittes J, Iacuzio D, Piedra P, Treanor J et al (2000) Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. *J Pediatr* 136: 168–175

27 Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, Bernstein DI, Hayden FG, Kotloff K, Zangwill K et al (1998) The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N Engl J Med* 338: 1405–1412

28 Tam JS, Capeding MR, Lum LC, Chotpitayasunondh T, Jiang Z, Huang LM, Lee BW, Qian Y, Samakoses R, Lolekha S et al (2007) Efficacy and safety of a live attenuated, cold-adapted influenza vaccine, trivalent against culture-confirmed influenza in young children in Asia. *Pediatr Infect Dis J* 26: 619–628

29 Vesikari T, Fleming DM, Aristegui JF, Vertruyen A, Ashkenazi S, Rappaport R, Skinner J, Saville MK, Gruber WC, Forrest BD et al (2006) Safety, efficacy, and effectiveness of cold-adapted influenza vaccine-trivalent against community-acquired, culture-confirmed influenza in young children attending day care. *Pediatrics* 118: 2298–2312

30 Ashkenazi S, Vertruyen A, Arístegui J, Esposito S, McKeith DD, Klemola T, Biolek J, Kühr J, Bujnowski T, Desgrandchamps D et al (2006) Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections. *Pediatr Infect Dis J* 25: 870–879

31 Fleming DM, Crovari P, Wahn U, Klemola T, Schlesinger Y, Langussis A, Øymar K, Garcia ML, Krygier A, Costa H et al (2006) Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. *Pediatr Infect Dis J* 25: 860–869

32 Ohmit SE, Victor JC, Rotthoff JR, Teich ER, Truscon RK, Baum LL, Rangarajan B, Newton DW, Boulton ML, Monto AS (2006) Prevention of antigenically drifted influenza by inactivated and live attenuated vaccines. *N Engl J Med* 355:. 2513–2522

33 Ambrose CS, Walker RE, Connor EM (2006) Live attenuated influenza vaccine in children. *Semin Pediatr Infect Dis* 17: 206–212
34 Bergen R, Black S, Shinefield H, Lewis E, Ray P, Hansen J, Walker R, Hessel C, Cordova J, Mendelman PM (2004) Safety of cold-adapted live attenuated influenza vaccine in a large cohort of children and adolescents. *Pediatr Infect Dis J* 23: 138–144
35 Redding G, Walker RE, Hessel C, Virant FS, Ayars GH, Bensch G, Cordova J, Holmes SJ, Mendelman PM (2002) Safety and tolerability of cold-adapted influenza virus vaccine in children and adolescents with asthma. *Pediatr Infect Dis J* 21: 44–48
36 Reichert TA (2002) The Japanese program of vaccination of schoolchildren against influenza: Implications for control of the disease. *Semin Pediatr Infect Dis* 13: 104–111
37 Sugaya N, Takeuchi Y (2005) Mass vaccination of schoolchildren against influenza and its impact on the influenza-associated mortality rate among children in Japan. *Clin Infect Dis* 41: 939–947
38 Halloran ME, Piedra PA, Longini IM Jr, Gaglani MJ, Schmotzer B, Fewlass C, Herschler GB, Glezen WP (2007) Efficacy of trivalent, cold-adapted, influenza virus vaccine against influenza A (Fujian), a drift variant, during 2003–2004. *Vaccine* 25: 4038–4045
39 Piedra PA, Gaglani MJ, Riggs M, Herschler G, Fewlass C, Watts M, Kozinetz C, Hessel C, Glezen WP (2005) Live attenuated influenza vaccine, trivalent, is safe in healthy children 18 months to 4 years, 5 to 9 years, and 10 to 18 years of age in a community-based, nonrandomized, open-label trial. *Pediatrics* 116:. e397–407
40 King JC Jr, Stoddard JJ, Gaglani MJ, Moore KA, Magder L, McClure E, Rubin JD, Englund JA, Neuzil K (2006) Effectiveness of school-based influenza vaccination. *N Engl J Med* 355: 2523–2532
41 Suguitan AL Jr, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K (2006) Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med* 3: e360

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MF59: A safe and potent oil in water emulsion adjuvant for influenza vaccines, which induces enhanced protection against virus challenge

Derek T. O'Hagan and Audino Podda

Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

Abstract

In pre-clinical studies, MF59 adjuvant offered improved protection against influenza virus challenge and significantly reduced the viral load in the lungs of challenged mice. The ability of MF59 to be an effective adjuvant for influenza vaccine in mice was shown in old mice (18 months of age) and also in mice that had been previously infected with influenza virus, situations closer to those in which influenza vaccines are used in humans. In pre-clinical studies with an influenza vaccine derived from a mammalian cell culture source, rather than eggs, MF59 has been shown to be the most potent adjuvant available for both antibody and T cell responses, and is significantly more potent than aluminum-based adjuvants. In humans, MF59 is a safe and potent vaccine adjuvant that has been included in a licensed influenza vaccine for elderly subjects in more than 20 countries (Fluad®). After 10 years of use, with more than 35 million doses distributed, the safety profile of the MF59-adjuvanted vaccine is well established through a large safety database. MF59 adjuvant has a significant impact on the immunogenicity of flu vaccines in the elderly, and also in adults who are chronically ill, an additional population who may need an improved influenza vaccine. MF59 has also been shown to have a significant impact on the immunogenicity of pandemic influenza vaccines, allowing a significant reduction in the dose of vaccine administered. Importantly, MF59 allows for more broad cross-reactivity against viral strains not included in the vaccine, for both seasonal and pandemic influenza vaccines. Moreover, the age range for which this adjuvant appears suitable can range from the elderly, down to very young children. Recent data establish the safety and potency of MF59 adjuvant in young children, in combination with influenza vaccine.

Introduction

MF59 is a safe and potent emulsion-based vaccine adjuvant that has been licensed in more than 20 countries (Fluad®), for more than 10 years, for use in an influenza vaccine focused on elderly subjects. The safety profile of MF59 is well established clinically through a large safety database (20,000 subjects) and through pharmacovigilance evaluations of greater than 35 million doses that have been distributed. The MF59 adjuvant has a significant

impact on the immunogenicity of flu vaccines in the elderly, who generally respond poorly to traditional influenza vaccines, due to age-related impairment of their immune responses, called immunosenescence. However, moving beyond the elderly population, the MF59 adjuvant has also been shown to have a significant impact on the immune response to flu vaccines in adults who are chronically ill with a range of diseases and, consequently, also respond poorly to traditional flu vaccines. Moreover, Fluad® also shows enhanced immunogenicity in very young subjects, while also displaying a similar reactogenicity profile to licensed vaccines in this population. Moving beyond seasonal flu vaccines, MF59 has also been shown to have a significant impact on the immunogenicity of potential pandemic flu vaccines and has enabled vaccines to achieve titers that might be expected to offer protection, with relatively low doses of vaccine. Moreover, the addition of MF59 to the vaccine allows for more broad cross-reactivity against viral strains not actually included in the vaccine. This is a key attribute, since it is difficult to predict exactly which strain might emerge and cause a pandemic. MF59 adjuvant recently received approval for licensure in Europe for all 27 member states for inclusion in a pandemic vaccine (Focetria®). This same vaccine is also under consideration for approval as a pre-pandemic vaccine (Aflunov®). Beyond its use in influenza vaccines, MF59 adjuvant has also been shown to be a potent adjuvant for a wide range of alternative vaccines, including those based on recombinant proteins, particulate antigens and protein polysaccharide conjugates. In most studies in which a comparison has been made, MF59 has been shown to be more potent for both antibody and T cell responses than aluminum-based adjuvants. Moreover, clinical evaluations have established that the MF59 adjuvant is safe in a wide range of subjects from only a few days old, to greater than 100 years of age. Hence MF59 has broad potential to be used as a safe and effective vaccine adjuvant for a broad range of vaccines, to be used in populations with a wide age range.

Emulsions as adjuvants

Emulsions are defined as liquid dispersions of two immiscible phases, usually an oil and water, either of which may comprise the dispersed phase or the continuous phase to provide water in oil, or oil in water emulsions, respectively. Emulsions are generally unstable and need to be stabilized by surfactants, which lower interfacial tension, and prevent coalescence of the dispersed droplets. Stable emulsions can be prepared through the use of surfactants that orientate at the interface between the two phases and reduce interfacial tensions, since surfactants comprise both hydrophobic and hydrophilic components. Although charged surfactants are excellent stabilizers, non-ionic surfactants are widely used in pharmaceutical emulsions due to their lower toxicity and their lower sensitivity to the destabiliz-

ing effects of formulation additives. Surfactants can be defined by their ratio of hydrophilic to hydrophobic components (hydrophile to lipophile balance, HLB), which gives information on their relative affinity for water and oil phases. At the high end of the scale, surfactants are predominantly hydrophilic and can be used to stabilize oil in water (o/w) emulsions. In contrast, oil-soluble surfactants are at the lower end of the scale and are used mainly to stabilize water in oil (w/o) emulsions. Polysorbates (Tweens) are commonly used surfactants with HLB values in the 9–16 range, while sorbitan esters (Spans) have an HLB in the range of 2–9. Extensive pharmaceutical experience has shown that a mixture of surfactants offers maximum emulsion stability, probably due to the formation of more rigid films at the interface. The physicochemical characteristics of emulsions, including droplet size, viscosity, etc, are controlled by a variety of factors, including the choice of surfactants, the ratio of continuous to dispersed phases and the method of preparation. For an emulsion to be used for administration as an injection, stability and viscosity are important parameters, as too is sterility of course. In general, stability is enhanced by having smaller sized droplets, while viscosity is decreased by having a lower volume of the dispersed phase.

Emulsions have a long history of use as adjuvants in both human and animal vaccines. Almost 70 years ago, Freund demonstrated the adjuvant effect of mineral (paraffin) oil combined with mycobacterial cells, and this adjuvant came to be known as Freund's complete adjuvant (FCA) [1]. The w/o emulsion, without bacterial cells (Freund's incomplete adjuvant, FIA) was subsequently used in veterinary vaccines [2] and even in humans. Recent studies have explored structure activity relationships for w/o emulsion adjuvants of the FIA type [3]. Although w/o emulsions containing mineral oils like FIA have been used as vaccine adjuvants in humans, including influenza vaccines [4], they are generally considered as too reactogenic for human use in prophylactic vaccines [5]. Nevertheless, long-term follow-up has established that there are no significant long-term adverse effects following FIA use in humans, although local reactogenicity was very common [6]. More recently, w/o emulsions with high oil content, based on mineral and non-mineral oils have been evaluated as vaccine adjuvants for malaria and HIV vaccines [7]. Clinical trials have demonstrated that these newer generation w/o emulsions induce potent immune responses, but also induce a significant number of local reactions, which can occasionally be severe [8]. Due to the reactogenicity of w/o emulsions, o/w approaches were evaluated as alternatives, and were initially promoted as delivery systems for immune potentiators [9].

The development of MF59 o/w adjuvant for flu vaccines

In the 1980s, a number of groups worked on the development of novel adjuvant formulations, including emulsions, ISCOMs, liposomes and mic-

roparticles [10]. These approaches had the potential to be more potent and effective adjuvants than insoluble aluminum salts, which were the only adjuvants included in licensed human vaccines at that time. Many of the novel adjuvant approaches contained immune potentiators of natural or synthetic origin, which were included to enhance the potency of the adjuvant. However, the inclusion of immune potentiators often raised concerns about the safety of the adjuvant technology. Based on the long history of emulsions as adjuvants, including FIA, several groups investigated the development of improved emulsion formulations as adjuvants. Syntex developed an o/w emulsion adjuvant (Syntex adjuvant formulation, SAF) using the biodegradable oil, squalane, to deliver a synthetic immune potentiator, called *N*-acetyl-muramyl-L-threonyl-D-isoglutamine (threonyl-MDP) [9]. The closely related immune potentiator, *N*-acetyl-L-alanyl-D-isoglutamine (MDP), had been originally identified in 1974 as the minimal structure isolated from the peptidoglycan of mycobacterial cell walls, which had adjuvant activity [11]. However, MDP was pyrogenic and induced uveitis in rabbits [12], making it unacceptable as an adjuvant for human vaccines. Therefore, various synthetic derivatives of MDP were produced, in an effort to identify an adjuvant molecule with an acceptable safety profile; threonyl-MDP was one of these synthetic compounds. More recently, it has been shown that MDP actually activates immune cells through interaction with the nucleotide-binding domain, which acts as an intracellular recognition system for bacterial components [13]. In addition to threonyl-MDP, SAF also contained a pluronic polymer surfactant (L121), which was included to help bind antigens to the surface of the emulsion droplets. Unfortunately, clinical evaluations of SAF as an adjuvant for an HIV vaccine showed it to have an unacceptable profile of reactogenicity [14]. As an alternative to SAF, Chiron Vaccines used squalene, a similar biodegradable oil, to develop an o/w emulsion as a delivery system for an alternative synthetic MDP derivative, muramyl-tripeptide phosphatidylethanolamine (MTP-PE). MTP-PE was lipidated to allow it to be more easily incorporated into lipid like formulations and to reduce toxicity [15]. Unfortunately, clinical testing also showed that emulsions of MTP-PE displayed an unacceptable level of reactogenicity, which made them unsuitable for routine clinical use [16, 17]. Although the emulsion formulation of MTP-PE enhanced antibody responses against influenza vaccine in humans, the level of adverse effects observed made this adjuvant unsuitable for widespread clinical use [16]. Nevertheless, additional clinical studies undertaken at the same time highlighted that the squalene-based emulsion alone (MF59), without any added immune potentiator, was well tolerated and had comparable immunogenicity to the formulation containing the MTP-PE [17, 18]. These observations resulted in the further development of the MF59 o/w emulsion vehicle alone as an injectable adjuvant.

In pre-clinical studies with influenza vaccine, it was confirmed that the immune potentiator, MTP-PE, was not required for MF59 to be an effective adjuvant [19]. A key early study highlighted the ability of MF59 adjuvant to

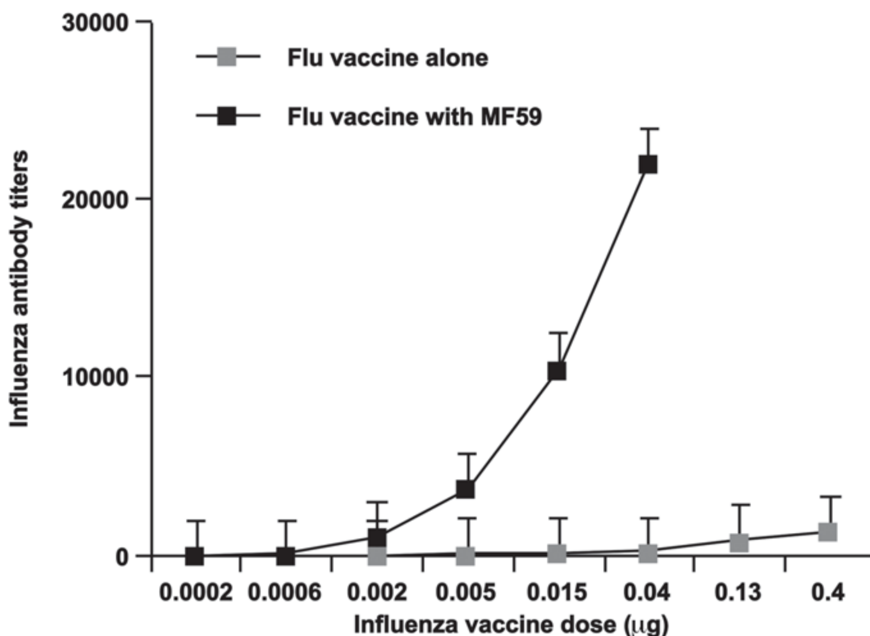


Figure 1. Serum antibody titers in mice at immunized with decreasing dose of influenza vaccine with and without MF59 adjuvant.

enhance protective immunity to flu virus challenge [20]. The use of MF59 adjuvant allowed a dose reduction of flu vaccine (50–200-fold lower doses) and improved protection against challenge for more than 6 months after vaccination [20]. MF59 induced enhanced antibody titers in comparison to flu vaccine alone, even at very low antigen dose (Fig. 1). Moreover, the addition of MF59 to flu vaccine offered improved survival against challenge with influenza virus in mice (Fig. 2) and also reduced viral titers in the lungs of challenged mice (Fig. 3). The enhanced protection afforded by the inclusion of MF59 in the vaccine was long lived and allowed a significant dose reduction in the amount of antigen needed to induce protection (Figs 2 and 3). Moving beyond the mouse model, MF59 was also shown to be an effective adjuvant for flu vaccine in a range of alternative pre-clinical animal models [19]. Importantly, in follow up studies, it was shown that MF59 was able to enhance the immune response to flu vaccines in both young and old animals [21]. Old mice (18 months old in these studies) typically have poor responses to flu vaccines, as do elderly humans, but the inclusion of MF59 in the vaccine restored the response of the old mice back up to the level of response achieved in young mice (Tab. 1). Moreover, MF59 was also shown to induce a potent T cell response to the flu vaccine, both in young and old mice (Tab. 2). Pushing the mouse model further, MF59 was also shown to be

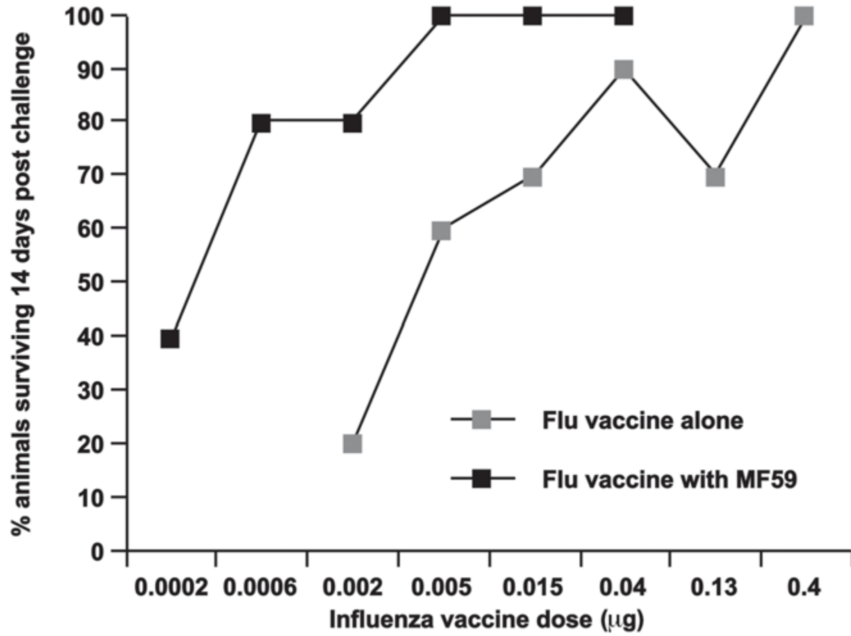


Figure 2. Survival after influenza virus challenge in mice immunized with decreasing doses of influenza vaccine with and without MF59.

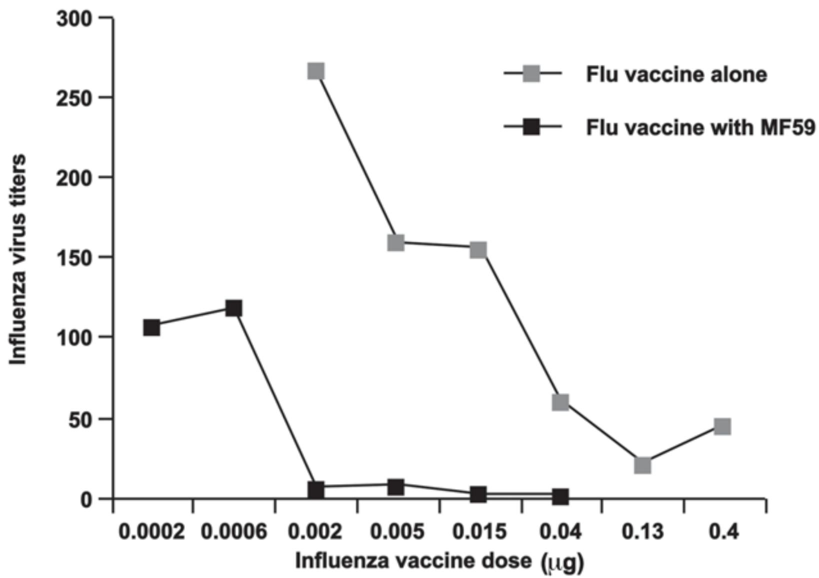


Figure 3. Influenza virus titers post challenge in the lungs of mice immunized with increasing doses of influenza vaccine with and without MF59 (virus titer in naive challenged mice was 346 ± 104).

Table 1. Antibody titers after immunizations with influenza vaccine with and without MF59 in naive mice.

Virus strain	Antibody titers							
	Young				Old			
	Vaccine alone	Vaccine with MF59	Fold increase	p value	Vaccine alone	Vaccine with MF59	Fold increase	p value
A/Beijing	26,470	318,909	12	<0.0001	1253	23,826	19	0.0001
A/Texas	8252	107,461	13	<0.0001	127	11,260	89	0.0033
B/Panama	1074	33,306	31	<0.0001	15	3795	253	0.0058

Geometric mean antibody titers in young (8 weeks) or old mice (18 months). p value, ANOVA (Fisher's PSLD)

Table 2. T-cell proliferative responses from splenocytes of mice immunized with influenza vaccine with and without MF59.

Virus strain	Simulation index							
	Young				Old			
	Vaccine alone	Vaccine with MF59	Fold increase	p value	Vaccine alone	Vaccine with MF59	Fold increase	p value
A/Beijing	13.34	24.41	1.83	0.2056	3.93	11.88	3.02	0.0596
A/Texas	13.66	28.27	2.07	0.1562	4.36	12.53	2.87	0.0351
B/Panama	7.84	13.04	1.66	0.1699	2.25	6.87	3.05	0.0215

Values are shown as geometric mean stimulation indices in young (8 weeks) or old mice (18 months). p value, ANOVA (Fisher's PSLD)

Table 3. Antibody titers immunization with influenza vaccine with and without MF59 in mice pre-infected with A/Taiwan virus

Virus strain	Antibody titer[a]							
	Young				Old			
	PBS	Vaccine alone	Vaccine with MF59	p value	PBS	Vaccine alone	Vaccine with MF59	p value
A/Beijing	10	2622	2588	0.7656	14	150	582	0.0132
A/Taiwan	25,520	124,400	165,049	0.1469	4319	13,024	31,863	0.0287
B/Panama	10	810	888	0.4138	10	19	142	0.0091

Geometric mean antibody titers in young (8 weeks) or old mice (18 months). p values shown for difference between hemagglutinin (HA) alone and MF59+HA.

an effective adjuvant in old mice, which had previously been infected with influenza (Tab. 3), a situation more similar to that found in humans, who are often re-infected annually with circulating flu strains [21]. These pre-clinical studies highlighted the huge potential of MF59 to be used as an adjuvant for an improved flu vaccine, potentially allowing antigen dose reduction, while enhancing protective antibody and T cell responses, for extended time periods. The ability of MF59 adjuvant to offer a significant reduction in the protective dose for flu vaccines has subsequently become very important in the pandemic flu vaccine setting.

The small droplet size of MF59 adjuvant emulsion, generated through the use of a microfluidizer in the preparation process, is crucial to the potency of the adjuvant, but also enhances emulsion stability and allows the formulation to be sterile filtered for clinical use. Overall, our early clinical experience with o/w emulsions serves to highlight the need for careful selection of immune potentiators, to be included in adjuvant formulations, should they prove necessary, or desirable. Nevertheless, the experience with MF59 also shows that o/w emulsions can be highly effective adjuvants, with an acceptable safety profile, which may not need the addition of immune potentiators.

The mechanism of action of MF59 adjuvant

Early studies designed to determine the mechanism of action of MF59 focused on the possibility of the creation of a ‘depot’ effect for co-administered antigen, since there had been suggestions that emulsions may retain antigen at the injection site. However, early work showed that an antigen depot was not established at the injection site and that the emulsion was cleared rapidly [22]. The lack of an antigen depot with MF59 was confirmed in later studies [23], which also established that MF59 and antigen were cleared independently. Subsequently, it was thought that perhaps the emulsion acted as a ‘delivery system’ and was responsible for promoting the uptake of antigen into antigen-presenting cells (APC). This theory was linked to earlier observations with SAF, which contained a pluronic surfactant that was thought to be capable of binding antigen to the emulsion droplets to promote antigen uptake [9]. However, studies with recombinant antigens showed that MF59 was an effective adjuvant, despite no evidence of binding of the antigens to the oil droplets. Moreover, an adjuvant effect was still observed if MF59 was injected up to 24 h before the antigen and up to 1 h after, confirming that direct association was not required for an adjuvant effect [22]. Nevertheless, administration of MF59 24 h after the antigen resulted in a much reduced adjuvant effect, suggesting that the emulsion was activating immune cells, which were then able to better process and present the co-administered antigen. A direct effect of MF59 on cytokine levels *in vivo* was also observed in separate studies, suggesting that the

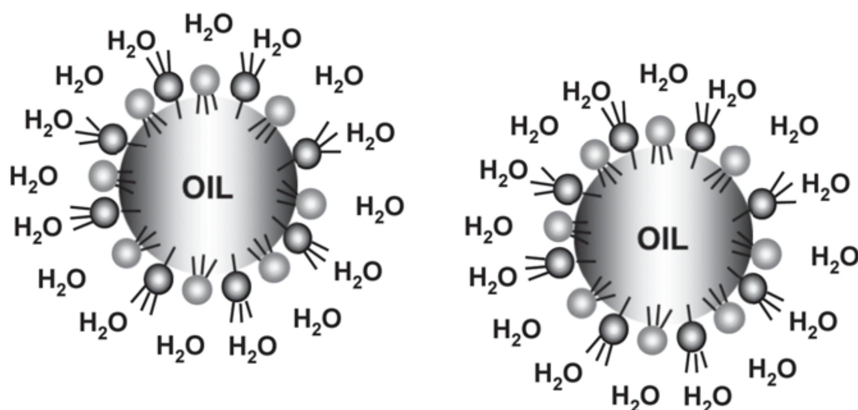
delivery method alone was too simplistic an explanation [24]. To gain a better understanding of the mechanism of action of MF59, we recently studied the early steps of the immune response on human cells *in vitro* and in mouse muscle *in vivo*. We have shown that there are at least two human target cells for MF59, monocytes and granulocytes, and that MF59 has a range of effects, including increased antigen uptake, the release of chemoattractants and cell differentiation. The observation of increased antigen uptake is in line with previous findings in mice [25]. The most readily induced chemoattractant was the chemokine, CCL2, which is involved in cell recruitment. Previous work had shown a reduction of MF59-induced cell recruitment into the muscle in CCR2-deficient mice [26], which is consistent with our observations on human cells. Moreover, ongoing experiments on gene expression profiles at the injection site are also consistent with the key role of chemokines. In addition, CCL2 was also found in serum after injection of MF59 into mouse muscle, providing further consistency between *in vitro* and *in vivo* observations. MF59 also induces phenotypic changes on human monocytes that are consistent with a maturation process towards immature dendritic cells (DCs). These observations will be reported in detail separately [27], but so far there appears to be an impressive consistency between data obtained *in vitro* with human cells, and the *in vivo* data from mouse. These observations suggest that MF59 induces a local pro-inflammatory environment within the muscle, which promotes the induction of potent immune responses to co-administered vaccines.

Hence, we conclude that during vaccination, adjuvants like MF59 augment the immune response at a range of intervention points. Through induction of chemokines, they increase recruitment of immune cells to the injection site, they augment Ag uptake by monocytes at the injection site, and they enhance differentiation of monocytes into DCs, which represent the gold-standard cell type for priming naive T cells. A particularly important feature of MF59 is that it strongly induces the homing receptor CCR7 on maturing DCs, thus facilitating their migration into draining lymph nodes where they can trigger the adaptive immune response specific to the vaccine antigen. Nevertheless, further studies are necessary to better define the precise mechanism of action of MF59 and these studies are ongoing.

The composition of MF59

MF59 is a low oil content o/w emulsion. The oil used for MF59 is squalene, which is a naturally occurring substance found in plants and in the livers of a range of species, including humans. Squalene is an intermediate in the human steroid hormone biosynthetic pathway and is a direct synthetic precursor to cholesterol. Therefore, squalene is biodegradable and biocompatible, since it is naturally occurring. Shark liver oil comprises 80% squalene and shark liver provides the natural source of the squalene,

Appearance: milky white oil in water (o/w emulsion)



Composition: 0.5% Polysorbate 80, water soluble surfactant
0.5% Sorbitan Triolate, oil soluble surfactant
4.3% Squalene oil
Water for injection
10 nM Sodium citrate buffer

Density: 0.9963 g/ml **Droplet Size:** ~150nm (sterile filtration)

Viscosity: close to water, easy to inject

Figure 4. The composition of MF59 emulsion adjuvant

which is used to prepare MF59. MF59 also contains two non-ionic surfactants Tween 80 and Span 85, which are designed to optimally stabilize the emulsion droplets. Figure 4 shows the composition of MF59 adjuvant. Citrate buffer is also used in MF59 to stabilize pH. Although single vial formulations can be developed with vaccine antigens dispersed directly in MF59, MF59 can also be added to antigens immediately prior to their administration. Although a less favorable option, combination prior to administration may be necessary to ensure optimal antigen stability for some antigens, but not for flu.

Manufacturing of MF59

Details of the manufacturing process for MF59 at the 50-liter scale have previously been described [28]. The process involves dispersing Span 85 in the squalene phase and Tween 80 in the aqueous phase, before high speed mixing to form a coarse emulsion. The coarse emulsion is then passed repeatedly through a microfluidizer to produce an emulsion of uniform

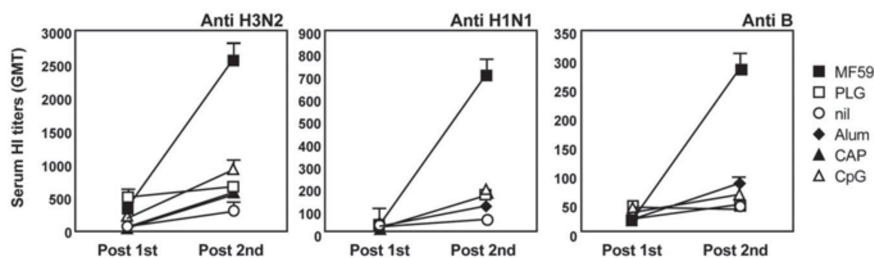


Figure 5. Serum hemagglutination (HA) titers against the three stains of influenza virus included in vaccines (H3N2, H1N1 and B) for flu cell culture vaccine in combination with various adjuvants. The adjuvants evaluated included MF59, aluminum salt (Alum), calcium phosphate (CAP), poly lactide co-glycolide microparticles (PLG), CpG oligonucleotide (CpG) and vaccine alone without adjuvant (nil). MF59 was the most potent adjuvant for all three strains.

small droplet size (165 nm), which can be sterile filtered and filled into vials. Methods have also been published previously to allow the preparation of MF59 on a small scale, for use in research studies [29]. MF59 is extensively characterized by various physicochemical criteria after preparation.

Pre-clinical experience with MF59

Pre-clinical experience with MF59 is extensive and has been reviewed on several occasions previously [28, 30, 31]. MF59 has been shown to be a potent adjuvant in a diverse range of species, in combination with a broad range of vaccine antigens, to include recombinant protein antigens, isolated viral membrane antigens, bacterial toxoids, protein polysaccharide conjugates, peptides and virus-like particles. MF59 is particularly effective for inducing high levels of antibodies, including functional titers (neutralizing, bactericidal and opsonophagocytic titers) and is generally more potent than Alum.

In a recent study, we directly compared MF59 and Alum for several different vaccines and confirmed that MF59 was more potent, although Alum performed well for bacterial toxoid antigens, particularly diphtheria toxoid [32]. MF59 has also shown enhanced potency over alum when directly compared in non-human primates with protein polysaccharide conjugate vaccines [33] and with a recombinant viral antigen [29].

In pre-clinical studies, MF59 is the most potent adjuvant for flu vaccines, in comparison to various alternatives (Fig. 5). In a recent study, we compared a number of adjuvants for flu vaccine in mice, and showed that MF59 significantly outperforms alternatives, including Alum, for both antibody and T cell responses [34]. Moreover, we have recently shown that MF59 offers enhanced protection against challenge with pandemic flu strains in mice (Unpublished observations, Kanta Subbarao), which is consistent with

our earlier work on interpandemic strains [20]. In addition to immunogenicity studies, extensive pre-clinical toxicology studies have been undertaken with MF59, in combination with a range of different antigens in a number of species. In these studies, it has been shown that MF59 is neither mutagenic, nor teratogenic, and did not induce sensitization in an established guinea pig model to assess contact hypersensitivity. The favorable toxicological profile established for MF59 allowed extensive clinical testing for MF59 with a number of different vaccine candidates, and the approval of a flu vaccine containing MF59 in Europe in 1997.

Clinical experience with MF59 adjuvant

Fluad® in elderly subjects

Fluad®, which was initially licensed as an interpandemic influenza vaccine in Italy in 1997 and is now licensed in more than 20 countries, contains MF59, which was the first novel adjuvant accepted for human use after the registration of insoluble alum salts in the first part of the 20th century. The registration of Fluad® was based on the results of a large clinical development plan, which showed, in more the 20,000 subjects, that the MF59-adjuvanted vaccine was well tolerated and more immunogenic than conventional non-adjuvanted influenza vaccines. The adjuvant is generally well tolerated, with only a low incidence of local mild reactions following immunization, and with no increases in incidence following subsequent immunizations [35]. The adjuvanted influenza vaccine was initially developed for vaccination of the elderly to fill the medical need for an improved influenza vaccine for this age group, in which conventional influenza vaccines are cost effective, but do not provide optimal protection [36]. For this reason, most of the early clinical trials with Fluad® were performed in elderly subjects. In this population, the adjuvanted vaccine consistently induced enhanced geometric mean titers, seroconversion rates and seroprotection rates The increased immunogenicity of Fluad® was particularly important in subsets of the elderly population, who have a higher risk of developing influenza and its most severe complications; this includes subjects with a low pre-immunization titer and subjects affected by chronic cardiovascular and respiratory diseases [35, 37]. An increased response against heterovariant influenza strains, different from the strains included in the vaccine, was also consistently observed. This will be particularly beneficial whenever the vaccine antigens do not match perfectly those of the circulating viruses [35, 38–40]. More recent data have indicated that younger adults may also benefit from immunization with Fluad®, particularly populations at high risk of influenza-related complications, such as HIV-positive subjects (Fig. 6) and those affected by chronic diseases (Fig. 7) [41, 42]. Importantly, the addition of MF59 to the influenza antigens did not affect the safety profile of the vaccine, which was very well

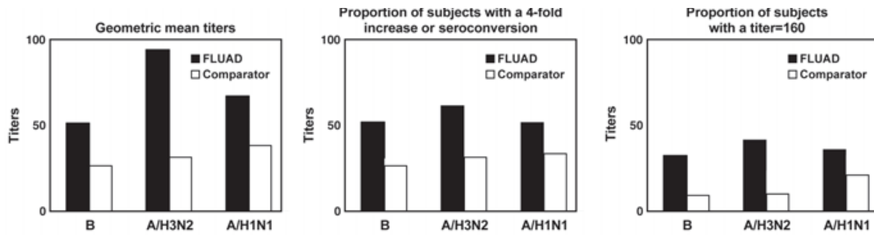


Figure 6. Comparison of hemagglutination inhibition antibody titers in HIV-1-seropositive individuals immunized with MF59 adjuvanted vaccine (Fluad®) or with a non-adjuvanted comparator. Against all three stains in the vaccine, MF59 induced higher geometric mean titers, a higher proportion of individuals seroconverted and a higher proportion of subjects achieved a hemagglutination titer of >160.

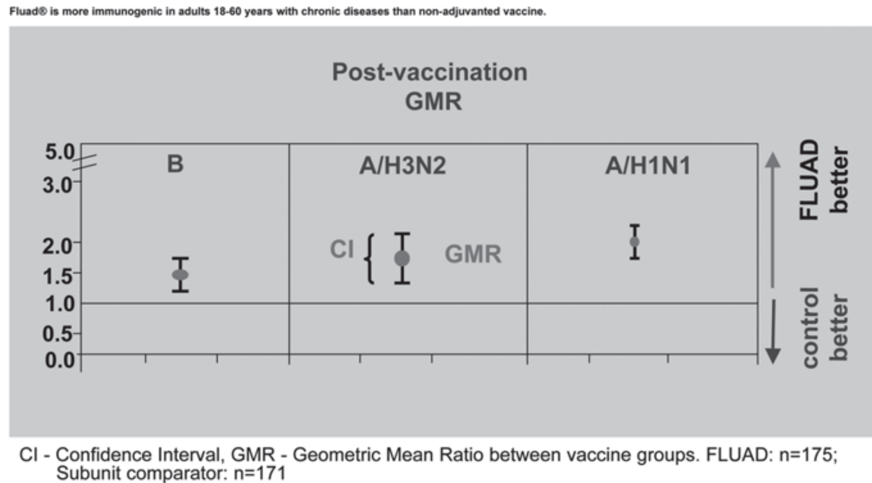


Figure 7. Fluad® is more immunogenic in adults 18–60 years with chronic diseases than non-adjuvanted vaccines. Geometric mean ratio of hemagglutination inhibition response in adults (18–60 years of age) with chronic diseases who were immunized with an MF59-adjuvanted subunit influenza vaccine *versus* a similar group immunized with a vaccine without MF59. Geometric mean ratios were higher for the MF59-adjuvanted group (Fluad®), for all three vaccine strains.

tolerated in all clinical trials performed in elderly and non-elderly subjects [35]. Most encouragingly, clinical effectiveness data have now begun to accumulate to indicate that the increased immunogenicity of MF59 also translates into improved protection. Puig-Barbera et al. [43] described the improved effectiveness of MF59 in preventing emergency admissions for pneumonia, while superior clinical protection against influenza-like illness has been reported [44]. The data showing improved clinical effectiveness

of MF59-adjuvanted influenza vaccine were recently reviewed [45], and showed significantly reduced influenza-like disease, and reduced hospitalization rates for pneumonia, cardiovascular and cerebrovascular disease in the elderly. Importantly, MF59 can compensate for the reduced efficacy of vaccines, which occasionally happens due to a mismatch between the antigenic composition and the circulating influenza strains [46]. It has been shown on several occasions both in the elderly and in non-elderly subjects, that MF59 induces a significantly enhanced response against heterovariant virus strains not included in the vaccine [35, 38–40, 47].

With more than 35 million doses distributed, the safety of Fluad® is supported by extensive pharmacovigilance data showing that vaccination with Fluad® is associated with a very low frequency of adverse reactions [48, 49]. In fact, the safety profile of Fluad® is broadly similar to the profile for non-adjuvanted flu vaccines [49]. Moreover, it has been shown that the rate of Guillain-Barre syndrome after Fluad® immunization is within the normal range of rates found in the US after immunization with conventional non-adjuvanted influenza vaccines [49]. In addition, a recent study highlighted that immunization with MF59 adjuvant neither raises the levels of pre-existing antibodies, nor induces new antibody responses against squalene, although antibodies to squalene are already naturally occurring in many subjects [50].

Fluad® in young children

In a recent clinical trial, we evaluated the potency of an MF59-adjuvanted subunit influenza vaccine in young children (6–36 months of age) and directly compared it with a licensed split influenza vaccine product. The MF59-adjuvanted vaccine (Fluad®) was significantly more immunogenic than the comparator for all three strains included in the vaccine. Moreover, the MF59-adjuvanted vaccine met all three EMEA CHMP criteria to gain vaccine approval for healthy adults (seroconversion, seroprotection and mean geometric increase in response), while the comparator vaccine did not (unpublished data). In addition, the Fluad® vaccine offered significantly enhanced protection against heterovariant strains not included in the vaccine, in this vulnerable population (Pellegrini et al., MF59 adjuvant enhances cross protective antibody responses in young children against heterovariant influenza strains; in preparation). Importantly, Fluad® was well tolerated in this young population and had a similar reactogenicity profile to the split vaccine product (T. Vesikari, personal communication).

Nevertheless, despite these encouraging data, the safety of MF59 adjuvant needs to be carefully evaluated in this young population, prior to consideration for product approval. However, the data on the safety of MF59 in toddlers, infants and newborns are very encouraging and suggest that the adjuvant will have an acceptable safety profile in young children [51, 52].

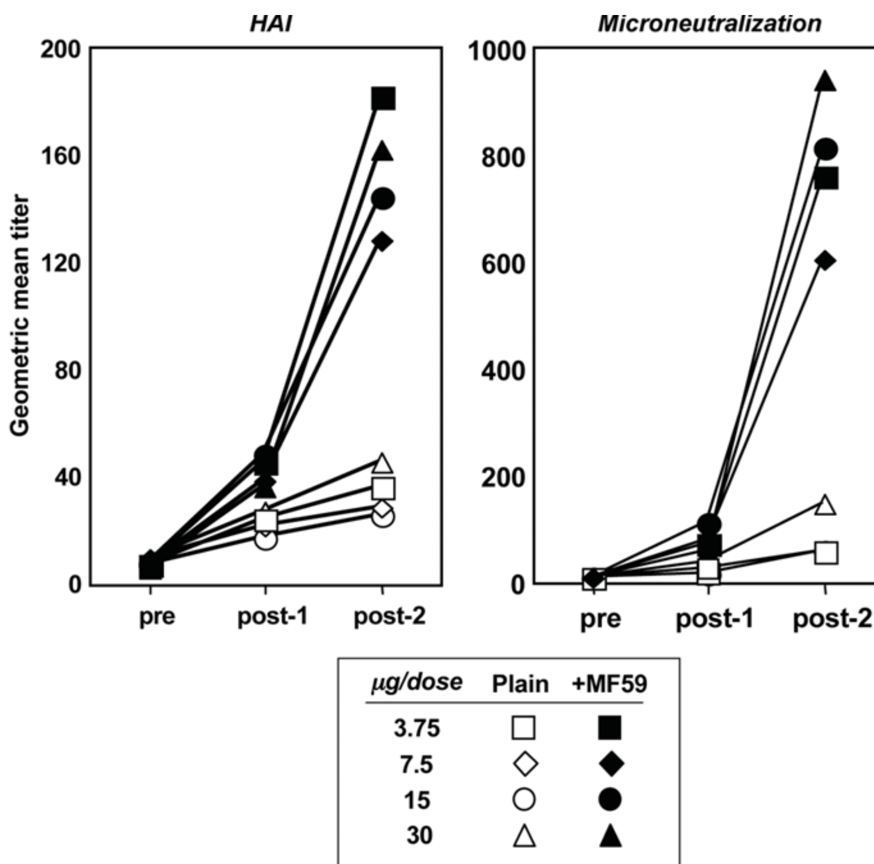


Figure 8. Serum hemagglutination inhibition and microneutralization geometric mean titers against H9N2 influenza at varying vaccine doses (3.75–30 mg) with and without MF59 adjuvant after one and two vaccine doses (post-1 and post-2) in healthy adults (18–34 years of age). All dose levels of vaccine were significantly more potent with MF59 than without (plain). Moreover, one dose of vaccine with MF59 adjuvant was comparable to two doses of the plain vaccine.

Pandemic flu vaccines containing MF59 (Focetria® and Aflunov®)

Unprecedented efforts are being undertaken worldwide to develop plans to mitigate the dire public health consequences of an influenza pandemic. The development of effective vaccines is of paramount importance for these plans. Recent studies have established that, since humans are naive to potential pandemic influenza strains, they respond poorly to conventional vaccines and large doses are needed to induce acceptable levels of antibodies [53]. In contrast, the response to potential pandemic flu vaccines is much improved by the addition of MF59 adjuvant [54–56] (Fig. 8). MF59

also allows for significant dose sparing, which is essential to increase the pandemic vaccine production capacity. Unfortunately, traditional adjuvants, like Alum do not appear to be potent for pandemic influenza vaccines [57]. In contrast, hemagglutinin (HA) antigens derived from several potentially pandemic strains, including H5N3, H9N2 and H5N1, have been combined with MF59 and induced a significant enhancement of antibody titers compared to non-adjuvanted vaccines ([55, 56, 58] and A. Banzhoff A, personal communication). Importantly, adjuvantation with MF59 allowed a significant reduction in the antigen concentration per dose (Fig. 8). This finding has significant implications in terms of production capacity for pandemic vaccines [58]. Notably, 7.5 μg MF59-adjuvanted H5 HA and 3.75 μg MF59-adjuvanted H9 HA were significantly more immunogenic than 30 μg of the respective plain HAs [56, 58]. As already shown for the interpandemic vaccine, broader cross-neutralization against heterovariant pandemic strains was an additional benefit of an MF59-adjuvanted vaccine [59]. These data were used to gain approval for a new pandemic influenza vaccine containing the MF59 adjuvant (Focetria®) in May 2007 in all 27 member states of the European Union, and to submit for approval as a pre-pandemic vaccine (Aflunov®). Focetria® will be manufactured to contain the influenza strain declared by the World Health Organization as a pandemic strain. Recent data have shown that the addition of MF59 to a potential pre-pandemic flu vaccine (Aflunov®) allows the vaccine to meet all three CHMP criteria for immunogenicity for interpandemic flu vaccines in adults at a 7.5-μg dose level. Moreover, the vaccine was shown to induce high levels of neutralizing antibodies in more than 80% of subjects immunized, while being very well tolerated in all populations immunized (Banzhoff et al., unpublished data). Moreover, the addition of the adjuvant induced potent $CD4^+$ T cell-mediated immune responses after a single immunization (Del Giudice et al., unpublished data). The use of a pre-pandemic flu vaccine would be likely to significantly reduce the catastrophic public health consequences of a pandemic, since large subsets of the population might be already protected or at least primed against the pandemic virus at the initiation of the pandemic threat. The dose-sparing aspect of MF59 is particularly attractive for pandemic flu vaccines, given the current limited capacity worldwide for influenza vaccine production, which is not sufficient to deal with a pandemic, given the high-dose requirements for unadjuvanted influenza vaccines [60].

Although clinical trials with interpandemic and pandemic vaccines represent the great majority of the clinical experience with MF59, several trials have been performed also with other MF59-adjuvanted investigational vaccines, including HIV, HSV, CMV, HBV and HCV. These studies have provided additional evidence of the safety, tolerability and adjuvanticity of MF59 [61–63]). The clinical experience with these vaccines has been previously reviewed [30]. Of note, MF59 has been used as an adjuvant for pediatric vaccines such as the CMV and HIV vaccines. Seronegative toddlers immunized with the CMV gB vaccine showed antibody titers that were

higher than those found in adults naturally infected with CMV. Moreover, the MF59-adjuvanted vaccine was well tolerated in this age group [51]. Additionally, an MF59-adjuvanted HIV vaccine was evaluated in newborns, born to HIV-positive mothers [52, 64, 65]. The vaccine was very well tolerated and, despite the presence of maternal antibodies, induced an antibody response in 87% of the immunized infants [52, 65]. Moreover, the MF59 vaccine was significantly more potent than alum for the induction of cell-mediated immune responses (proliferative T cell responses) against homologous and heterologous strains of HIV [64].

In summary, clinical testing of MF59 adjuvant has resulted in the registration in more than 20 countries of an effective and well-tolerated influenza vaccine for use in the elderly population. In addition, clinical trials have demonstrated that MF59 can be safely administered with a range of antigens, to diverse age groups, including the pediatric population.

Combination of MF59 with immunopotentiators

Although MF59 is generally a more potent adjuvant than alum [22], it cannot be expected to be a suitable adjuvant for all vaccines; there is no "universal adjuvant". However, MF59 is particularly effective for enhancing antibody and T cell proliferative responses [22, 28]. However, it is not a potent adjuvant for the induction of Th1 cellular immune responses in pre-clinical models involving naive mice [22, 28]. Potent Th1 responses may be required to provide protective immunity against some viruses and additional intracellular pathogens. Th1 immune potentiators, including CpG oligonucleotides [66] have been successfully added to MF59 to improve its potency and to alter the kind of response induced [67]. Although the formulation of MF59 can be modified to promote the association of CpG with the oil droplets [67], more recent studies suggest that this may not be necessary, and simple addition of CpG to MF59 may be sufficient in some situations [34]. However, careful consideration is required in relation to which immune potentiators to add to MF59 emulsion and how best to formulate them. Although pre-clinical studies showed that the potency of MF59 was enhanced by the inclusion of MTP-PE [68], our early experience in the clinic showed that MTP-PE added to MF59 gave an unacceptable level of reactogenicity [16, 17]. Unfortunately, the animal models available at the time were not able to predict the poor tolerability of MTP-PE in humans. Fortunately, subsequent data showed that the inclusion of MTP-PE was not necessary to enhance the immunogenicity of antigens combined with MF59.

In addition to immune potentiators, alternative delivery systems, including microparticles can also be added to MF59 to enhance its potency [69]. However, the level of enhancement achieved would need to be highly significant and enabling for vaccine efficacy, to justify development of such a complex formulation.

The use of MF59 in prime/boost settings

As an alternative to the inclusion of immune potentiators in MF59 to promote a Th1 response, MF59 can also be used as a booster vaccine with proteins once a Th1 response has already been established by immunization with DNA [70]. Recently, this strategy has been shown to be highly promising for the development of a vaccine against HIV, since all arms of the immune response, including CTL responses, T helper responses and neutralizing antibodies were induced by this combination approach [71–74]. A similar approach of DNA prime and protein boost with MF59 has also shown significant promise in non-human primates as a vaccine strategy against HCV [75]. Alternatively, MF59 and protein antigens can also be used to boost Th1 responses primed by immunization with attenuated viral vectors. The concept of an attenuated viral vector prime followed by MF59 boost has been established in the clinic using canarypox vectors, as a strategy for both HIV [76] and CMV [77].

Conclusion

In an extensive range of pre-clinical and clinical studies MF59 has proven to be a safe and potent vaccine adjuvant, resulting in the licensure of an MF59-adjuvanted influenza vaccine in more than 20 countries. The ability of MF59 to induce significantly enhanced titers against potential pandemic flu strains at low antigen doses appears highly promising, as too does the ability of MF59 to offer neutralization against heterologous strains. These data were used to gain approval for a new pandemic influenza vaccine containing the MF59 adjuvant (Focetria®) in May 2007 in all 27 member states of the European Union. Focetria® will be manufactured to contain the influenza strain declared by the World Health Organization as a pandemic strain. The dose-sparing aspect of MF59 is particularly attractive, given the current limited capacity worldwide for influenza vaccine production, which is not sufficient to deal with a pandemic, given the high dose requirements for unadjuvanted influenza vaccines [60].

The encouraging safety and tolerability profile of MF59, in combination with immunogenicity data, suggest that MF59 is an appropriate adjuvant for use in pediatric populations. In a trial in neonates, MF59 was shown to be more potent than an Alum-adjuvanted comparator vaccine, while being similarly well tolerated [52]. An MF59-adjuvanted CMV vaccine was also shown to be safe, potent and well tolerated in toddlers [51]. Moreover, preclinical data have firmly established that MF59 is a more potent adjuvant than Alum for a wide range of vaccines, including recombinant proteins and protein polysaccharide conjugates [32]. The potential for MF59 to be used in recombinant vaccines was recently highlighted in a study on a new generation serogroup B meningococcus vaccine candidate [78]. Previously, MF59

had been shown to enhance immune responses to protein polysaccharide conjugate vaccines, including against *Neisseria meningitides* serogroup C [33]. Hence, MF59 has broad potential to be used as a vaccine adjuvant for a range of vaccines, in diverse age groups, including infants. Although M59 has been mainly used in elderly adults to date, recent data have highlighted the significant potential of MF59 to be used in an improved influenza vaccine for young children. We confidently expect that the adjuvant will gain approval for wider use in diverse population groups in the forthcoming years.

References

1 Freund J, Casals J, Hosmer EP (1937) Sensitization and antibody formation after injection of turbecle bacili and paraffin oil. *Proc Soc Exp Biol Med* 37: 509–513

2 Hilleman MR (1966) Critical appraisal of emulsified oil adjuvants applied to viral vaccines. *Prog Med Virol* 8: 131–182

3 Jansen T, Hofmans MP, Theelen MJ, Schijns VE (2005) Structure-activity relations of water-in-oil vaccine formulations and induced antigen-specific antibody responses. *Vaccine* 23: 1053–1060

4 Salk JE, Laurent AM, Bailey ML (1951) Direction of research on vaccination against influenza; new studies with immunologic adjuvants. *Am J Public Health* 41: 669–677

5 Edelman R (1980) Vaccine adjuvants. *Rev Infect Dis* 2: 370–383

6 Page W (1993) Long-term followup of Army recruits immunized with Freund's incomplete adjuvanted vaccine. *Vaccine* Research 2: 141–149

7 Aucouturier J, Dupuis L, Deville S, Ascarateil S, Ganne V (2002) Montanide ISA 720 and 51: A new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev Vaccines* 1: 111–118

8 Audran R, Cachat M, Lurati F, Soe S, Leroy O, Corradin G, Druilhe P, Spertini F (2005) Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. *Infect Immun* 73: 8017–8026

9 Allison AC, Byars NE (1986) An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity. *J Immunol Methods* 95: 157–168

10 Vogel FR, Pruett MF (1995) A compendium of vaccine adjuvants and excipients. In: MF Powell, MJ Newman (eds): *Vaccine Design: The Subunit and Adjuvant Approach.* Plenum Press, New York, 141–228

11 Ellouz F, Adam A, Ciorbaru R, Lederer E (1974) Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem Biophys Res Commun* 59: 1317–1325

12 Waters RV, Terrell TG, Jones GH (1986) Uveitis induction in the rabbit by muramyl dipeptides. *Infect Immun* 51: 816–825

13 Fritz JH, Ferrero RL, Philpott DJ, Girardin SE (2006) Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* 7: 1250–1257

14 Kenney RT, Edelman R (2004) *New Generation Vaccines.* Marcel Dekker, New York

15 Wintsch J, Chaignat CL, Braun DG, Jeannet M, Stalder H, Abrignani S, Montagna D, Clavijo F, Moret P, Dayer JM et al (1991) Safety and immunogenicity of a genetically engineered human immunodeficiency virus vaccine. *J Infect Dis* 163: 219–225
16 Keitel W, Couch R, Bond N, Adair S, Van Nest G, Dekker C (1993) Pilot evaluation of influenza virus vaccine (IVV) combined with adjuvant. *Vaccine* 11: 909–913
17 Keefer MC, Graham BS, McElrath MJ, Matthews TJ, Stablein DM, Corey L, Wright PF, Lawrence D, Fast PE, Weinhold K et al (1996) Safety and immunogenicity of Env 2-3, a human immunodeficiency virus type 1 candidate vaccine, in combination with a novel adjuvant, MTP-PE/MF59. NIAID AIDS Vaccine Evaluation Group. *AIDS Res Hum Retroviruses* 12: 683–693
18 Kahn JO, Sinangil F, Baenziger J, Murcar N, Wynne D, Coleman RL, Steimer KS, Dekker CL, Chernoff D (1994) Clinical and immunologic responses to human immunodeficiency virus (HIV) type 1SF2 gp120 subunit vaccine combined with MF59 adjuvant with or without muramyl tripeptide dipalmitoyl phosphatidylethanolamine in non-HIV-infected human volunteers. *J Infect Dis* 170: 1288–1291
19 Ott G, Barchfeld GL, Van Nest G (1995) Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59. *Vaccine* 13: 1557–1562
20 Cataldo DM, Van Nest G (1997) The adjuvant MF59 increases the immunogenicity and protective efficacy of subunit influenza vaccine in mice. *Vaccine* 15: 1710–1715
21 Higgins DA, Carlson JR, Van Nest G (1996) MF59 adjuvant enhances the immunogenicity of influenza vaccine in both young and old mice. *Vaccine* 14: 478–484
22 Ott G, Barchfeld GL, Chernoff D, Radhakrishnan R, van Hoogevest P, Van Nest G (1995) MF59: Design and evaluation of a safe and potent adjuvant for human vaccines. In: MF Powell, MJ Newman (eds.): *Vaccine Design: The Subunit and Adjuvant Approach*. Plenum Press, New York, 277–296
23 Dupuis M, McDonald DM, Ott G (1999) Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice. *Vaccine* 18: 434–439
24 Valensi JP, Carlson JR, Van Nest GA (1994) Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. *J Immunol* 153: 4029–4039
25 Dupuis M, Murphy TJ, Higgins D, Ugozzoli M, Van Nest G, Ott G, McDonald DM (1998) Dendritic cells internalize vaccine adjuvant after intramuscular injection. *Cell Immunol* 186: 18–27
26 Dupuis M, Denis-Mize K, LaBarbara A, Peters W, Charo IF, McDonald DM, Ott G (2001) Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis. *Eur J Immunol* 31: 2910–2918
27 Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A (2008) The adjuvants aluminium hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation towards dendritic cells. *J Immunol* 180: 5402–5412
28 Ott G (2000) The Adjuvant MF59: A ten year perspective. In: D O'Hagan (ed):

Vaccine Adjuvants: Preparation Methods and Research Protocols. Humana Press, Totowa, 211–228
29 Traquina P, Morandi M, Contorni M, Van Nest G (1996) MF59 adjuvant enhances the antibody response to recombinant hepatitis B surface antigen vaccine in primates. *J Infect Dis* 174: 1168–1175
30 Podda A, Del Giudice G (2003) MF59-adjuvanted vaccines: Increased immunogenicity with an optimal safety profile. *Expert Rev Vaccines* 2: 197–203
31 Podda A, Del Giudice G, O'Hagan DT (2005) MF59: A safe and potent adjuvant for human use. In: V Schijns, DT O'Hagan (eds): *Immunopotentiators in Modern Vaccines*, Chapter 9. Elsevier Press, Amsterdam, 149
32 Singh M, Ugozzoli M, Kazzaz J, Chesko J, Soenawan E, Mannucci D, Titta F, Contorni M, Volpini G, Del Guidice G, O'Hagan DT (2006) A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens. *Vaccine* 24: 1680–1686
33 Granoff DM, McHugh YE, Raff HV, Mokatrin AS, Van Nest GA (1997) MF59 adjuvant enhances antibody responses of infant baboons immunized with Haemophilus influenzae type b and Neisseria meningitidis group C oligosaccharide-CRM197 conjugate vaccine. *Infect Immun* 65: 1710–1715
34 Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, Tavarini S, Scheffczik H, Ugozzoli M, Singh M, Kazzaz J et al (2008) Combination adjuvants for the induction of potent, long-lasting antibody and T cell responses to influenza vaccine. *Vaccine* 26: 552–561
35 Podda A (2001) The adjuvanted influenza vaccines with novel adjuvants: Experience with the MF59-adjuvanted vaccine. *Vaccine* 19: 2673–2680
36 Strassburg MA, Greenland S, Sorvillo FJ, Lieb LE, Habel LA (1986) Influenza in the elderly: Report of an outbreak and a review of vaccine effectiveness reports. *Vaccine* 4: 38–44
37 Banzhoff A, Nacci P, Podda A (2003) A new MF59-adjuvanted influenza vaccine enhances the immune response in the elderly with chronic diseases: Results from an immunogenicity meta-analysis. *Gerontology* 49: 177–184
38 Minutello M, Senatore F, Cecchinelli G, Bianchi M, Andreani T, Podda A, Crovari P (1999) Safety and immunogenicity of an inactivated subunit influenza virus vaccine combined with MF59 adjuvant emulsion in elderly subjects, immunized for three consecutive influenza seasons. *Vaccine* 17: 99–104
39 De Donato S, Granoff D, Minutello M, Lecchi G, Faccini M, Agnello M, Senatore F, Verweij P, Fritzell B, Podda A (1999) Safety and immunogenicity of MF59-adjuvanted influenza vaccine in the elderly. *Vaccine* 17: 3094–3101
40 Del Giudice G, Hilbert AK, Bugarini R, Minutello A, Popova O, Toneatto D, Schoendorf I, Borkowski A, Rappuoli R, Podda A (2006) An MF59-adjuvanted inactivated influenza vaccine containing A/Panama/1999 (H3N2) induced broader serological protection against heterovariant influenza virus strain A/Fujian/2002 than a subunit and a split influenza vaccine. *Vaccine* 24: 3063–3065
41 Iorio AM, Francisci D, Camilloni B, Stagni G, De Martino M, Toneatto D, Bugarini R, Neri M, Podda A (2003) Antibody responses and HIV-1 viral load in HIV-1-seropositive subjects immunised with either the MF59-adjuvanted influenza vaccine or a conventional non-adjuvanted subunit vaccine during highly active antiretroviral therapy. *Vaccine* 21: 3629–3637

42 Baldo V, Baldovin T, Floreani A, Carraro AM, Trivello R (2007) MF59-adjuvanted influenza vaccine confers superior immunogenicity in adult subjects (18–60 years of age) with chronic diseases who are at risk of post-influenza complications. *Vaccine* 25: 3955–3961
43 Puig-Barbera J, Diez-Domingo J, Perez Hoyos S, Belenguer Varea A, Gonzalez Vidal D (2004) Effectiveness of the MF59–adjuvanted influenza vaccine in preventing emergency admissions for pneumonia in the elderly over 64 years of age. *Vaccine* 23: 283–289
44 Iob A, Brianti G, Zamparo E, Gallo T (2005) Evidence of increased clinical protection of an MF59-adjuvant influenza vaccine compared to a non-adjuvant vaccine among elderly residents of long-term care facilities in Italy. *Epidemiol Infect* 133: 687–693
45 Puig-Barbera J, Gonzalez Vidal D (2007) MF59-adjuvanted subunit influenza vaccine: An improved interpandemic influenza vaccine for vulnerable populations. *Expert Rev Vaccines* 6: 659–665
46 Skowronski DM, Masaro C, Kwindt TL, Mak A, Petric M, Li Y, Sebastian R, Chong M, Tam T, De Serres G (2007) Estimating vaccine effectiveness against laboratory-confirmed influenza using a sentinel physician network: Results from the 2005–2006 season of dual A and B vaccine mismatch in Canada. *Vaccine* 25: 2842–2851
47 Baldo V, Baldovin T, Floreani A, Fragapane E, Trivello R (2007) Response of influenza vaccines against heterovariant influenza virus strains in adults with chronic diseases. *J Clin Immunol* 27: 542–547
48 D'Agosto V, Berardi S, Burroni D, Hennig R (2006) Tolerability and safety of an MF59–adjuvanted subunit influenza vaccine (FLUAD®). *IVW 2006 – The Second International Conference on Influenza Vaccines for the World*
49 Schultze V, D'Agosto V, Hennig R, Novicki D, Wack A, Zorn J (2008) Safety of MF59 adjuvant. *Vaccine* (in press)
50 Del Giudice G, Fragapane E, Bugarini R, Hora M, Henriksson T, Palla E, O'Hagan D, Donnelly J, Rappuoli R, Podda A (2006) Vaccines with the MF59 adjuvant do not stimulate antibody responses against squalene. *Clin Vaccine Immunol* 13: 1010–1013
51 Mitchell DK, Holmes SJ, Burke RL, Duliege AM, Adler SP (2002) Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers. *Pediatr Infect Dis J* 21: 133–138
52 McFarland EJ, Borkowsky W, Fenton T, Wara D, McNamara J, Samson P, Kang M, Mofenson L, Cunningham C, Duliege AM et al (2001) Human immunodeficiency virus type 1 (HIV-1) gp120–specific antibodies in neonates receiving an HIV-1 recombinant gp120 vaccine. *J Infect Dis* 184: 1331–1335
53 Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M (2006) Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* 354: 1343–1351
54 Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: A randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 357: 1937–1943
55 Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, Ypma E, Zambon M (2003) Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3

A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine* 21: 1687–1693

56 Atmar RL, Keitel WA, Patel SM, Katz JM, She D, El Sahly H, Pompey J, Cate TR, Couch RB (2006) Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. *Clin Infect Dis* 43: 1135–1142

57 Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, Hoschler K, Zambon MC (2006) Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: Phase I randomised trial. *Lancet* 367: 1657–1664

58 Nicholson K, Colegate A, Podda A, Stephenson I, Wood J, Ypma E, Zambon M (2001) Confronting a potential H5N1 pandemic: A randomised controlled trial of conventional and MF59 adjuvanted influenza A/Duck/Singapore/97 (H5N3) surface antigen vaccine. *Lancet* 9272: 357

59 Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood J, Zambon M, Katz J (2005) Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF-59–adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: A potential priming strategy. *J Infect Dis* 191: 1210–1215

60 Daems R, Del Giudice G, Rappuoli R (2005) Anticipating crisis: Towards a pandemic flu vaccination strategy through alignment of public health and industrial policy. *Vaccine* 23: 5732–5742

61 Heineman TC, Clements-Mann ML, Poland GA, Jacobson RM, Izu AE, Sakamoto D, Eiden J, Van Nest GA, Hsu HH (1999) A randomized, controlled study in adults of the immunogenicity of a novel hepatitis B vaccine containing MF59 adjuvant. *Vaccine* 17: 2769–2778

62 Langenberg AG, Burke RL, Adair SF, Sekulovich R, Tigges M, Dekker CL, Corey L (1995) A recombinant glycoprotein vaccine for herpes simplex virus type 2: Safety and immunogenicity [published erratum appears in *Ann Intern Med* (1995) 123: 395]. *Ann Intern Med* 122: 889–898

63 Corey L, Langenberg AG, Ashley R, Sekulovich RE, Izu AE, Douglas JM Jr, Handsfield HH, Warren T, Marr L, Tyring S et al (1999) Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: Two randomized controlled trials. Chiron HSV Vaccine Study Group. *JAMA* 282: 331–340

64 Borkowsky W, Wara D, Fenton T, McNamara J, Kang M, Mofenson L, McFarland E, Cunningham C, Duliege AM, Francis D et al (2000) Lymphoproliferative responses to recombinant HIV-1 envelope antigens in neonates and infants receiving gp120 vaccines. AIDS Clinical Trial Group 230 Collaborators. *J Infect Dis* 181: 890–896

65 Cunningham CK, Wara DW, Kang M, Fenton T, Hawkins E, McNamara J, Mofenson L, Duliege AM, Francis D, McFarland EJ, Borkowsky W (2001) Safety of 2 recombinant human immunodeficiency virus type 1 (hiv-1) envelope vaccines in neonates born to hiv-1–infected women. *Clin Infect Dis* 32: 801–807

66 Klinman DM (2004) Use of CpG oligodeoxynucleotides as immunoprotective agents. *Expert Opin Biol Ther* 4: 937–946

67 O'Hagan DT, Singh M, Kazzaz J, Ugozzoli M, Briones M, Donnelly J, Ott G

(2002) Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen. *Vaccine* 20: 3389–3398

68 Burke RL, Goldbeck C, Ng P, Stanberry L, Ott G, Van Nest G (1994) The influence of adjuvant on the therapeutic efficacy of a recombinant genital herpes vaccine. *J Infect Dis* 170: 1110–1119

69 O'Hagan DT, Ugozzoli M, Barackman J, Singh M, Kazzaz J, Higgins K, VanCott TC, Ott G (2000) Microparticles in MF59, a potent adjuvant combination for a recombinant protein vaccine against HIV-1. *Vaccine* 18: 1793–1801

70 Cherpelis S, Srivastava I, Gettie A, Jin X, Ho DD, Barnett SW, Stamatatos L (2001) DNA vaccination with the human immunodeficiency virus type 1 SF162DeltaV2 envelope elicits immune responses that offer partial protection from simian/human immunodeficiency virus infection to CD8(+) T-cell-depleted rhesus macaques. *J Virol* 75: 1547–1550

71 Otten GR, Schaefer M, Greer C, Calderon-Cacia M, Coit D, Kazzaz J, Medina-Selby A, Selby M, Singh M, Ugozzoli M et al (2003) Induction of broad and potent anti-HIV immune responses in rhesus macaques by priming with a DNA vaccine and boosting with protein-adsorbed PLG microparticles. *J Virol* 77: 6087–6092

72 Otten G, Schaefer M, Doe B, Liu H, Srivastava I, zur Megede J, O'Hagan D, Donnelly J, Widera G, Rabussay D et al (2004) Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine* 22: 2489–2493

73 Otten GR, Schaefer M, Doe B, Liu H, Megede JZ, Donnelly J, Rabussay D, Barnett S, Ulmer JB (2006) Potent immunogenicity of an HIV-1 gag-pol fusion DNA vaccine delivered by *in vivo* electroporation. *Vaccine* 24: 4503–4509

74 Otten GR, Schaefer M, Doe B, Liu H, Srivastava I, Megede J, Kazzaz J, Lian Y, Singh M, Ugozzoli M et al (2005) Enhanced potency of plasmid DNA microparticle human immunodeficiency virus vaccines in rhesus macaques by using a priming-boosting regimen with recombinant proteins. *J Virol* 79: 8189–8200

75 O'Hagan DT, Singh M, Dong C, Ugozzoli M, Berger K, Glazer E, Selby M, Wininger M, Ng P, Crawford K et al (2004) Cationic microparticles are a potent delivery system for a HCV DNA vaccine. *Vaccine* 23: 672–680

76 AIDS Vaccine Evaluation Group 022 Protocol Team (2001) Cellular and humoral immune responses to a canarypox vaccine containing human immunodeficiency virus type 1 Env, Gag, and Pro in combination with rgp120. *J Infect Dis* 183: 563–570

77 Bernstein DI, Schleiss MR, Berencsi K, Gonczol E, Dickey M, Khoury P, Cadoz M, Meric C, Zahradnik J, Duliege AM, Plotkin S (2002) Effect of previous or simultaneous immunization with canarypox expressing cytomegalovirus (CMV) glycoprotein B (gB) on response to subunit gB vaccine plus MF59 in healthy CMV-seronegative adults. *J Infect Dis* 185: 686–690

78 Giuliani MM, Adu-Bobie J, Comanducci M, Arico B, Savino S, Santini L, Brunelli B, Bambini S, Biolchi A, Capecchi B et al (2006) A universal vaccine for serogroup B meningococcus. *Proc Natl Acad Sci USA* 103: 10834–10839

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Non-recent history of influenza pandemics, vaccines, and adjuvants

Maria Lattanzi

Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

Abstract

Mankind has been affected by influenza epidemics and pandemics for many centuries. As for many others infectious diseases, measures to reduce the heavy burden of this disease through prevention were first developed during the 20th century. The purpose of this chapter is to describe the non-recent history of influenza, including the origin of the disease, the pandemics and the epidemics. Moreover, we also discuss the first attempts to develop safe and efficacious vaccines, including the negative experience with the swine influenza vaccination and the numerous attempts to improve the efficacy of influenza vaccines using adjuvants carried out since the 1950s.

Introduction

The influenza virus derived its name in the 14th–15th Century when a Florentine family used the word “influence” to suggest an unusual conjunction of planets at times of epidemics of cough, colds and fever. The word “influenza” was thus derived as a description name for the epidemics due to “influences” “ab occulta coeli influentia” [1].

The real cause of the “influenza” was discovered in 1933 with the first isolation of the flu virus by Smith. Since then, the knowledge of the virus has increased exponentially and reaches to the finest molecular details. Just a few years after the isolation of the virus, the first flu vaccines were developed and their use has become an annual routine.

In the Middle Ages, the Germans thought influenza was caused by eating too many sour apples and salt fish. Now we know much more about the flu virus, but our knowledge on how to take control of pandemic and epidemic influenza is still primitive and poor, and this virus continues even today to be one of the major threats for human health and one of the most important public health concerns [1].

In annual seasonal influenza epidemics, as much as 5–15% of the population is affected by upper respiratory tract infections, which impose a

considerable economic burden due to indirect and direct costs due to hospitalizations, other healthcare costs and a loss of productivity [2].

Influenza pandemics can affect up to 70% of the population, with a dramatic increase in morbidity and mortality that could lead to the failure of healthcare systems as a large proportion of the population is affected at any one time.

Although a single pandemic leads to a higher toll that a single epidemic, the cumulative disease burden of epidemics overall is greater than that of pandemics [3]. In this chapter we focus on the non-recent history of influenza, the origin of the disease, the pandemics for which we have records starting from 1580, the first vaccine, the negative experience with the swine influenza vaccination and the numerous attempts to improve the efficacy of influenza vaccines using adjuvants that were carried out since 1950s.

History of influenza

Historical records carry evidence of the circulation of the influenza virus starting from the ancient Greeks. Although it is difficult to interpret old literature, it could be that Hippocrates' writings of 412 BC are the first reports of possible influenza [4]. In the Middle Ages several widespread outbreaks that were probably due to influenza occurred in Europe. However, the first report of an influenza epidemic, where symptoms can be regarded with confidence as probably due to influenza, occurred in 1173–1174 [5], while the first influenza pandemic agreed by all authors occurred in 1580. This pandemic originated in Asia during the summer of that year, spread to Africa, and then to Europe along two corridors from Asia Minor and North-West Africa [6]. The whole Europe was infected from south to north in a 6-month period, and infection subsequently spread to America [6, 7]. Illness rates were high; 8000 deaths were reported from Rome, and some Spanish cities were decimated. It was said that only one twentieth of the people escaped the illness [7].

According to the dictionary definition, a pandemic is simply a widespread epidemic, but when referring to influenza, a pandemic now signifies a worldwide epidemic caused by a new subtype of influenza A virus. Thus, although only during the virological era we can recognize a pandemic with certainty, at least ten pandemics have been agreed by all the reviewers since the first one in 1580 and another three have been classified as possible [8].

Numerous references to influenza pandemics and epidemics were made for the 17th century in America and Europe. From the beginning of the 18th century, the quality and quantity of data increased and medical historians were drawn to comment on the number of infected persons, whether they were considering an epidemic or a pandemic, the countries involved and the possible origins of the virus strains involved.

The first agreed influenza pandemic of the 18th century began in AD 1729 [5, 6, 9, 10]: the outbreak started in Russia in the spring, spread westwards in expanding waves to embrace all Europe within a 6-month period [6], and encompassed the whole known world over a 3-year period with high death rates. Distinct waves of infection were recorded; the later were more severe than the first [10–12].

The next pandemic occurred after a gap of some 40 years between 1781 and 1782 [6, 9]. Most authors agree that the outbreak began in China in the autumn, spread to Russia and from there westwards in widening circles to encompass the whole of Europe in a period of 8 months [13]. There is evidence of extensive seeding in both Russia and North America in the early months of the pandemic, followed by extensive outbreaks. The attack rate was reported to be high, particularly among young adults [14]: at the peak of the pandemic, 30,000 fell ill each day in St. Petersburg; two-thirds of the population of Rome became ill; and the outbreak is reported to have raged through Britain during the summer of 1782.

The first pandemic of the 19th century began in the winter of 1830 in China, from where it spread southwards by sea to reach the Philippines, India and Indonesia, and across Russia into Europe. The contagion spread into North America to cause outbreaks in 1831–1832, recurred in Europe at the same time and recurred again in Europe in 1832–1833 [13]. All authors comment on the high attack rate of the population, but the mortality rate was not exceptionally high [10].

Since the pandemic of 1889–1892, data have been more reliable and more thoroughly reviewed, and since 1957 the causal viruses were available for analysis.

The epidemic of 1889–1892 stands out not only in severity, but also in its wavelike extension in annual recurrences into the immediately succeeding years. In London, the epidemic increased in severity with each successive wave so that mortality was highest in 1892 [15]. Another notable feature of this epidemic was the occurrence in 1890 of an increase in the proportion of deaths in the 20–40-year-old age group, ordinarily considered to be a unique characteristic of the 1918 pandemic.

The greatest pandemic of the 20th century was the pandemic of 1918, popularly known as "Spanish flu", which is believed to be caused by an influenza A/H1N1 virus, which is closely related to the virus later found in pigs [16], and which remains an infection of this species to the present time. Epidemiological evidence suggests that the Spanish flu originated in the US in March and April 1918 and was transported by American troops to Europe, when it first reached epidemic levels in France in April 1918. Over the next several months, influenza spread throughout the whole of Europe, Middle-East, India, East and South East Asia, Australia and Africa in three subsequent waves. In the US, the 1918 influenza pandemic killed approximately 600,000 people – 0.5% of the population, about ten times more than the number of Americans who died in the First World War. In England and

Wales the official deaths numbered 200,000. In a few places, such as Samoa and Alaska some 25% or more of the population died. No figures exist for many parts of the world, but the pandemic is estimated to have infected 50% of the world's population, 25% suffered a clinical infection and the total mortality was 40–50 million. The 1918 influenza pandemic killed more people in less time than any other disease before or since.

Since the 1918 pandemic, there have been two major outbreaks of influenza A in the 20th century. In 1957 influenza was induced by the "Asian" influenza virus A/H2N2, and in 1968, influenza was induced by the "Hong Kong" influenza virus A/H3N2. Neither pandemic was as bloodthirsty as in 1918; nevertheless, in the US alone 40,000 and 30,000 deaths were attributed to the "Asian" and to the "Hong Kong" pandemics, respectively.

In addition to the three major influenza pandemics, a few smaller influenza epidemics as well as flu "scares" occurred in the 20th century. Of these, the most famous is the outbreak of "Swine flu" that happened in 1976 at Fort Dix, NJ. The recovery of this virus raised concerns about a worldwide pandemic and a mass vaccination program was launched in the US (see below).

There is no periodicity to the occurrence of pandemics and no basis for predicting where and when a new outbreak may arise [10]. Since 1889 pandemics of influenza have occurred at intervals of 28/29 and 10/11 years and in fact several suppositions on the possibility of predicting an influenza pandemic on the basis of cyclical recurrence of the pandemic strains or solar activity or other factors, are only speculative. In the 20th century, Hope-Simpson published an article [17] in which he suggested that antigenic shifts of influenza A virus, and accordingly a pandemic, coincided with sunspot, and showed some examples of this from 1930 to 1970. But in 1979 sunspots activity took place but there was no pandemic. So we should be prepared for unforeseen influenza pandemic in any given year [1].

First influenza vaccines

Efforts to develop influenza virus vaccines began soon after influenza A and B viruses were recognized as the etiological agents of influenza. The first papers on immunogenicity of influenza vaccines were published in the second half of 1930s [18]. The vaccine tested was a crude, poorly purified preparation of the Puerto Rico 8 (PR8) strain obtained from the lungs of infected mice and inactivated with formalin.

Subsequently, it was noticed that the inoculation and passaging of the flu strains on the chorio-allantoic membranes of developing chick embryos lead to an increase in the production process and to a more immunogenic vaccine.

The main input and interest in the development of influenza vaccines came from the US military during World War II, in part because of the dev-

astation caused in both military and civilian population by the 1918–1919 influenza pandemic, during the late stages of World War I.

In very few years, whole-virus inactivated bivalent (type A PR8 strain and type B Lee strain), and trivalent vaccines (type A PR8, and Weiss strains and type B Lee strain) were developed and tested in large field trials, mainly among healthy military recruits and college students [19, 20]. These field trials lead to the approval of the first commercial vaccines in the US in 1945.

The results of all those field trials suggested that vaccination with concentrated inactive virus induced an increase in antibodies against flu and a distinct effect in reducing the incidence of influenza.

It is very impressive to notice how the vast majority of knowledge on the effects of influenza vaccination was already described in those papers written in the early 1940s!

It was noted, indeed, that the increase in virus neutralizing capacity following vaccination was related to the pre-vaccination level. In general, the lower the neutralizing capacity before vaccination the greater was the increase in antibodies after vaccination. Additionally, it was observed that the mean neutralizing capacity following vaccination was highest in the group who possessed the highest antibody levels before vaccination, even though the mean increase in neutralizing capacity in this group was lowest at all. Moreover, it was shown that there was a correlation between neutralizing antibody levels against influenza and the occurrence of the disease. It was found that the higher the level of antibodies, the less likely it was that disease would develop, although an exact quantification of this correlation was not possible at that time [21].

Those first experiences showed also that, while vaccination prevented the development of clinical infection to a considerable degree, it also reduced the severity of the disease [21]. It is of interest that no significant differences in incidence were observed between control and vaccinated subjects when the diagnosis of common cold or local respiratory infection were analyzed. Moreover, the incidence of local respiratory infections in either group did not vary significantly from the pre-epidemic level. These facts suggested the specificity of action of the influenza vaccine [21].

Furthermore, the same authors also suggested how to best use vaccination in a public health setting to maximize potentialities of such a tool. It was proposed to control the epidemic recurrence of influenza by revaccination at intervals shorter than the epidemic periodicity, or at intervals determined by immunological surveys. "This would appear a more practical method of administering vaccine for prophylaxis than to vaccinate in the face of an outbreak, after its identification, particularly since influenza spreads with such rapidity that the epidemic might be well under way before it would be possible to vaccinate a significant proportion of the population" [21].

The process used to make the current commercially available inactivated influenza virus vaccines share certain key features with those pioneer-

ing early vaccines. The influenza vaccine viruses are still replicated in the allantoic cavities of embryonated hen's eggs and the inactivation of the harvested influenza viruses is done even now using either formalin or beta-propriolactone.

However, early influenza vaccines were relatively crude preparations of chick embryo allantoic fluid virus and were frequently contaminated with bacterial endotoxin, which quite often evoked febrile reactions after vaccinations and were contraindicated in children.

Although the extensive field trials that established influenza vaccines as safe and reasonably effective for prevention of influenza in military population began in the mid 1940s, 1957 was the first year in which there was widespread civilian use of influenza vaccines in US; approximately 65 million doses were distributed in anticipation of the onset of the Asian influenza pandemic. From 1957 to the second half of the 1970s polyvalent and bivalent vaccines incorporating either multiple strains of influenza A and B or only the most current influenza A and B strain had been marketed. At that time, the rationale for polyvalent vaccine usage was based on the inability to predict epidemic strains likely to be prevalent and the belief that a broad base of protection would result from multiple antigenic stimulations [22].

Whole-virus vaccines produced with more or less the same technologies as those pioneer vaccines have been used since the 1970s . However, at that time, yearly vaccine formulation depended upon less-than-adequate viral surveillance, relatively cumbersome method of vaccine production and, often, no established knowledge of effectiveness. Furthermore, those vaccines produced a disturbingly high percentage of side reactions. Therefore, during the 1960s, influenza vaccines never enjoyed wide popularity among private physicians, nor they were promoted universally by public health officials. They have been used sparingly in perhaps 10–20% of the at risk population [22].

All those reasons, but especially the high incidence of reactogenicity induced by these vaccines, prompted attempts to produce a less reactogenic vaccine. Subvirion preparations were developed to meet this medical need, retaining the immunogenic properties of the whole-virus preparations but with greatly reduced reactogenicity [23]. Subvirion (or split) vaccines are prepared using a solvent (such as ether or a detergent) to dissolve or disrupt the viral lipid envelope. They contain membrane proteins, the antigens hemagglutinin (HA) and neuraminidase (NA), and parts of the lipid envelope, but no RNA molecules (the genetic material). These vaccines have been shown to induce fewer side effects in the vaccinees but are equally immunogenic as whole-virus vaccine. Split virus vaccines are suitable for the use in adults and children (Fig. 1).

Additional purification steps can be taken to reduce the amount of viral proteins (essentially influenza matrix protein and nucleoprotein) to produce a subunit or purified surface antigen vaccine. Internal proteins of the virus can be removed leaving only HA and NA, which are the only two antigens

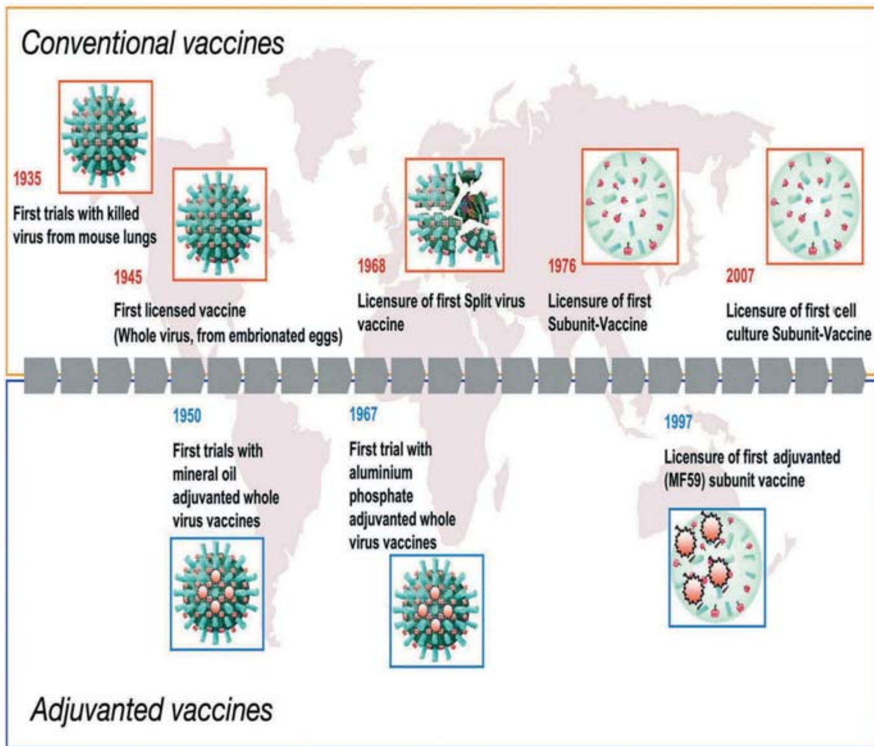

Figure 1. Milestones of inactivated influenza vaccine development

necessary to elicit a protective antibody response. Volunteers given subunit vaccines experienced fewer adverse reactions than those given whole virus vaccines [24].

Due to their good tolerability and immunogenicity, split and subunit vaccines have been the most widely used influenza vaccine during the last few decades.

The swine influenza outbreak

A negative experience with influenza and influenza vaccination comes from the Swine influenza A outbreak that took place in Fort Dix, New Jersey (USA), at the beginning of 1976.

The severity of the 1918 influenza A (H1N1) "Spanish" pandemic and evidence for a cycle of pandemics aroused concern that the 1918 disaster could recur. In the early 1970s the only A virus to circulate was an H3N2 subtype. Thus, when, in 1976, cases of influenza in soldiers, mostly recruits, at Fort Dix were associated with isolation of influenza A (H1N1) serotype

(which in 1976 was labeled A/New Jersey/76 Hsw1N1) [25], there was great concern that this might mark the beginning of a new dreadful pandemic, like the Spanish one.

Thankfully, A/New Jersey/76 virus was detected only from 19 January to 9 February, and did not spread beyond Fort Dix. In this time period, up to 230 soldiers were infected, including 13 soldiers with severe respiratory illness and 1 death. A/Victoria/75 (H3N2) spread simultaneously in the same setting, and caused illness (301 cases documented by viral isolation, serological responses or both), and persisted until March. Despite intensive investigation, the source of the virus, the exact time of its introduction into Fort Dix, and factors limiting its spread and duration remained unknown [26–28].

The appearance of a new flu virus subtype had always presaged the disappearance of the prevalent subtype of virus and rapid spread of the new strain coincident with pandemic disease. When the new virus was identified as the putative agent of 1918 pandemic, concern evoked caution. The chance that the new virus might possess the virulence attributed to the 1918 virus was one of the main reasons to undertake mass immunizations in the US.

Supported by a US$35 million appropriation form the Congress, manufacture of vaccine and intensive surveillance for new swine influenza cases began. Between April and October 1976 the most extensive trials ever performed with an influenza vaccine were conducted on 7500 volunteers.

National Influenza Immunization Program (NIIP) was designed to provide A/New Jersey influenza vaccine for almost the entire adult population in the USA. Only children at high risk of serious illness for influenza virus infection recieved the vaccine due the unacceptable toxicity of whole virus vaccine in younger children confirmed from the clinical trials.

Between 1 October 1976, and 15 December 1976, 43 million people were immunized, including nearly 50% of the high-risk group, the greatest protection ever afforded in that group and twice the number of persons in that group usually immunized.

Four vaccine manufacturers produced over 100 million doses: approximately 66 million doses of monovalent A/New Jersey/76 (Hsw1N1) vaccine (reassortant virus X-53a) and 35.5 million doses of bivalent A/New Jersey/76 (Hsw1N1) and A/Victoria/75 (H3N2) vaccine. Whole virus was used in 93% of the monovalent vaccine and in 14% of the bivalent vaccine (from two producers), while the remaining doses were split or subunit vaccines (from the other two producers).

Vaccine manufacturers and their insurers had foreseen possible liability issues of this unprecedented vaccination campaign, and were able to force the Government to assume primary liability responsibility.

The vaccination program incorporated a national wide surveillance system to evaluate possible adverse vaccine reactions. On 16 December 1976, the apparent association between vaccination and the onset of Guillain-Barré syndrome (GBS) (and the continuing absence of swine influenza virus in man) led to the suspension of the NIIP, and to the expansion of

GBS surveillance nationally to determine as quickly as possible whether the influenza vaccinations were related to GBS. By January of 1977, more than 500 cases of GBS had been reported with 25 deaths [29].

Initial reports indicated that, in persons older than 18 years, the risk of getting GBS was five to six times greater in vaccinated than in unvaccinated individuals [30]. This report was criticized during subsequent court proceedings in which victims sued for damages because it did not contain the basic data for calculation of incidence and estimation of risk. Indeed, no control group of similar size was followed as carefully at that time. So the true dominator is not known.

A definitive study has subsequently been made [31]. This study essentially corroborated the earlier report, but refined the analysis. Vaccine associated risk (4.9–5.9 per million) was found to last for at least 6 weeks following vaccination but not beyond 8 weeks. Subsequent studies on seasonal influenza vaccines found low relative risks of 1.4 in 1978–1979, 0.6–1.4 in 1979–1980 and 1980–1981 and 1.1 in 1980–1988; these relative risks were not significantly different form 1 (no association). Moreover, a marked decline in the incidence of GBS was also reported from the 1990 to 2003 [32, 33]. Therefore, it has been proposed that the elicitation of GBS is unique to swine influenza vaccine. However, it has to be considered that the revelation of increased risk could be unique to the massive swine influenza immunization program and to the subsequent intensive surveillance rather that to the vaccine itself.

In conclusion, the NIIP for "swine flu" is an experience that we need to learn from to avoid the scientific, technical, human, and political problems encountered at that time and that can probably be avoided with proper planning.

Early adjuvanted influenza vaccines

Quite soon after the development of the first inactivated influenza vaccines, scientists, with the sponsor of the US Army, tried to find a way to improve the immunogenicity of those vaccines through the addition of adjuvants.

The most powerful adjuvant used in research is the Freund's complete adjuvant (FCA), a water-in-oil emulsion containing 50% mineral oil, Arlacel A (a surfactant) and killed mycobacteria. FCA generates side effects far too severe to be used in human vaccine applications. However, the first adjuvants used in influenza vaccines were substantially based on a slightly modified FCA.

In 1950, the first clinical trials of mineral oil-adjuvanted influenza vaccines were performed, after preclinical testing in more than 70 monkeys that were carefully studied histologically and serologically for more than 1 year. In the subsequent years, extensive field clinical trials were sponsored by the Commission on Influenza, Armed Forces Epidemiological Board and man-

aged by J. Salk at Fort Dix in 1951–1953 [34]. These trials included 18 000 Army personnel given adjuvanted influenza virus vaccine, 4000 given standard (aqueous) vaccine and 22 000 given formalinized saline as a control.

The vaccines used in these studies were prepared by commercial biological firms. Virus suspensions were inactivated with formalin (1:4000) and merthiolate (1:10 000) was added as a preservative. Mineral oil-adjuvanted vaccines were made by emulsifying one volume of virus concentrate with an equal volume of a mixture containing nine parts light medicinal mineral oil (Drakeol 6-VR) and one part emulsifier (purified Arlacel A).

Results of these trials showed that effectiveness of influenza vaccines could be greatly increased by emulsification in light mineral oil [35], but also that reactogenicity increased. Short-term results reported that about 3% of recipients developed delayed local cystic reactions requiring surgical intervention at the site of inoculation. Moreover, a higher incidence of allergic manifestations, almost entirely urticaria, was reported. Those allergic reactions were due to a sensitization to penicillin by the adjuvant, because penicillin had been used in its manufacture and not totally removed by purification processes.

However, the major concerns about mineral oil-adjuvanted vaccines were not related to the described reactogenicity, but to the long-term safety issues like carcinogenicity and hypersensitivity, due to the fact that mineral oil products were categorized as non-metabolizable and non-excretable. Thus, although the effectiveness of the flu vaccine was enhanced by the mineral-oil adjuvant, doubts about the long-term safety of the oil adjuvant preparation generally prevented its acceptance for routine use [36].

To address the long-term safety issues of the adjuvanted vaccines used at Fort Dix in 1951–1953, a 9-year and subsequently an extra 8-year follow-up was performed on mortality after vaccination [37, 38]. Both studies concluded that there was no suggestion of a mortality effect attributable to the adjuvant. All the findings were essentially negative with respect to malignant neoplasms, allergic diseases, and collagen diseases. In addition, there was no evidence that men known to have had the cyst-like reaction at the site of inoculation, or thought to have had allergic reactions mediated by the adjuvanted vaccine had experienced a higher mortality risk.

Those early adjuvanted flu vaccines were extensively studied in clinical trials during the 1950s and the 1960s because of the cardinal advantage over aqueous vaccines: such as higher and more durable levels of broadly reactive antibodies (Tab. 1) and the reduction of the antigen content [39, 40].

A couple of studies showed that the persistence of high levels of antibodies after a single dose of mineral oil-adjuvanted vaccine was at least 2–3 years [34, 41], but one trial found a 9-year persistence [40]. The ultimate goal of those trials was to evaluate the possibility of achieving a durable protection without the troublesome (even today!) necessity for annual revaccination. However, regardless the potency of the adjuvant, the emergence of drifted strains has not allowed skipping the annual use of flu vaccines.

Table 1. Comparative immunogenicity of one and two doses of aqueous and adjuvant influenza vaccines in healthy adults subjects [39].

Polyvalent vaccines	Test strain and antibody levels		
	Swine	PR8	PR301
Aqueous; aqueous			
Before second dose	30	29	102
After second dose	179	512	307
Adjuvant, adjuvant			
Before second dose	242	141	768
After second dose	1229	1741	1818

The reduced antigen content requirement using in mineral oil-adjuvanted vaccines could allow multiple antigens to be included in the same vaccine to achieve protection against more than one disease through a single injection.

Moreover, those in favor of the adoption of mineral oil-adjuvanted vaccines stressed the fact that those vaccines afforded the greatest promise for coping with a new pandemic strain [36]. Today, the fear for a new influenza pandemic is high, based on the historical records of the Spanish flu, but at that time, in 1968, only 50 years had passed and the vast majority of the key opinion leaders and policy makers had witnessed the 1918–1919 disaster.

To improve the safety profile of mineral oil-adjuvanted vaccines, other types of adjuvant were tested, like vegetable oil emulsions. However, those formulations were not considered as substitutes of the mineral oil products, because vegetable oils emulsions did not perform as effectively as mineral oil preparations with respect to height of titer achieved and duration of antibody at high levels [42].

Aluminum salts, the most widely used adjuvant for human vaccines, were tested as a way to increase influenza vaccine efficacy without safety concerns. Table 2 describes the differences between the aluminum salts used as vaccine adjuvants. Preclinical observations had shown that the adsorption of influenza virus HA on $AlPO_4$ enhanced their antigenic potency in mice [43]. Thus, a clinical trial on healthy military recruits was performed to compare immunogenicity of two influenza vaccines, with the same concentration of HA. The adsorbed vaccine contained 3.8 mg $AlPO_4$/ml. Results showed that the frequencies of antibody increase and the antibody levels achieved after administration of unadsorbed or adsorbed vaccine indicated that both preparations were antigenically potent with no significant differences. These results demonstrated that $AlPO_4$ fails to exert an adjuvant effect on HA vaccines given to adult human subjects [44].

However, despite the mentioned scientific evidence, during the 1960s and the 1970s many influenza vaccines commercially available in both Europe and US (either whole virus, split virus or subunit) were aluminum adsorbed

Table 2. Aluminum salts used as adjuvant in vaccines [49]

Chemical form of Al	Characteristics
Aluminum hydroxide $Al(OH)_3$	Crystalline aluminum hydroxide positively charged at physiological pH [isoelectric point (p*I*) = 11]
Aluminum phosphate $AlPO_4$	Amorphous aluminum hydroxyphosphate negatively charged at physiological pH (p*I* = 5–7).
Alum $KAl(SO_4)_2 \cdot 12H_2O$	Aluminum hydroxide that contains some sulfate anions as well as anions that are used in the buffer

Table 3. Aluminum and influenza vaccines: Historical view

Examples of aluminum-adsorbed influenza vaccines, commercially available during the 1970s in Europe or US	
Whole virus	Alorbat (ASTA Laboratories, Bielefeld, Germany) Agrippal (ISVT Sclavo, Siena, Italy)
Split virus	Begrivac (Behring, Marburg, Germany) Influenza vaccine (Cantacuzino Institute, Romania)
Subunit virus	Fluvirin (Medeva, Liverpool, UK)

(Tab. 3). Aluminum salts were removed in the early 1980s because a growing body of evidence showed that the adjuvant did not increased immunogenicity but slightly increased reactogenicity, especially in children [44–47].

Those unsuccessful early experiences did not stop the research for a safe and potent adjuvant to be used not only with influenza vaccines, but also with other human vaccines. During the 1980s, efforts toward generating potent but safe adjuvant formulations have centered on the development of oil-based adjuvants, like FCA widely used in preclinical research. To reduce the toxicity associated with Freund's-like adjuvants, formulations were developed that reduced the amount of oil from 50% to 4–5% and replaced all of the Freund's components with similar acting but less toxic components. In the most successful of these formulations, MF59, the mineral oil of Freund's adjuvant was replaced with the metabolizable oil squalene, and the Arlacel A surfactant was replaced with cleaner more-defined surfactants such as Tween 80 and Span 85. The reduction of the oil content to 5% results in the formation of oil-in-water emulsions rather than water-in-oil emulsions [48].

MF59 has been extensively studied in preclinical and clinical models with many different antigens. The largest experience is with subunit influenza antigens. MF59-adjuvanted influenza subunit vaccine with enhanced immunogenicity has now been available in Europe for approximately 10 years (Fig. 1).

Conclusion

Mankind has been affected by influenza epidemics and pandemics for many centuries. Over the centuries, we have seen attempts to develop safe and efficacious vaccines, including the negative experience with the swine influenza vaccination and the numerous attempts to improve the efficacy of influenza vaccines using adjuvants carried out since the 1950s. However, as has been the case for many others infectious diseases, it was only during the 20th century that we have succeeded in developing measures to reduce the heavy burden of this disease through prevention.

References

1 Ghendon Y (1994) Introduction to pandemic influenza through history. *Eur J Epidemiol* 10: 451–453
2 WHO (2003) Fact sheet no. 211. (http://www.who.int/mediacentre/factsheets/fs211/en/ Web site accessed 18 September 2006)
3 Fukuda K, Levandowski RA, Bridges CB, Cox NJ (2004) Inactivated influenza vaccines. In: SA Plotkin, WA Orenstein (eds): *Vaccines*, 4th edn. Saunders, Philadelphia, 339–370
4 Potter CW (1998) Chronicle of influenza pandemics. In: KG Nicholson, RF Webster, AJ Hay (eds): *Textbook of Influenza*. Blackwell Science, Oxford, 3–18
5 Hirsch A (1883) *Handbook of Geographical and Historical Pathology*. New Sydenham Society, London
6 Pyle GF (1986) *The Diffusion of Influenza: Patterns and Paradigms.* Rowan & Littlefield, New Jersey
7 Beveridge WIB (1991) The chronicle of influenza epidemics. *History Philos Life Sci* 13: 223–235
8 Potter CW (2001) A history of influenza. *J Appl Microbiol* 91: 572–579
9 Finkler D (1899) Influenza in twentieth century practice. In: TL Shipman (ed): *An International Encyclopaedia of Modern Medical Science*. Sampson Law & Marston, London, abstract 12, 21–32
10 Patterson KD (1987) *Pandemic Influenza 1700–1900; A Study in Historical Epidemiology.* Rowman & Littlefield, New Jersey
11 Brown MW (1932) Early epidemics of influenza in America. *J Med Rec* 135: 449–451
12 Beveridge WIB (1977) *Influenza: The Last Great Plague*. Heinemann, London
13 Pyle GF, Patterson K (1984) Influenza diffusion in European history: Patterns and paradigms. *Ecol Dis* 2: 173–184
14 Thompson ES (1890) *Influenza*. Pervical, London
15 Burnet FM, Clark E (1942) Influenza. A survey of the last 50 years in the light of modern work on the virus of epidemic influenza. *Monogr Walter Eliza Hall Inst Res Pathol Med* 69–99
16 Shope RE (1931) Swine influenza: Experimental transmission and pathology. *J Exp Med* 54: 349–359

17 Hope-Simpson R (1978) Sunspots and flu: A correlation. *Nature* 275: 86
18 Francis T Jr, Magill TP (1937) The antibody response of human subjects vaccinated with the virus of human influenza. *J Exp Med* 65: 251–259
19 Francis T Jr (1945) The development of the 1943 vaccination study of the commission on influenza. *Am J Hyg*iene 42: 1–11
20 Francis T Jr, Salk JE, Pearson HE, Brown PN (1945) Protective effect of vaccination against induced influenza A. *J Clin Invest* 24: 536–546
21 Salk JE, Menke WJ, Francis T Jr (1945) A clinical, epidemiological and immunological evaluation of vaccination against epidemic influenza. *Am J Hyg* 42: 57–93
22 Gregg MB (1974) Recent advances in influenza vaccines. *J Med* 16: 651–653
23 Cate TR, Couch RB, Parker D, Baxter B (1983) Ractogenicity, immunogenicity, and antibody persistence in adults given inactivated influenza virus vaccines – 1978. *Rev Infect Dis* 5: 737–747
24 Spila-Alegiani S, Salmaso S, Rota MC, Tozzi A, Raschetti R, the Italian SVEVA group (1999) Reactogenicity in the elderly of nine commercial influenza vaccines: Results from the Italian SVEVA study. *Vaccine* 17: 1898–1904
25 Kendal AP, Goldfield M, Noble GR, Dowdle WR (1977) Identification and preliminary antigenic analysis of swine influenza-like viruses isolated during an influenza outbreak at Fort Dix, New Jersey. *J Infect Dis* 136 (Suppl): S381–S385
26 Goldfield M, Bartley JD, Pizzuti W, Black HC, Altman R, Halperin WE (1977) Influenza in New Jersey in 1976: Isolation of influenza A/New Jersey/76 virus at Fort Dix. *J Infect Dis* 136 (Suppl): S347–S355
27 Top FH, Russell PK (1977) Swine influenza A at Fort Dix, New Jersey (January–February 1976). IV. Summary and speculation. *J Infect Dis* 136 (Suppl): S376–S380
28 Gaydos JC, Top FH, Hodder RA, Russer PK (2006) Swine influenza A outbreak, Fort Dix, New Jersey, 1976. *Emerg Infect Dis* 12: 23–28
29 Langmuir AD (1979) Guillain-Barrè syndrome: The swine influenza virus vaccine incident in the United States of America, 1976–1977; preliminary communication. *J R Soc Med* 72: 660–669
30 Schonberger LB, Bregman DJ, Sullivan-Bolyai JZ, Keenlyside RA, Ziegler DW, Retailliau HF, Eddins DL, Bryan JA (1979) Guillain-Barrè syndrome following vaccination in the national influenza immunization program, United States, 1976–1977. *Am J Epidemiol* 110: 105–123
31 Langmuir AD, Bregman DJ, Kurland LT, Nathanson N, Victor M (1984) An epidemiologic and clinical evaluation of Guillain-Barrè syndrome reported in association with the administration of swine influenza vaccines. *Am J Epidemiol* 119: 841–879
32 Lasky T, Terracciano GJ, Magder L, Koski CL, Ballesteros M, Nash D, Clark S, Haber P, Stolley PD, Schonberger LB, Chen RT (1998) The Guillain-Barrè syndrome and the 1992–1993 and 1993–1994 influenza vaccines. *N Engl J Med* 339: 1797–1802
33 Haber P, DeStefano F, Angulo FJ, Iskander J, Shadomy SV, Weintraub E, Chen RT (2004) Guillain-Barrè syndrome following influenza vaccination. *JAMA* 292: 2478–81

34 Salk JE (1953) Use of adjuvants in studies on influenza immunization. 3. Degree of persistence of antibody in human subjects two years after vaccination. *JAMA* 151: 1169–1175

35 Salk JE, Bailey ML, Laurel AM (1952) The use of adjuvants in studies on influenza immunization. II. Increased antibody formation in human subjects inoculated with influenza virus vaccine in a water-in-oil emulsion. *Am J Hyg* 55: 439–456

36 Davenport FM (1968) Seventeen years' experience with mineral oil adjuvant influenza virus vaccines. *Ann Allergy* 26: 288–292

37 Beebe GW, Simon AH, Vivona S (1964) Follow-up study on Army personnel who received adjuvant influenza virus vaccine 1951–1953. *Am J Med* Sci 247: 385–405

38 Beebe GW, Simon AH, Vivona S (1972) Long-term mortality follow-up of Army recruits who received adjuvant influenza virus vaccine in 1951–1953. *Am J Epidemiol* 95: 337–346

39 Hennessy AV, Davenport FM (1961) Relative merits of aqueous and adjuvant influenza vaccines when used in a two-dose schedule. *Public Health Rep* 76: 411–419

40 Davenport FM, Hennessy AV, Bell JA (1962) Immunologic advantages of emulsified influenza virus vaccines. *Mil Med* 127: 95–100

41 Davis DJ, Philip RN, Bell JA, Voegel JE, Jensen DV (1961) Epidemiological studies on influenza in familiar and general population groups. 1951–1956. III. Laboratory observations. *Am J Hyg* 73: 138–147

42 Stuart-Harris CH (1967) Scientific Publication No. 147, *PAIIO*, 573

43 Davenport FM (1968) Antigenic enhancement of ether-extracted influenza virus vaccines by $AIPO_4$. *Proc Soc Exp Biol Med* 127: 587–590

44 Davenport FM, Hennessy AV, Askin FB (1968) Lack of adjuvant effect of $AlPO_4$ on purified influenza virus hemagglutinins in man. *J Immunol* 100: 1139–1140

45 Werner J, Kuwert EK, Stegmaier R, Simbock H (1980) [Local and systemic antibody response after vaccination with 3 different types of vaccines against influenza. II. Neuraminidase inhibiting antibodies (author's transl)]. *Zentralbl Bakteriol* A 246: 1–9

46 D'Errico MM, Grasso GM, Romano F, Montanaro D (1988) [Comparison of anti-influenza vaccines: Whole adsorbed trivalent, trivalent subunit and tetravalent subunit]. *Boll Ist Sieroter Milan* 67: 283–289

47 Ionita E, Lupulescu E, Alexandrescu V, Matepiuc M, Constantinescu C, Cretescu L et al (1989) Comparative study of the immunogenicity of aqueous *versus* aluminium phosphate adsorbed split influenza vaccine C.I. *Arch Roum Pathol Exp Microbiol* 48: 265–273

48 Van Nest GA, Steimer KS, Haigwood NL, Burke RL, Ott G (1992) Advanced adjuvant formulations for use with recombinant subunit vaccines. In: F Brown, RM Chanock, HS Ginsberg, RA Lerner (eds): *Vaccines* 92. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 57–62

49 Baylor NW, Egan W, Richman P (2002) Aluminum salts in vaccines – US perspective. *Vaccine* 20: S18–S23

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Waiting for a pandemic

Rino Rappuoli and Giuseppe Del Giudice

Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

Abstract

After a quiet period of nearly 30 years, influenza strains with hemagglutinin types that have not been seen in humans previously started to jump from birds to man, suggesting the risk of a new influenza pandemic. However, in contrast to the situation with all the other influenza pandemics occurring in the 20th century and before, in the 21st century we have sophisticated technologies for diagnosis, therapy and prevention. Modeling of the possible spread of a pandemic suggests that vaccination is by far the only way to eliminate the risk of a new pandemic. In this chapter we review the development of new vaccines against H5N1 viruses, showing that effective vaccines adjuvanted with oil-in-water emulsion are about to be licensed and will soon be available. The race against an influenza pandemic has begun; it is a battle against time that mankind cannot afford to lose.

Introduction

Since 1580, at least ten influenza pandemics have occurred, with an average of one pandemic every 42 years. Analysis of the most recent and more accurate data predicts one pandemic every 30 years (see the chapter by Lattanzi). The last pandemic was in 1968, 40 years ago and therefore common sense and mathematical models predict that we should be prepared for a new pandemic. During the last 9 years, all the events that are expected to happen before a pandemic did happen. First, a new virus carrying the H5 antigen, that had never been in humans before jumped from chickens into man and killed six people in Hong Kong in 1997. This early outbreak was contained by culling chickens in the Hong Kong area. The virus momentarily disappeared and we forgot about it for a few years. However, the virus was not dead at all; it was successfully breading, multiplying and expanding in birds in South East Asia [1], until it suddenly blew up again with human cases in 2003 and 2004 in Vietnam, Thailand, Indonesia, and China. Clearly

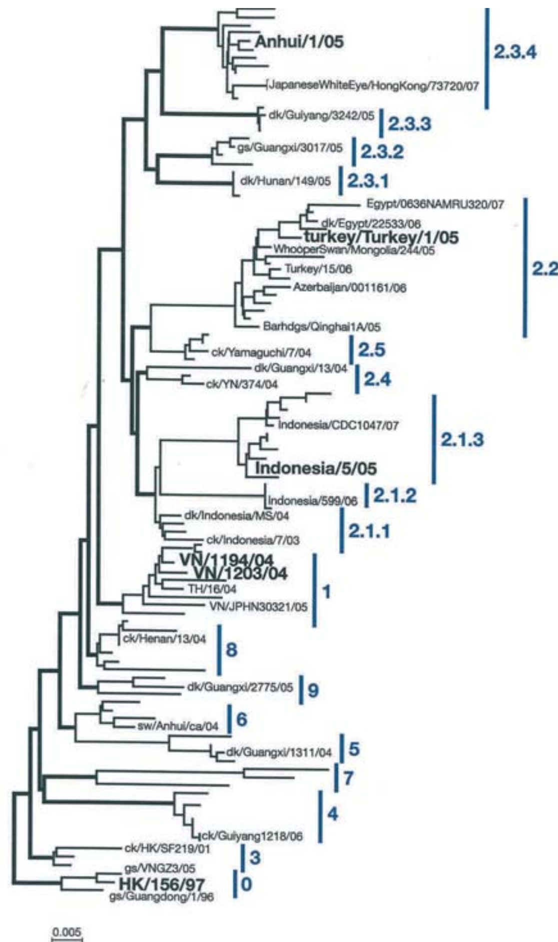

Figure 1. Evolution and nomenclature of the H5N1 virus isolates. Bold face indicates the most commonly studied viruses. Bold numbers on the right hand side indicate the clades and subclades.

the virus had escaped any control and was so widespread that since then culling hundreds of million of chickens in the areas of outbreak has provided only a temporary relief, but has never been able to control the spread of the virus. The virus, in fact, was spreading globally using migratory birds as vectors and soon appeared in the rest of Asia, Russia, Turkey, Egypt, and Nigeria. Today, the H5N1 virus is endemic in the bird population in Asia, Europe, and Africa. While spreading geographically, the virus has also been evolving and drifting antigenically, so that today we have many genetically distinct isolates of the H5N1 virus that can be classified into several clades and subclades (see Fig. 1). Up to 1 February 2008, the virus caused

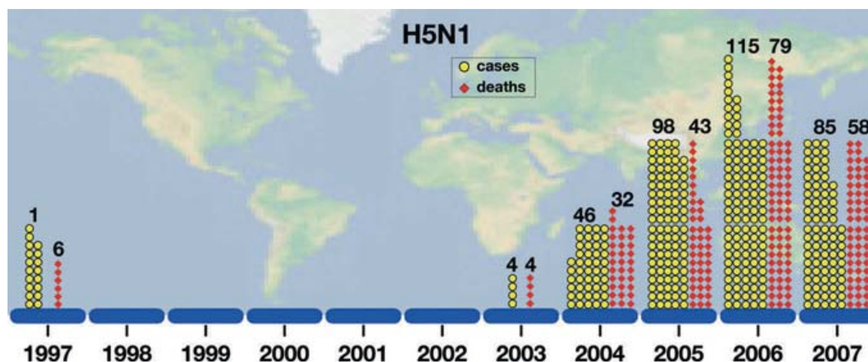

Figure 2. Yearly cases and deaths caused by the H5N1 virus from 1997 to 2007

Table 1. Cases and deaths caused by the H5N1 virus in different countries (WHO website, 1 February 2008)

Country	Total 2007	
	cases	deaths
Azerbaijan	8	5
Cambodia	7	7
China	27	17
Djibouti	1	0
Egypt	43	19
Indonesia	124	102
Iraq	3	2
Laos People's Democratic Republic	2	2
Myanmar	1	0
Nigeria	1	1
Pakistan	1	1
Thailand	25	17
Turkey	12	4
Viet Nam	102	48
Total	357	225

Total number of cases includes number of deaths. WHO reports only laboratory-confirmed cases.

357 reported cases and 225 deaths, with an overall mortality rate of 61% (see Fig. 2 and Tab. 1). All cases derived from close contacts with poultry, although in a few cases close contacts between people may have caused the infection. The virus continues to evolve, but is not yet able to be efficiently transmitted from human to human, and therefore the isolated viruses do not seem to be an immediate threat.

Table 2. Known influenza hemagglutinin types and species where they have been isolated

Hemagglutinin	Human	Swine	Equine	Avian
H1	+	+	–	+
H2	+	–	–	+
H3	+	+	+	+
H4	–	–	–	+
H5	–	–	–	+
H6	–	–	–	+
H7	–	–	+	+
H8–H16	–	–	–	+

In the meantime all predictions have been made. Some predict that the pandemic will come soon; others say that H5N1 will never cause a pandemic because the genetic background of the virus does not fit a pandemic strain. Others say that we may expect a pandemic from a virus different from H5N1. We should also consider the possibility that given the intense vaccination of the elderly population, the evolved healthcare system, the increased global surveillance, the 21st century may be the first period that will not see a pandemic influenza. Unfortunately, this scenario is unlikely to happen; in fact, the influenza virus is able to infect and evolve in several animal species (see Tab. 2), and considering that so far only 3 of the 16 known hemagglutinin (HA) types are present in human isolates, the virus seems to have enough fuel to continue to be a threat for mankind for the next several centuries.

In conclusion, although we have no certainty about when a pandemic is going to strike, these facts suggest that the risk of a pandemic should be taken into serious consideration, and that we should have a plan to be prepared for it. Considering that even a mild pandemic is predicted to cost to the USA economy alone more than 650 billion dollars and globally in excess of 3000 billion, a global investment of 30 billion (1% of the potential economic damage) seems a reasonable investment (insurance) against this risk.

Preclinical studies with vaccines against avian influenza

During the last few years, many academic laboratories, biotech companies and vaccine manufacturers have produced a plethora of vaccines potentially active against avian influenza. The most widely used are those manufactured in eggs using the same technology used to manufacture seasonal vaccines. These vaccines may be composed of whole inactivated virus, detergent-split virus or purified HA and neuraminidase (NA) subunits. These vaccines have already been tested in clinical trials and are described later on in this chapter. Here we describe the vaccine approaches for which results of clinical trials are not yet available or that have not yet been tested in humans.

The wild-type whole inactivated virus grown in cell culture (Baxter)

Baxter took the unusual approach of using fully virulent wild-type H5N1 viruses grown in Vero cells to make H5N1 vaccines. The rationale provided is that using a reverse genetic procedure, which delivers an attenuated non-dangerous virus that can be grown in biosafety level 2 laboratories, takes 3–4 weeks to generate the initial virus seed, while the wild-type virus can be used immediately for manufacturing. The caution with this approach is that thousands of liters of wild-type H5N1 virus even if grown under a biosafety 3 levels represent an extremely dangerous procedure, and in case of leakage may be enough to cause a pandemic in itself. A/Vietnam/1203/2004, A/Indonesia/05/2005 and other viruses were produced using this procedure [2]. The virus was first inactivated by a double procedure involving formaldehyde and UV treatment and then purified by sucrose gradient, ultracentrifugation, followed by diafiltration. The vaccine was immunogenic in preclinical studies, protective in mice, induced T cell responses and was cross protective. Preliminary clinical data have been reported at meetings but no published data are available yet.

Live attenuated vaccines (MedImmune/Astra)

Live attenuated H5N1 vaccines generated by reverse genetics and grown in eggs using the same procedure used for the live attenuated seasonal vaccine [3] have been reported. The preclinical studies have shown that the vaccines are immunogenic and induce protection and cross protection in mice even after one single dose [4]. However, these vaccines for the moment are not supposed to be used, i.e., not until a pandemic is declared, to avoid the possibility that the circulation of a live virus carrying the H5 gene may favor the evolution of a dangerous H5 strain and this may accelerate the pandemic.

Virus-like particles produced in baculovirus

Virus-like particles (VLPs) expressing the HA, NA, and M1 antigens of H5N1 virus have been produced in insect cell culture, using a baculovirus vector. The VLPs were immunogenic in mice. Recently, testing in a clinical trial in humans of an A/Indonesia/05/2005 H5N1 vaccine was reported [5]. Two doses of 15 and 45 μg were reported to induce a fourfold rise in neutralizing antibodies of 63% of subjects with the highest dose.

Vaccines based on M2

M2-based vaccines have been described in a number of studies. So far, nothing more than interesting preclinical reports are available [6–9]. Given the

absence of correlates of protection for vaccines based on M2, it is unlikely that it will be possible to see a fast development of these vaccines because in the absence of an efficacy trial it is virtually impossible to license these vaccines. It is possible, however, that in the long-term these components may find space first in improved seasonal vaccines and later in pandemic vaccines. The same may apply to vaccine constructs based on the internal conserved nucleoprotein (NP) [8, 10].

Clinical studies with vaccines against avian influenza

Many clinical studies have been performed during the last 10 years with vaccines against avian influenza. Below we describe mostly those studies that have been published, although in some cases we mention also data reported from meetings for which preliminary information is available. Unless otherwise stated in the text or in Table 3, immunogenicity was tested by hemagglutinin inhibition (HI) using horse red blood cells, by microneutralization (MN) using the RG A/Vietnam/1203/2004xAPR/8/34 influenza virus strain or by serum radial hemolysis (SRH) using turkey red blood cells coated with antigens derived from the tested virus (see the chapter by Montomoli). Seroconversion was considered positive when HI, or MN titers were <10 before immunization and >40 after immunization or when a fourfold increase in titer was observed in those subjects that had a pre-immunization titer >10. The overall data are reported in Table 3. SRH was considered positive when it was negative before immunization and had an area of hemolysis >25 mm^2 after immunization. Looking at the table, it is important to keep in mind that the data are not directly comparable since they have been produced at different times in different laboratories. HI studies in particular have been performed with horse erythrocytes using an assay that has never been validated across laboratories and for which no standards exist, so that large differences have been reported. Although MN also shows variability in different laboratories, it is probably a better assay for comparing results because it measures the ability of antibodies to neutralize viral infection, and this activity is biologically important.

The experience with MF59-adjuvanted H5N3 vaccine (Chiron/Novartis)

The first attempts to develop vaccines against H5N1 followed the 1997 outbreak in Hong Kong. At that time reverse genetics was not available, H5N1 could not be grown in eggs, reassortants could not be made and therefore only two attempts to make vaccines were made. One was a recombinant H5 subunit vaccine expressed in baculovirus produced by Protein Sciences (described below), the second one was a subunit vaccine prepared using a

non-virulent duck H5N3 virus that could grow in chicken eggs [11]. This H5N3 vaccine was a very interesting experience that provided most of the useful information we have today, and paved the way to further vaccine development. This vaccine was tested in human adult volunteers with and without the adjuvant MF59 (see the chapter by O'Hagan/Podda) at 7.5, 15 and 30 μg per dose (licensed vaccines contain 15 μg/dose). The data of the trial (reported in Fig. 4 in the chapter by Montomoli) were very surprising (Tab. 3). First, the conventional, non-adjuvanted vaccine did not elicit a significant protective antibody response (no HI response and only 10–45% MN). In retrospective we should not be too surprised by this result; while we use one dose of seasonal influenza vaccine in people that already have immunity against influenza, in the case of H5, people are naive to this antigen and therefore the vaccination needs to prime the immune system before it can induce a high antibody response. The really surprising finding instead was that the vaccine administered with the newly licensed MF59 adjuvant did induce a good immune response after one dose and a seroconversion by MN and SRH in nearly all subjects after two doses. Interestingly, the highest immune response was achieved with the 7.5 μg dose, which was the lowest used in the trial [11]. At 16 months after the primary immunization the test persons were given a boost with the same vaccine. Again the subjects vaccinated with the MF59-adjuvanted vaccine responded with a very strong and long-lasting immune response, while those vaccinated with the non-adjuvanted vaccine showed still a very low but detectable protective antibody response [12]. When the H5N1 virus started to circulate again in Thailand and Vietnam in 2003 and 2004, it was interesting to go back to the sera obtained from the trial and ask whether the people that had been immunized with the H5N3 virus had antibodies able to neutralize the H5N1 clade 1 viruses deriving from an antigenic drifting that had been taking place for 6–7 years. Figure 3 shows that, while the subjects vaccinated with the non-adjuvanted vaccines had no cross-clade neutralizing antibodies, the majority of the subjects vaccinated with MF59-adjuvanted vaccine had protective levels of antibodies against the heterologous clade 1 virus isolated in Vietnam and Thailand [13, 14]. In summary, the data had shown that MF59-adjuvanted vaccines could induce protective immunity against viruses not matching the vaccine strain and could cover the antigenic drift of the virus for 6–7 years. Finally, more recently, the people vaccinated with H5N3 in 1999 and boosted with H5N3 in 2001 were vaccinated in 2007 with a clade 1 vaccine. Preliminary data show that those who had been primed with the MF59-adjuvanted H5N3 vaccine produced very high level of antibodies against the clade 1 strain by 7 days after vaccination, while those primed without adjuvant also had a response but of much lower magnitude. People that had not been primed responded as expected only after two doses of vaccine and reached protective levels only 42 days after the first immunization. This latter study provided extremely important information showing that when people are primed with any H5N1 strain adjuvanted

Table 3. Clinical studies with vaccines against avian influenza.

Vaccine	Date	Dose	Adjuvant	Subjects	Pre-titers	Seroconversion* HI	Seroconversion* MN	Seroconversion* SRH	Seroconversion* Cross neutr. HI	Seroconversion* Cross neutr. MN	Symptoms
Chiron/Novartis H5N3 subunit Nicholson et al. [11]	1999	7.5	MF59	65	3%	60[5]	**80**[2]	**(100)/90**[3]	n.d.	7–14[4] (2 doses)	No major findings
		15				45	**100**	**(100)/82**			
		30				20	**100**	**(100)/80**			
		7.5	None			0	10	(0) 0		43–71 (3 doses)	
		15				0	18	(45) 0			
		30				9	36	(36) 9			
Baculovirus recombinant H5 Treanor et al. [17]	1999	0	None	147	Not reported	n.d.	4[1]	n.d.	n.d.	n.d.	No major findings
		25					17				
		45					28				
		90					52				
Sanofi split H5N1Vietnam 2004 Treanor et al. [16]	April 2005	0	None	450	3%	0	0	n.d.	n.d.	n.d.	Pain, dose-dependent up to 50%
		7.5				13	7				
		15				24	20				
		45				41	41				
		90				57	53				
Sanofi split Vietnam 2004 Bresson et al. [18]	July 2005	7.5	Aluminium hydroxide	300	~1%	28	16	n.d.	n.d.	n.d.	Pain, dose-dependent up to 68%
		15				44	18				
		30				66	41				
		7.5	None			43	20				
		15				44	22				
		30				53	27				

GSK split H5N1 Vietnam 2004 Leroux-Roels et al. [19]	March 2006	3.8	ASO3	400	~1%	**82**	**86**	n.d.	20–32%	67–77%	Pain, dose-independent up to 100% Pain, dose-dependent up to 68%
		7.5				**90**	**86**				
		4.5				**96**	**86**				
		30				**85**	**98**				
		3.8	None			4	22				
		7.5				16	37				
		7.5				35	53				
		30				41	65				
Novartis subunit H5N1 Vietnam 2004 Banzhoff [20]	April 2005	7.5	MF59	312 adults	n.d.	n.d.	**85**	**85**	n.d.	n.d.	Pain in 50–60% of subjects Pain in 20–25% of subjects
		15					**81**	**80**			
		75	MF59	173 elderly			**79**	**71**			
		15					**76**	**68**			
Sinovac whole virus H5N1 Vietnam 2004 Lin et al. [21]	July 2006	1.25	Aluminium hydroxide	120	3%	13[5]	9	n.d.	n.d.	n.d.	No major findings
		2.5				21	21				
		5				33	33				
		10				78	65				
Hungarian whole virus Vietnam 2004			Alum phosphate	146	0	645	n.d	n.d.	n.d.	n.d.	No major findings
Chiron/Novartis subunit H9N2 [23]	March 2005	3.75	MF59	96	n.d.	**100**	**100**	n.d.	n.d.		No major findings
		7.5				**92**	**92**				
		15				**100**	**100**				
		30				**100**	**100**				
		3.75	None			67	67				
		7.5				58	58				
		15				50	50				
		30				75	83				

*Seroconversion is regarded as positive when HI or MN were <10 before immunization and ≥ 40 after second immunization. When pre-titers were >10, fourfold rise in titer may be needed to score positive in seroconversion. SRH was considered positive when pre-immunization tests were negatives and post-immunization had a an area of hemolysis >25 mm^2.
[1]% with MN > 1/80; [2]% with NeuT > 1/32; [3]Seroconversion against H5N3 and H5N1, respectively; [4]Against Vietnam 2004 and Thailand 2004, respectively; [5]HI was done with chicken or turkey red blood cells.

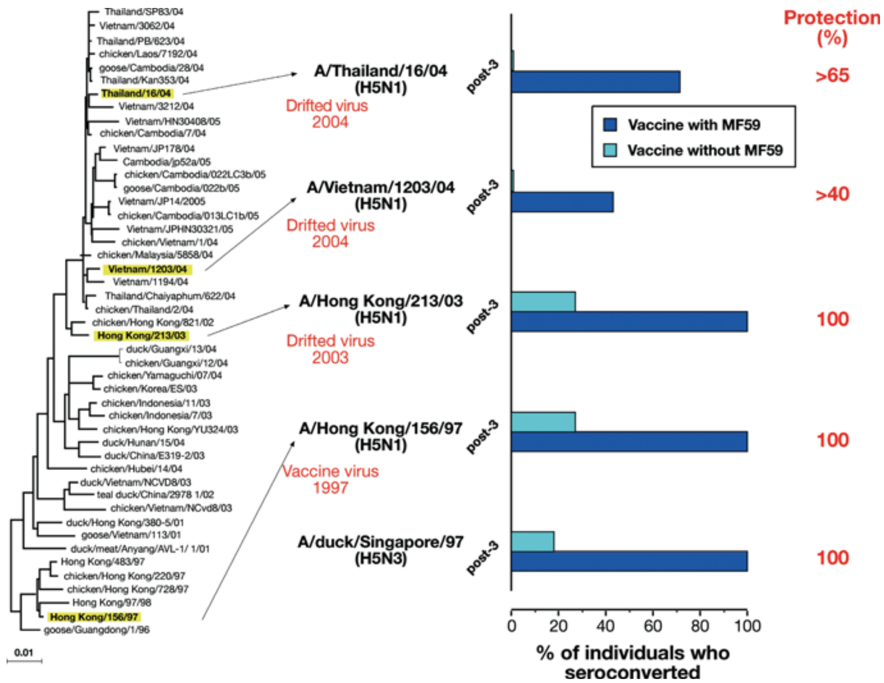

Figure 3. Phylogenetic tree of the H5N1 viruses circulating from 1997 to 2004 (left). On the right are reported the % of people with neutralizing titers ≥40 against the virus strains. The graph shows that vaccination with MF59-adjuvanted clade 0 vaccine induces protective antibodies against clade 1 strains, while the non-adjuvanted vaccine is unable to do so.

with MF59, not only do they develop a cross-protective immune response that may cover the strains that can come during the 6–7 years following vaccination, but also that at any time it is sufficient to give a boost with one dose of adjuvanted vaccine to get an almost immediate protection against the new strain. These data are of paramount importance in designing pre-pandemic vaccination strategies that are discuss later on the policy section of this chapter.

Recombinant HA (Protein Sciences)

The recombinant HA cloned from the H5N1 strain isolated in Hong Kong in 1997 was produced in baculovirus-infected cells and purified. Placebo, 25, 45 or 90 μg protein were used to immunize 147 subjects. Two doses were given, at day 0 and 21 or 42. An additional blood sample was taken at day 56. Respectively, 4, 17, 28 and 52% of the subjects had a MN > 1/80 after the second immunization (Tab. 3). The trial showed that, although some

immunity to H5N1 could be induced, large amounts of antigen were necessary. More recently these subjects were boosted with an H5N1 vaccine from Sanofi Pasteur without adjuvant. Preliminary available data suggest the 90 μg of adjuvant-free vaccine can boost the antibody response, but with a strength lower and a kinetic slower than that provided by MF59-adjuvanted vaccine. No data on cross-neutralizing antibodies have been reported [15].

Non-adjuvanted H5N1split vaccine (Sanofi Pasteur)

The real race to develop H5N1 vaccines started in 2004 and 2005 when, after the quiet period between 1998 and 2002, H5N1 started to cause new human cases in Vietnam and Thailand (see Fig. 2). This time it became clear that H5N1 not only had not gone away, but it was there to stay, and the global community started to panic fearing that the pandemic could come before the world had the time to be prepared. The first egg-based H5N1 vaccine was produced by Sanofi Pasteur. This was a split vaccine. The virus strain was generated by reverse genetics containing the HA and NA gene segments from the influenza A/Vietnam 1203/2004 and all the other genes from the strain A/PR/8/34 that grows well in embryonated eggs and is normally used to generate vaccine strains. The HA gene was further modified to replace the six basic amino acids at the cleavage site between domain 1 and 2 of the HA, which are known to be associated with increased virulence. Thanks to these modifications the new virus carried the HA and NA genes of the pathogenic H5N1 virus but was non-virulent and could be grown in a biosafety level 2 laboratory. After growth in eggs, the virus was purified by ultracentrifugation, inactivated with formaldehyde and split by addition of Triton X-100. The vaccine was finally ready after sterile filtration and additional steps of purification. Clinical trials started in April 2005 [16]; 450 subjects, with a median age of 39 years, were enrolled in a randomized, placebo-controlled, double-blind, multicenter trial. Groups of approximately 100 people received 7.5, 15, 45 or 90 μg vaccine antigen, 36 people received a placebo intramuscularly (i.m.) at days 0 and 28. A blood sample was also taken 28 days after the second dose. Safety of the vaccine was fine overall, with local pain and tenderness being the most frequently reported symptoms. These symptoms occurred after both doses, were dose-dependent and were reported by 40–50% of the subjects that received the highest vaccine doses. The immunogenicity results were clearly disappointing and completely aligned with the non-adjuvanted group of the H5N3 vaccine study [11] and with those from the Protein Sciences study with recombinant H5N1 [17]. Seroconversion using HI and MN were observed only after the second dose in approximately 50% of the subjects that received the high dose of 45 and 90 μg (see Tab. 3). Clearly these results indicated that, while a vaccine against H5N1 was possible, without an adjuvant up to 180 mg per subject were required. With this vaccine, the global

influenza vaccine manufacturing capacity (approximately 400 M doses of trivalent vaccine/year) was able to provide a vaccine for not more than 200 million people/year. This vaccine has been licensed in the USA by FDA.

Split vaccine adjuvanted with alum (Sanofi Pasteur)

In July 2005 Sanofi Pasteur performed a trial in Europe using a similar split vaccine in a randomized, multicenter, open label trial in 300 subjects aged 18–40 years [18]. The strain used was influenza A/H5N1/Vietnam/2004 prepared by reverse genetics by the National Institute for Biological Standards and Control (NIBSC). The vaccine was adjuvanted by aluminum hydroxide. Three doses 7.5, 15 and 30 μg were injected i.m. at days 0 and 21, a further blood sample was obtained at day 42. HI and MN were measured as in the previous trial. The results were disappointing. Neither the vaccine without the adjuvant nor the one adjuvanted with alum were able to induce substantial HI or MN seroconversions (Tab. 3). Some dose-dependent immunogenicity was observed; however, seroconversion was usually achieved in less than 50% of the cases and the only group that had an HI seroconversion above 50% was the one with 30 μg and alum. However, this group had very low seroconversion in MN, raising questions on the how the HI titers should be interpreted. In conclusion, this study confirmed what had already been well described in the 1960s and 1970s, that is, that the aluminum is not a good adjuvant for the influenza vaccine (see the chapter by Lattanzi).

AS03 adjuvanted H5N1 split vaccine (Glaxo SmithKline)

The split vaccine was made using the strain A/Vietnam/1194/2004, NIBRG-14 grown in eggs. The strain was generated by reverse genetics and contained the HA and NA gene segments from the H5N1 Vietnam 2004 and the other gene segments from the A/PR/8/34 strain. In this case as well, the HA gene was engineered to remove the six basic amino acids, so that the strain could be grown under biosafety level 2 conditions. The trial started in March 2006 and 400 subjects 18–60 years old were enrolled in an observer-blind, randomized trial [19]. Groups of 50 received 3.8, 7.5, 15 or 30 μg of vaccine alone or adjuvanted with the AS03 oil in water emulsion (5% DL-α-tacopherol and 95% squalene oil in water containing 2% Tween 80), a composition very similar to that of MF59 (see the chapter by O'Hagan/Podda). Two i.m. doses, at days 0 and 21 were given. Blood was also taken at day 42. Overall, safety was fine, with local pain being the most frequently reported symptom, and was dose dependent in up to 68% of the non-adjuvanted groups and dose independent in virtually all subjects (90–96%) in the adjuvanted groups. HI and MN were measured against the homologous vaccine strain and also against the heterologous, clade 2

A/Indonesia/5/2005 strain. As shown in Table 3, data obtained with the AS03-adjuvanted vaccine were very similar to those previously described for the MF59-adjuvanted H5N3 vaccine: seroconversion measured by MN and HI was achieved in 86–98% and 85–96% of the subjects, respectively, independently of the dose used. The data obtained with the vaccine without adjuvant were as disappointing as those previously obtained with the non-adjuvanted H5N3 and H5N1 vaccines: seroconversion was dose dependent and reached a maximum of 65% and 41% in MN and HI, respectively, at the maximum dose used. Interestingly, in this case, CHMP criteria of seroconversion rates in at least 40% of the subjects were achieved also after the first dose in those subjects that received 7.5 μg or more of adjuvanted vaccine. The vaccine was also shown to induce seroconversion against the heterologous clade 2 Indonesia strain, which was observed in 67–77% of the subjects when using the MN, but could be detected in only 20–32% of the subjects when using the HI.

Subunit vaccine adjuvanted with MF59 (Novartis)

The subunit vaccine was produced in eggs using the strain A/Vietnam/1194/2004, NIBRG-14 and according to the procedures used for the seasonal influenza vaccine [20]; 7.5 and 15 μg were used to immunize 312 adults and 173 elderly subjects i.m., at days 0 and 21. A blood sample was taken also at day 42. Table 3 shows that both doses induced seroconversion in the majority of the subjects (80–85% of the adults and 68–79% of the elderly seroconverted by MN or SRH). These data confirmed that the data obtained with H5N3 also apply to H5N1. These are also the first data available of the immunogenicity of the vaccine in the elderly population, and they indicate that the adjuvanted vaccine is suitable for the immunization of 65 years and older subjects [20].

Whole inactivated virus vaccine adjuvanted with aluminum hydroxide (Sinovac)

The strain A/Vietnam/1194/2004, NIBRG-14 grown in eggs, inactivated by formalin, concentrated and purified by chromatography, and formulated with aluminum hydroxide was used to immunize 120 subjects [21]. The randomized, placebo-controlled, double-blind study, started in July 2006 in 18–60 years old subjects in Beijing, China; 1.25, 2.5, 5 and 10 μg antigen per dose were used for immunization at day 0 and 28. A blood sample was also obtained at day 42. The vaccine was reported to be safe; however, the number of adverse events reported is so low that it is likely that the standards for reporting in this trial were different from the other trials. The HI titers were measured using turkey erythrocytes, and therefore cannot be compared to

any of the other data. In any case a dose-dependent seroconversion was observed in 13–78% of the subjects (Tab. 3). MN seroconversion occurred in only 9–65% of the subjects. So far, this is the only published trial using whole inactivated virus. The data (as judged from the MN seroconversion) are not very exciting. A second whole virus vaccine produced by the Hungarian Center for Allergy and Immunology reported 64% seroconversion by HI. However, even in this case HI was measured by chicken erythrocytes and therefore data cannot be compared with the others. [22]. Many other claims were made with whole inactivated viral vaccines by several independent groups. However, so far, none of them has provided robust data able to support the claims made often in press releases.

H9N2 vaccine adjuvanted with MF59 (Novartis)

The vaccine strain was a reassortant containing the HA and NA gene segments from the influenza A/chicken/Hong Kong/G9/97 and the remaining gene segments from the A/PR/8/34 strain. The virus was grown in eggs and the vaccine produced according to the procedures used for the seasonal subunit vaccine. In April 2005, 96 subjects (12 per group) received 3.75, 7.5, 15, or 30 μg vaccine with or without MF59 adjuvant. Immunization was i.m., at days 0 and 28. A further blood sample was taken at day 56.

The results, reported in Table 3, confirmed the data obtained with all the other trials performed with H5N1 and H5N3 [11–13]. Basically, nearly all subjects immunized with the adjuvanted vaccine achieved seroconversion both in HI and MN, independently of the dose used, showing that 3.75 μg are still able to induce a full immune response. In marked contrast, the non-adjuvanted vaccine showed a much lower, dose-dependent response [23].

Policy

During the 1997–2007 period we have seen the most diverse reactions of policymakers towards the potential risk of a pandemic influenza. The approaches have been from totally ignoring the problem (1998–2003), to panic (2005–2006), to a more recent balanced rational approach. Below are the possible options that policymakers have. A summary of the different options is reported in Figure 4.

Options 1 and 2: Vaccinating pandemic survivors

Options 1 is based on the strategy to wait for the WHO to declare the beginning of the pandemic, identify the strain causing the pandemic, rush to manufacture the vaccine and then vaccinate people with two doses of vaccine.

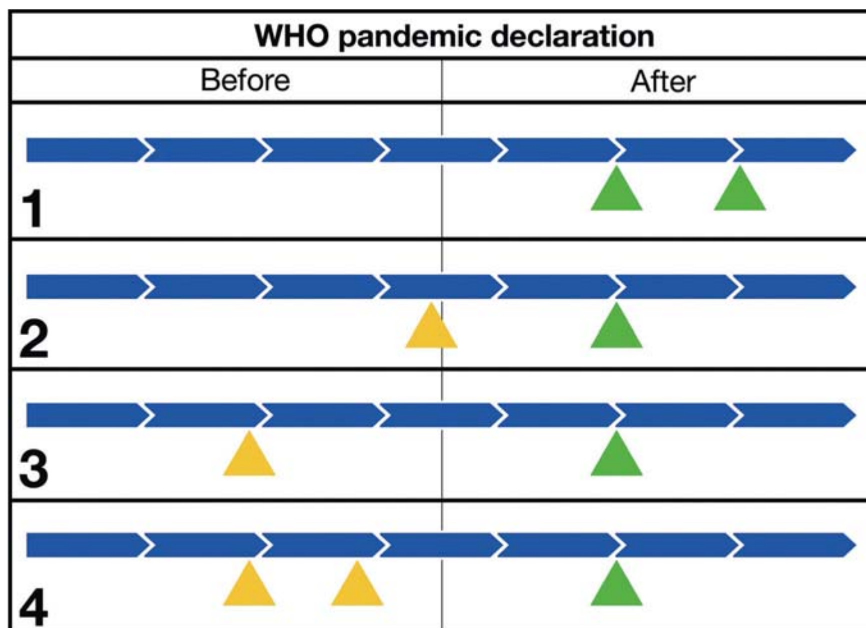

Figure 4. Options available to policymakers to vaccinate against a pandemic influenza virus. Options 1–4 are described in the text. Yellow triangles show immunizations before the beginning of the pandemic; green triangles indicate vaccination after the beginning of the pandemic.

Option 2 is to stockpile enough vaccine to administer one dose when WHO declares the pandemic and then manufacture the vaccine using the pandemic strain and give the second dose. Clearly these options have the insurmountable limit that the vaccination campaign becomes effective when it is too late, and therefore vaccination can only help those people that have already survived the pandemic. In fact, while all modeling studies [24–26] predict that in only 3 months a pandemic will sweep a country, preparing and delivering two doses of vaccines will take at least 8–10 months, independently of the capacity and technology available (3–4 weeks to prepare the seed strain, 3–6 months to manufacture any significant amount of vaccine, 1 month to build immunity). These two options have been very popular between 2004 and 2007 when vaccines had not yet been tested in clinical trials, safety data were not yet available, and cross protection had not been convincingly demonstrated. Both options are based on the assumption that the vaccines need to be made using the same strain causing the pandemic and that cross protection is not effective. Option 2 has the additional disadvantage that if the strain causing the pandemic is of a different clade of the one stockpiled it would be not useful. Today we know that, while non-adjuvanted or alum-adjuvanted vaccines have not been shown to induce cross protection among

different clades of H5N1, the vaccines adjuvanted with oil in water emulsions have been shown to induce cross protection- and therefore it is possible to vaccinate without waiting for the strain that causes the pandemic.

Options 3 and 4: Pre-pandemic vaccination

Option 3 is to vaccinate people with one dose of adjuvanted vaccine before the pandemic starts and then to give a boost with the pandemic strain once the pandemic starts. This option is based on the observation that, while priming with one dose is not able to produce a fully protective and long lasting immunity, it will be able to prime the immune system and induce a memory that can be boosted later with the pandemic strain. This option, although not ideal would have the great merit to prime the immune system against an H5 virus so that the basic assumptions for pandemic to happen (which is the circulation of the virus in an unprimed population) would be eliminated. If option 3 was adopted, we should no longer have to fear a pandemic caused by an H5 virus, because this virus would circulate in a primed population and in the worse case scenario the virus would be able to cause only a bad influenza season.

Option 4 is to induce fully protective and cross protective titers of antibodies and memory B and T cells by vaccinating the population with two doses of adjuvanted vaccine using any H5 virus and still have the option to boost the immunity with a pandemic vaccine when the pandemic is declared. Today, this is possible using the vaccines adjuvanted with MF59 or AS03. In fact, these vaccines have been shown to induce highly protective level of antibodies against the vaccine strain, which are cross reactive with strains from different clades, and to induce a memory that when boosted by any distantly related H5 virus it provides, in just 7 days, a fully protective response against the new virus. This implies that regardless of the virus strain used for the pre-pandemic vaccination, the appropriate immunity will be there when needed. In fact, the boost can be provided by a new immunization as shown in Figure 4, or by the infection with the pandemic strain that will be able to induce a protective immunity before it induces the disease.

In conclusion, pre-pandemic vaccination is the only option that we have to make sure that we are in the position to prevent and control the risk of a pandemic. In this way, the vaccine would block the disease or would significantly affect its morbidity/mortality.

Barriers to pre-pandemic vaccination

If pre-pandemic vaccination has the potential to eliminate the risk of a pandemic, why are policymakers not rushing to implement it? There are several barriers between the policymakers and the implementation. The first one is

that pre-pandemic vaccines have not yet been licensed and therefore they are not yet available. This barrier is about to disappear because the Novartis and GSK vaccines are likely to be licensed in Europe in the near future and will be submitted for registration in the USA shortly. The second barrier has been that, while cross protection had been amply demonstrated with H5N3, and in pre-clinical studies, human data on H5N1 were not yet available. Today, these data are available and strong. Perhaps the strongest data derive from the boost in 2007 using a clade 1 virus of the people immunized in 1999 and 2001 with H5N3. These data show that when the appropriate memory is present, a few days after boost are sufficient to induce a fully protective response against a drifted virus. This can be achieved by vaccinating with an adjuvanted vaccine or by infection.

In conclusion, the technically sound barriers to pre-pandemic vaccination are disappearing and we see no reason why this should not be implemented. However, there are probably even more important barriers that are likely to be in the way of making the right decisions. The first and most important is that a decision to actively vaccinate people implies taking a risk, while waiting is not risky for the policymakers. (However, it is a risk for the population.) The major fear of the policymakers is safety. What happens if they recommend a vaccination and then we have a safety problem with the vaccine? This is reasonable concern that should be addressed. Policymakers look at the swine flu experience of 1977 (see the chapter by Lattanzi) and they do not want to find themselves in a similar situation. They are absolutely right in this; no compromise is acceptable for safety in a pre-pandemic situation. On the other hand, there are a number of reassuring data that should make policymakers confident. First, the swine flu vaccination was mostly done with a whole inactivated virus, which at that time was known to be not pure and to induce a lot of side effects. (That is the reason why split and subunit vaccines were developed and replaced the whole virus in seasonal vaccines.) Today, we have the option to use vaccines that are not based on whole, inactivated virus. Second, the safety of some of the adjuvants has been amply demonstrated. MF59 has been safely given to >30 million people, and passive surveillance showed that there is no risk of Guillain Barré (reported cases were similar to those of non-adjuvanted vaccines). In conclusion, all the barriers to pre-pandemic vaccination are disappearing and we are confident that our generation will be the first one in centuries that will be able to prevent an influenza pandemic.

References

1 Deng G, Li Z, Tian G, Li Y, Jiao P, Zhang L, Liu Z, Webster RG, Yu K (2004) The evolution of H5N1 influenza viruses in ducks in southern China. *Proc Natl Acad Sci USA* 101: 10452–10457
2 Kistner O, Howard MK, Spruth M, Wodal W, Brühl P, Gerencer M, Crowe BA,

Savidis-Dacho H, Livey I, Reiter M et al (2007) Cell culture (Vero) derived whole virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses. *Vaccine* 25: 6028–6036

3 Luke CJ, Subbarao K (2006) Vaccines for pandemic influenza. *Emerg Infect Dis* 12: 66–72

4 Suguitan, AL Jr, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K (2006) Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med* 3: e360

5 Novavax Inc (2007) *Press release Novavax announces preclinical results for seasonal influenza vaccine program*

6 De Filette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, Resch S, Fiers W (2005) Universal influenza A vaccine: Optimization of M2-based constructs. *Virology* 337: 149–161

7 De Filette M, Fiers W, Martens W, Birkett A, Ramne A, Löwenadler B, Lycke N, Jou WM, Saelens X (2006) Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine* 24: 6597–6601

8 Jimenez GS, Planchon R, Wei Q, Rusalov D, Geall A, Enas J, Lalor P, Leamy V, Vahle R, Luke CJ et al (2007) Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. *Hum Vaccines* 3: 157–164

9 Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, Hogan RJ, Wu Z, Benton KA, Tumpey TM, Epstein SL (2007) Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. *Emerg Infect Dis* 13: 426–435

10 Livingston BD, Higgins D, Van Nest G (2006) Evolving strategies for the prevention of influenza infection: potential for multistrain targeting. *BioDrugs* 20: 335–340

11 Nicholson KG, Colgate AE, Podda A, Stephenson I, Wood J, Ypma Y, Zambon MC (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: A randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 357: 1937–1943

12 Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, Ypma E, Zambon M (2003) Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine* 21: 1687–1693

13 Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, Zambon MC, Katz JM (2005) Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: A potential priming strategy. *J Infect Dis* 191: 1210–1215

14 Schwatz B, Gellin B (2005) Vaccination strategies for an influenza pandemic. *J Infect Dis* 191: 1207–1209

15 Zangwill K, Campbell J, Noah D, Treanor J (2007) Evaluation of a third dose of subvirion H5N1 influenza vaccine (rgA/Vietnam/1203/04xPRB) in healthy adults. In: *Options for the Control of Influenza VI* (Toronto, June 17–23, 2007). Abstract no. P737

16 Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M (2006) Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* 354: 1343–1351
17 Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, Wolff M, Rabinovich G, Blackwelder W, Katz JM (2001) Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. *Vaccine* 19: 1732–1737
18 Bresson JL, Perronne L, Leunay O, Gerdil C, Saville M, Wood J, Hoschler K, Zambon MC (2006) Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: Phase I randomized trial. *Lancet* 367: 1657–1664
19 Leroux-Roels I, Borkowski A, Vanwolleghem T, Dramé M, Clement F, Hons E, Devaster JM, Leroux-Roels G (2007) Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: A randomised controlled trial. *Lancet* 370: 580–589
20 Banzhoff A (2007) Antigen-sparing effect of MF59 in Fluad-H5N1. In: *Third WHO meeting on evaluation of pandemic influenza prototype vaccines in clinical trials*, 15–16 February 2007, WHO, Geneva
21 Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, Gao Q, Zhang Z, Liu Y, Wang Z et al (2006) Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: A Phase I randomised controlled trial. *Lancet* 368: 991–997
22 Vajo Z, Kosa L, Visontay I, Jankovics M, Jankovics I (2007) Inactivated whole virus influenza A (H5N1) vaccine. *Emerg Infect Dis* 13: 807–808
23 Atmar RL, Keitel WA, Patel SM, Katz JM, She D, El Sahly H, Pompey J, Cate TR, Couch RB (2006) Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. *Clin Infect Dis* 43: 1135–1142
24 Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S, Burke DS (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437: 209–214
25 Germann TC, Kadau K, Longini IM Jr, Macken CA (2006) Mitigation strategies for pandemic influenza in the United States. *Proc Natl Acad Sci USA* 103: 5935–5940
26 Flahault A, Vergu E, Coudeville L, Grais RF (2006) Strategies for containing a global influenza pandemic. *Vaccine* 24: 6751–6755

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Modeling influenza pandemic and interventions

Caterina Rizzo[1,2] and Marta Luisa Ciofi degli Atti[1]

[1]National Center of Epidemiology, Surveillance, and Health promotion, Infectious Disease Unit, Istituto Superiore di Sanità, Roma, Italy; [2]Department of Pharmaco-Biology, University of Bari, Italy

Abstract

Modeling is an important aspect of pandemic preparedness, and different approaches have been conducted to date. The epidemic dynamics and the assessment of containment or mitigation strategies, such as vaccination, border restriction, antiviral treatment of cases and prophylaxis of household or school/workplace contacts of index cases can be predicted by employing classical compartmental models [susceptible, exposed but not yet infectious, infectious, recovered and no longer susceptible (SEIR), possibly with age and/or geographic component]. Other authors have implemented the evaluation of realistic, individually targeted, public health intervention strategies that requires highly detailed models, such as the individual based model (IBM). According to the predictive models used, an influenza pandemic would spread worldwide over a period of 2–7 months, depending on the basic reproductive number (a measure on how many people an infectious individual infects on average), and reducing transmission would entail combining control measures, specifically, reducing contacts and performing both therapeutic and prophylactic use of antivirals and vaccination. International border restrictions are unlikely to delay the spread by more than 2–3 weeks unless more than 99% effective. Similar results were obtained for social distancing measures, such as school closure. Treatment of clinical cases and prophylaxis of close contacts can lower transmission, reducing the clinical attack rate by 40–50%, but it would be logistically challenging, requiring a stockpile for more than 25% of the population. Vaccine stockpiled in advance, even if less efficacious than pandemic vaccines, could significantly reduce the cumulative attack rates. In Italy, the combination of the described measures would lead to 10–15 million of cases being avoided, depending on vaccine effectiveness (taken as 50% or 70%). Mathematical models are necessary to plan and evaluate interventions based on different strategies. They represent a relevant tool to assist public health decision-making in preparing the response to a new influenza pandemic.

Introduction

Three influenza pandemics occurred in the 20th century. Their mortality impact ranged from devastating (the 1918 ‘Spanish’ A/H1N1 influenza), to moderate (the 1957 Asian A/H2N2 pandemic) to rather mild (the 1968

'Hong Kong' A/H3N2 virus) [1, 2]. Recently, the emergence of the highly virulent A/H5N1 avian influenza strain [3], which has proven its ability to pass directly from birds to humans [4], and could potentially acquire the capacity for efficient person-to-person transmission, has raised concern over the risk of a future influenza pandemic [5]. This virus or a closely related one is considered to be the leading contender as the source of the next human influenza pandemic [6, 7]. Moreover, the increasing mobility of the population at a global level and the greater speed of means of transport would make the control of the spread of infection particularly problematic.

Measures to be adopted in case of a pandemic

To harmonize interventions to be adopted in case of an influenza pandemic, countries have been urged to strengthen their preparedness plans [8] following the 2005 WHO recommendations [9]. For this reasons, a number of countries have developed or updated a national influenza preparedness plan including measures to be adopted in case of a pandemic. A range of measures have been suggested, including those that involve personal actions (hand-washing and mask wearing), non-pharmaceutical interventions (e.g., international air travel restriction, border closure, social distancing measures, quarantine) [10], and pharmaceutical interventions (antiviral drugs and vaccines) [6]. A few of the measures are relatively straightforward to implement (e.g., hand-washing), while others are going to be difficult (timely mass use of antiviral drugs for index and close contacts of cases) and yet others are costly and potentially highly disruptive (border closures) [10]. However, the application of these measures has the objective to reduce the number of people who are infected, need medical care and die during an epidemic. By lowering the numbers of affected people, the measures can also lessen the secondary consequences of epidemics such as the impact of mass absenteeism on key functions (e.g., delivering healthcare, food supplies, fuel distribution, the utilities, etc).

The experience gathered from past pandemics could be useful to orientate public health decision-making in the case of an influenza pandemic. Models are a useful tool to plan and evaluate interventions based on different strategies. However, models are only as reliable as the estimates of the biological and epidemiological parameters they require; in the case of pandemic influenza, it is possible to use data from past pandemics to implement some important parameters (such as the length of latent and infectious period of the new pandemic influenza virus). Past pandemics, however, widely differ between countries in terms of severity and in terms of groups of individuals most affected and experiencing most transmission [11, 12]. For instance, in Italy, as in other European countries [12], the pandemic season developed a more destructive conflagration in the second season of

Table 1. Multinational comparison of the mortality impact of the A/H3N2 Hong-Kong pandemic, based on all-cause excess mortality estimates.

	Italy[a]	England & Wales[a]	France[a]	Australia[b]	Japan[a]	USA[c]	Canada[c]
Overall mortality impact per 100,000	107	77	72	64	49	27	12
Percent mortality increase over baseline	24%	20%	21%	16%	20%	8%	6%

[a, b, c]Mortality estimates for the major pandemic season, which timing varied by country:
[a]the second season of A/H3N2 virus circulation, 1969–1970.
[b]the second season of A/H3N2 virus circulation, 1970.
[c]the first season of A/H3N2 virus circulation, 1968–1969.

circulation of A/H3N2 virus (i.e., in 1969–1970), 1 year after the pandemic strain was first introduced in Italy [13]. The pandemic season seems to have had a greater impact in Italy with an estimated excess mortality of 38 (20 000 deaths) for pneumonia and influenza and of 107 (57 000 deaths) for all cause. This unexpected large mortality impact, for pneumonia and influenza and all cause excess mortality, was threefold higher compared to that in the United States and onefold higher than in other European countries (Tab. 1) [11].

Despite all the uncertainties, several countries have considered such previous statistics in quantitative estimates of the likely pattern and speed of spread of a pandemic and the possible impact of different interventions, using mathematical modeling [14–19].

Different type of mathematical models

As mentioned above, epidemic dynamics and the assessment of containment or mitigation strategies, can be predicted by employing classical compartmental models, i.e., SEIR [16, 20–22], or by implementing the evaluation of realistic, individually targeted, public health intervention strategies using highly detailed models [17, 18], such as IBM.

The classical SEIR model is based on the assumptions that were considered mandatory in traditional mathematical models of diseases, such as the assumption of homogeneous mixing, in which the population is structured according to age and residence on the basis of Census data or specific survey [20]. IBM provides a system in which the precise spatial locations of individuals and movement patterns can be used to evaluate mitigation measures [23]. These models also allow the most detailed representation of social contacts between individuals, and are therefore considered more

reliable than SEIR. As a first step in the construction of an IBM, contacts are progressively "structured" by co-locating individuals into the diverse environments where they are expected to have contacts. Transmission can occur within households, schools and workplaces that represent the structured component of the model, or through random contacts, that represent the unstructured component of the model. The available IBM has achieved sophisticated descriptions of the structured component of contacts, but the unstructured component continues to be unclear.

Epidemiological parameters

Parameters used in the models influence the time estimated for the pandemic to evolve, although the estimates are similar considering that studies based on deterministic SEIR models on a global [22] or local [24] scale or individual-based models [17, 18, 25]. In fact, most of the modeling studies are calibrated using parameters derived from the observed rates of seroconversion and illness due to the pandemic strains that circulated during the 20th century, particularly to scale infectivity. In 1918, during the first pandemic wave, the attack rate of clinical influenza was highest in children (40%) and then fell gradually with age, reaching 9% in people aged 75 years or more. An average attack rate of 34% was reported during the 1957 pandemic, with an age distribution similar to that observed during the first pandemic wave of 1918 [26]. The age distribution of attack rates during the 1968 pandemic was similar in children, ranging between 41% and 43%, but decreased less markedly with age, remaining above 30% in all other age groups [26].

The three basic parameters used to describe the natural history of infection are: (i) person-to-person transmission rate, which is assumed to vary by age of susceptible and infectious individuals and with the time since infection; (ii) the length of the latent period (time elapsed from infection to the onset of infectivity); and (iii) the length of the infectious period.

Past modeling studies have largely assumed distributions of the incubation and infectious periods reported in two historical papers [27, 28]. However, data for these estimates, used to estimate the duration of infectiousness are lacking. In fact, researchers have assumed constant infectiousness from the end of infection latency to recovery from infection. Recently, some authors [17, 24] have derived new estimates of the incubation period and development of infectiousness over time for human influenza based on data from experimental studies in which viral shedding was measured in volunteers challenged with wild-type influenza viruses [29]. The profiles obtained were consistent with those of a prospective household-contacts survey conducted in France [30, 31], with peak infectivity between the days 2–3 after infection, and infectivity lasting a maximum of 10 days [17, 24].

Table 2. Comparison of average arrival time and peak time of mathematical modelling studies in Europe.

Studies	Region	Average arrival time in days	Average peak time in days
Flahault et al. [22]	World	90	150
Ferguson et al. [32]	UK	30	50–65[a]
Germann et al. [18]	USA	–	64–117[a]
Colizza et al. [21]	Western Europe	50–90*	140–450[a]
Ciofi degli Atti et al. [48]	Italy	–	112

[a]Depending on R_0 values

Seeding the epidemic

According to the predictive models used, an influenza pandemic would spread worldwide over a period of 2–7 months, depending on the basic reproductive number (R_0, commonly known as the average number of secondary infections produced when one infected individual is introduced into a host population where everyone is susceptible [20]), and reducing transmission would entail combining control measures, specifically, reducing contacts and performing both therapeutic and prophylactic use of antiviral, and vaccination [16–18, 21, 22, 25, 32, 33].

Depending on the type of model used to estimate the spread and the impact of an influenza pandemic, the epidemic could began with a fixed starting number of infected individuals (e.g., a single [25] or 76 [18] randomly assigned infective on day 0) or modeling a simple global compartmental transmission model, using international travel data, to estimate the number of infected persons expected to arrive in a country per day [17, 21]. Using this second method, which is not a spatial method but is more appropriate than assuming a fixed starting number of infected individuals or a constant importation rate through time [17], the probability of importing infection in Europe or in the USA in the first 30 days is very small [17, 21] (Tab. 2).

An example of simulation model: The case of Italy

To estimate the expected reduction in the attack rate for different mitigation strategies in Italy, a deterministic mathematical model of influenza transmission with a stochastic simulation component was used. The model used to predict the spread of pandemic influenza in Italy and to evaluate the impact of vaccination, antiviral for prophylaxis (AVP) and social distancing measures is an SEIR deterministic model. The model included a stochastic component that takes into account all of the random effects that

are important during a pandemic's initial and final stages, when the number of infected individuals would be low. Precisely, whenever the deterministic prediction of the number of infected individuals in an age class/region was below the threshold value of 10, it was replaced by a Poisson variable with the same mean. In this way all the simulations were different and in some of them stochastic extinction may occur due to the fact that the total number of infected may drop to 0 in the initial phases of the epidemics. Analyzing the results of the model, we have considered only results regarding those simulations in which sustained transmission is observed and used to evaluate the effect of possible containment strategies.

In each simulation, the pandemic began with the introduction of 5 infected adults in the Lazio Region, where Rome's international airport is located. The results were obtained by averaging over 200 simulations for each scenario.

Using this type of model, analytical calculation of R_0 is not feasible [25]. For this reason, we proceeded by simulation, randomly choosing 5 infective individual, as described above, and then continuing the number of secondary infections. The mean R_0 was 1.8, which, when applying the contact matrix, corresponds to a cumulative infected attack rate of 35%. How the results would change depending on different levels of pathogen transmissibility, with a resulting R_0 of 1.6 or 2.0, was then explored with a sensitivity analysis.

Demographic parameters of the model

In the SEIR model used, the population is structured according to age and region of residence. In the case of Italy, the national population (56 995 744 inhabitants) was distributed in 20 regions, according to national demographic data obtained from the 2001 Census [34]. Six age classes were defined: infants 0–2 years of age, children 3–14 years of age, teenagers 15–18 years of age, young adults 19–39 years of age, adults 40–64 years of age and elderly aged 65 and older. The contact matrices have been defined considering separately household, school/work-place and "random" contacts, and using data on household composition, school attendance, employment condition. The distribution of household sizes and the average numbers of children in households of different sizes were simulated to be consistent with 2001 Census [34]. For greater flexibility of the model, the contact matrix includes compartments of individuals with lower contact rates, as often assumed in epidemic models, especially for sexually transmitted diseases [20]. Data on transportation were defined using data on national airline traffic [35].

Epidemiological parameters of the model

The three basic parameters used to describe evolutions between the different stages of infection were: (i) person-to-person transmission rate, which

is assumed to vary by age of susceptible and infectious individuals and with the time since infection; (ii) the length of the latent period (time elapsed from infection to the onset of infectivity); and (iii) the length of the infectious period. In the model, as an assumption, for symptomatic individuals the incubation period was equal to the latent period [36–41]. As influenza virus infection is not always symptomatic, in the model 30% of infected individuals would not be sufficiently ill to be identifiable [25]. Based on the literature [17, 25, 42, 43], in the model an incubation period of 1 day and an infectious period of 3.9 days were assumed.

Intervention scenarios

In the intervention scenarios, two doses of vaccine were administered, 1 month apart. The target population was divided into four categories, according with the Italian National Plan for preparedness and response to an influenza pandemic [44]: (i) personnel providing essential services (15% of the 25–60-year-old working population) [45]; (ii) elderly persons (≥65 years); (iii) children and adolescents aged 2–18 years; and (iv) adults (40–64 years). A vaccination coverage of 60% of the target population, based on the 2005–2006 national influenza coverage [46], was assumed. A period of 2 weeks would be necessary for administering the vaccine to each target category.

For vaccine effectiveness (VE) two different assumptions were made: (i) VE of 70% for all categories; and (ii) VE of 50% for all categories; for both assumptions, we assumed that the VE would be reached 15 days after the second dose. Moreover, different scenarios of vaccine availability were considered: in one scenario, adequate VE would be reached 4 months after the first national case; in the second scenario, it would be reached after 5 months. An adequate VE at 4 months would be feasible only if the first dose contained an avian virus precursor of the pandemic strain [6], followed by a dose of pandemic vaccine; the actual VE of this regimen was assumed to be equal to that of two doses of the pandemic vaccine.

The treatment of the index case was assumed not to affect virus transmission [6]. However, the use of antiviral for prophylaxis (AVP) of uninfected individuals was assumed to reduce susceptibility by 30% and infectiousness by 70% [47]. The administration of one course of antiviral drugs for the entire epidemic period was considered; however, since the feasibility of actually doing this would be limited, the opportunity to limit AVP to 16 weeks from the onset of the first Italian case was also considered. AVP was assumed to reduce the transmission rate among household contacts, based on the consideration that those household contacts already infected at the time of AVP treatment would have a reduced infectiousness, so that it would be as if only a fraction of them were actually infected; those not yet infected when beginning AVP would benefit from both lower susceptibility and lower infectiousness.

As non-pharmaceutical measures, the nationwide closing of all schools for 3 weeks, public offices for 4 weeks, and public gathering places (e.g., restaurants, cinemas, and churches) for 8 weeks, were considered. These measures would be introduced simultaneously 4 weeks after the start of the pandemic, in the country. In the model, school closure would reduce the contacts among children and teenagers (the school component of the transmission rate) by 75%; workplace closure would reduce the job component of the transmission rate by 16%; closure of public gathering places would reduce the random component of the transmission rate by 50%.

The baseline scenario

In the absence of control measures, the epidemic peak would be reached approximately 4 months after the identification of the first case, with a total of 3 million cases during the peak week. The epidemic would be over in 7 months, with a cumulative infected attack rate (AR) of 35% (approximately 20 million cases). The dynamics of the epidemic were similar in all age groups, whereas the cumulative infected AR varied markedly by age group. The incidence would be particularly high among 15–18-year olds, with a cumulative infected AR of 54% (Fig. 1).

Due to the stochastic component of the model, the introduction of few infectious individuals in the population did not always result in an outbreak; in fact, in around 40% of the simulations, the number of infected individuals in the early stages of the pandemic was insufficient for sustaining transmission, and the epidemic expired spontaneously. When the simulation produced an epidemic, the 5–95% percentile values of the AR estimates were within 11%.

The value of mitigation measures

For $R_0 = 1.8$, the highest reduction (from 35% to 4.2%) would be obtained by implementing social distancing measures, providing AVP for the entire epidemic, and assuming that vaccination begins 4 months after the first national cases with a VE of 70% (when combining measures, we assumed that vaccination would be provided to all categories). This would allow for 17 million cases to be avoided by vaccinating around 26 million individuals and by providing AVP to approximately 3 million individuals. The cumulative infected AR would be higher (11%) if assuming that vaccination begins 5 months after the first national cases, avoiding 13 million cases by treating 25 million individuals and 7 million individuals with vaccine and AVP, respectively. Providing AVP for 16 weeks, instead of for the entire epidemic period, would increase the cumulative infected AR to 8.4% or 16.6% if starting vaccination 4 or 5 months after the first national cases, respectively.

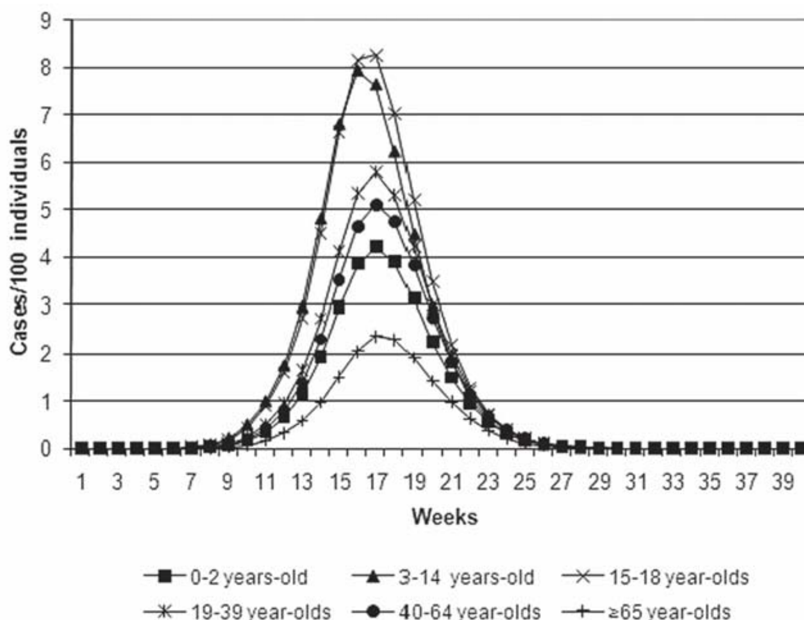

Figure 1. Weekly attack rate, by age group, with no control measures

However, this would determine an important reduction in the number of treated individuals (approximately 150,000). Combining control measures would also increase the probability of stochastic extinction during the initial phases of the epidemic, due to a low number of infectious individuals. A VE of 50% for all categories considered would affect the cumulative infected AR estimates, but only when considering vaccination starting 4 months after the identification of the first national cases. In fact, the cumulative infected AR would be 6–8% higher than the AR assuming a VE of 70%, with a remarkable difference in terms of the number of avoided cases (Tabs 3 and 4).

For $R_0 = 1.6$, the epidemic could be mitigated with moderate efforts; all strategies would be successful independently of the timing of vaccination, of the duration of providing AVP, and of the timing of social distancing measures. For $R_0 = 2$, this combination of control measures would result in a decrease in the cumulative infected AR, from 42% to approximately 20%.

Discussion

All studies conducted to date suggest that combining measures would be more effective, especially when using the pre-pandemic vaccine. This holds

Table 3. Effectiveness of combined control measures on the dynamics of an influenza pandemic in Italy with an R_0 of 1.8 and an attack rate of 35%, with 70% vaccine effectiveness (VE)

Interventions		Attack rate[a]	Avoided cases	Treated individuals	
				With vaccine	With antiviral
First vaccine dose at 5 months for all categories[b]					
Social distancing measures	Antiviral for 16 weeks	16.6% (10.5–21.2)	10,371,848	25,837,926	159,521
	Antiviral for the entire epidemic	11.3% (5.5–15.8)	13,408,909	25,837,928	7,177,152
First vaccine dose at 4 months for all categories[b]					
Social distancing measures	Antiviral for 16 weeks	8.4% (5.3–10.2)	15,070,696	25,837,928	152,056
	Antiviral for the entire epidemic	4.2% (1.4–6.4)	17,477,424	25,837,928	2,673,736

[a]Value in parentheses represent the 5–95 percentile values of the attack rate estimates.
[b]The target population was divided into four categories: (i) personnel providing essential services (15% of the 25–60-year-old working population) [45]; (ii) elderly persons (≥65 years); (iii) children and adolescents aged 2–18 years; and (iv) adults (40–64 years).

even if considering the pre-pandemic vaccine less effective than the pandemic vaccine [17, 18, 25].

Dynamic of an influenza pandemic in absence of interventions

Depending on the R_0 values considered, from mild to high transmissibility scenarios, modeling studies have estimated that the first cases of influenza in a future pandemic would be imported to Europe within 30–90 days of its emergence elsewhere in the world [11, 17, 22]. The incidence would peak 50–150 days after importation of first cases. [17, 18, 21, 22]. All the results of the considered studies showed that infection would spread simultaneously to nearly the entire country considered, with no clear spatial pattern. According with these studies, the results reported for Italy [48] showed that, with an R_0 value of 1.8, the incidence would peak after 112 days. The epidemic, in Italy, would be over in 215 days.

However, all the dynamics of an influenza pandemic described by mathematical models do not take in account the fact that the past influenza pandemics in Europe and Italy occurred over two consecutive winters, with the highest AR in the second winter [11, 12, 49]. This two-wave pattern is probably an effect of the closing of schools during the summer [11]. Thus, models probably depict a "worst case scenario", which could be useful in evaluating control measures [18].

Table 4. Effectiveness of combined control measures on the dynamics of an influenza pandemic in Italy with an R_0 of 1.8 and an attack rate of 35%, with 50% vaccine effectiveness (VE)

Interventions		Attack rate[a]	Avoided cases	Treated individuals	
				With vaccine	With antiviral
First vaccine dose at 5 months for all categories[b]					
Social distancing measures	Antiviral for 16 weeks	20.9% (16.9–24.1)	7,907,818	25,837,926	159,520
	Antiviral for the entire epidemic	15.4% (11.5–18.7)	11,059,485	25,837,928	9,763,649
First vaccine dose at 4 months for all categories[b]					
Social distancing measures	Antiviral for 16 weeks	16.0% (15.1–16.7)	10,715,666	25,837,928	154,130
	Antiviral for the entire epidemic	11.0% (8.6–12.2)	13,580,818	25,837,928	6,983,830

[a]Value in parentheses represent the 5–95 percentile values of the attack rate estimates.
[b]The target population was divided into four categories: (i) personnel providing essential services (15% of the 25–60-year-old working population) [45]; (ii) elderly persons (≥65 years); (iii) children and adolescents aged 2–18 years; and (iv) adults (40–64 years).

Pharmaceutical interventions

Modeling studies have shown that vaccination and the use of antiviral drugs seem to be the most effective measures for pandemic control, especially when rapidly implemented [17, 18, 25]. In the Italian case, considering an R_0 value of 1.8, the cumulative infected AR would be 4.2%, but this would require an extremely high number of AVP doses. Providing AVP for 16 weeks only would increase cumulative infected AR to 8.4%, which is similar to that observed during severe seasonal epidemics [50], with a considerable reduction in the number of doses provided. All the modeling studies published to date have shown a successful control of pandemics, except the highest level of transmissibility, using vaccination [17, 18, 25]. However, as reported in other studies [18, 25, 48], independently of the VE, vaccination of children and young adults would considerably reduce the incidence also in other age groups (i.e., resulting in "herd immunity"), probably because of the important role of children and adolescents in the spread of influenza, as also observed in inter-pandemic periods [50, 51]. All the modeling studies, published to date, support the idea that, during a pandemic, vaccinating children should be a higher priority than vaccinating elderly [18, 25, 48]. For the high level of transmissibility, the timing of vaccination is also crucial. Only the administration of the first vaccine dose within 2 months from the first world case [17] would control the pandemic. However, this would be possible only if vaccines against "high pandemic risk" avian influenza strains (such as A/H5N1) were stockpiled before the

pandemic, since to develop a vaccine against a new, potentially pandemic influenza virus would take approximately 6–8 months [52].

Regarding antiviral drugs, AR reduction were obtained when treating close contacts of index cases (i.e., household contacts and close contacts in the school or workplace) with AVP only in the earliest stages of an outbreak [17, 25]. However, to obtain a reduction in the cumulative AR for a high transmissibility scenario ($R_0 > 2$), interventions based solely on AVP use are inadequate to mitigate an influenza pandemic, even in the case of adequate stockpile of antiviral drugs supplies, for approximately 25% of the population [17, 45].

Non-pharmaceutical interventions

The simulations show that appropriate and prompt measures, when combined, could be effective in containing an influenza pandemic [16–18, 25]. Timing is also essential, and measures that at first glance appear to be less important, such as increasing social distancing, could be extremely useful in delaying the epidemic peak and thus providing more time for vaccines to be produced. Implementing such measures, however, would entail organizing a variety of both medical and non-medical resources, and some measures, such as the closing of schools, would also have a social impact. After the identification of a new human-to-human transmissible influenza virus strain somewhere in the world, efforts to prevent the spread to unaffected countries are expected. The role of global air travel restriction has been extensively studied, but it still remains controversial. Rvachev and Longini [28] developed a deterministic model to study the role of global air travel restriction in the 1968–1969 influenza pandemic. Recently, other studies have indicated models to update population levels [53, 54], incorporate more recent air travel patterns [53, 54], adjust seasonality parameters [53, 54], add stocasticity to the model [55], and extend it to more cities [55]. These models found that an influenza pandemic at the present time, when compared to 1968, would spread faster, and that the international air travel restrictions alone will not contain a pandemic, but can delay the epidemic peak (about 2–3 weeks) [56]. In contrast, other authors [21, 55] have concluded that international travel restriction do little to reduce the rate of spread globally, slowing down by only a few days or weeks the overall evolution of a pandemic. All these studies suggest that unless there is almost complete cessation of international travel to a country, the attempts at border closure will be unsuccessful in preventing disease entry [55].

Similar results were obtained for social distancing measures and quarantine [10, 17]. The model implemented in Italy [16, 48] found that social distancing measures, including closing of all schools for 3 weeks, public offices for 4 weeks, and public gathering place for 8 weeks, were not effective in reducing the cumulative AR, but would delay the epidemic peak by

2 weeks. These results are consistent with that reported in previous studies, where social distancing measures would delay the epidemic peak by 1–3 weeks [17].

Conclusion

The transmissibility of a future pandemic virus is uncertain, so is important to explore different possible scenarios. Mathematical modeling studies have shown the importance of the combination of different mitigation measures for pandemic control. For the highest level of transmissibility, in particular, only the very rapid treatment with antiviral drugs and vaccines can significantly reduce transmission. For these reason pandemic preparedness is crucial: a large stockpile of a vaccine with potential pandemic influenza antigens, coupled with the capacity to rapidly make a better-matched vaccine based on the human strain, would be the best strategy to control an influenza pandemic. In Italy, it has been shown [12], for example, that using this strategy combined with antiviral for 16 weeks, and with social distancing measures would avoid 10 to 15 million of cases, depending on VE (50% or 70%) (Tabs 3 and 4).

For all these reasons, modeling tools can help to determine and prioritize the measures most likely to mitigate an influenza pandemic, and can be very useful in informing health authorities, at the national and international levels, in the absence of data from actual intervention trials, which would be unfeasible outside, and impractical within, the context of an actual pandemic.

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References

1 Glezen WP (1996) Emerging infections: Pandemic influenza. *Epidemiol Rev* 18: 64–76
2 Simonsen L (1999) The global impact of influenza on morbidity and mortality. *Vaccine* 17 (Suppl 1): S3–10
3 Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD et al (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 430: 209–213
4 Anonymous (2006) Epidemiology of WHO-confirmed human cases of avian influenza A (H5N1) infection. *Wkly Epidemiol Rec* 81: 249–257

5 Anonymous (2006) Outbreak news. Avian influenza, Turkey – Update. *Wkly Epidemiol Rec* 81: 42–43
6 Monto AS (2006) Vaccines and antiviral drugs in pandemic preparedness. *Emerg Infect Dis* 12: 55–60
7 Stephenson I, Gust I, Kieny MP, Pervikov Y (2006) Development and evaluation of influenza pandemic vaccines. *Lancet Infect Dis* 6: 71–72
8 Influenza Team ECDPaC (2007) Pandemic preparedness in the European Union – Multi-sectoral planning needed. *Eur Surveill* 12: E070222.1
9 Anonymous (2005) *WHO global influenza preparedness plan: The role of WHO and recommendations for national measures before and during pandemics*. WHO/CDS/CSR/GIP/2005.5
10 Glass RJ, Glass LM, Beyeler WE, Min HJ (2006) Targeted social distancing design for pandemic influenza. *Emerg Infect Dis* 12: 1671–1681
11 Rizzo C (2007) Trends for influenza-related deaths during pandemic and epidemic seasons, Italy, 1969–2001. *Emerg Infect Dis* 13: 694–699
12 Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L (2005) Multinational impact of the 1968 Hong Kong influenza pandemic: Evidence for a smoldering pandemic. *J Infect Dis* 192: 233–248
13 Rocchi G, Ragona G, de Felici A, Muzzi A (1974) Epidemiological evaluation of influenza in Italy. *Bull World Health Organ* 50: 401–406
14 Chun BC (2005) Modelling the impact of pandemic influenza. *J Prev Med Pub Health* 38: 379–385
15 Doyle A, Bonmarin I, Levy-Bruhl D, Strat YL, Desenclos JC (2006) Influenza pandemic preparedness in France: Modelling the impact of interventions. *J Epidemiol Community Health* 60: 399–404
16 Epico working group. (2007) Modelling scenarios of diffusion and control of pandemic influenza, Italy. *Euro Surveill* 12: E070104
17 Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS (2006) Strategies for mitigating an influenza pandemic. *Nature* 442: 448–452
18 Germann TC, Kadau K, Longini IM Jr, Macken CA (2006) Mitigation strategies for pandemic influenza in the United States. *Proc Natl Acad Sci USA* 103: 5935–5940
19 Hak E, Meijboom MJ, Buskens E (2006) Modelling the health-economic impact of the next influenza pandemic in The Netherlands. *Vaccine* 24: 6756–6760
20 Anderson RM, May RM (1992) *Infectious Diseases of Humans: Dynamics and Control*. Oxford University Press, New York
21 Colizza V, Barrat A, Barthelemy M, Valleron AJ, Vespignani A (2007) Modeling the worldwide spread of pandemic influenza: Baseline case and containment interventions. *PLoS Med* 4: e13
22 Flahault A, Vergu E, Coudeville L, Grais RF (2006) Strategies for containing a global influenza pandemic. *Vaccine* 24: 6751–6755
23 Riley S (2007) Large-scale spatial-transmission models of infectious disease. *Science* 316: 1298–1301
24 Carrat F, Luong J, Lao H, Salle AV, Lajaunie C, Wackernagel H (2006) A ‘small-world-like’ model for comparing interventions aimed at preventing and controlling influenza pandemics. *BMC Med* 4: 26
25 Longini IM Jr, Nizam A, Xu S, Ungchusak K, Hanshaoworakul W, Cummings

DA, Halloran ME (2005) Containing pandemic influenza at the source. *Science* 309: 1083–1087
26 Glezen WP, Simonsen L (2006) Commentary: Benefits of influenza vaccine in US elderly – New studies raise questions. *Int J Epidemiol* 35: 352–353
27 Elveback LR, Fox JP, Ackerman E, Langworthy A, Boyd M, Gatewood L (1976) An influenza simulation model for immunization studies. *Am J Epidemiol* 103: 152–165
28 Rvachev LA, Longini IMJ (1985) A mathematical model for the global spread of influenza. *Math Biosci* 75: 3–22
29 Treanor JJ, Hayden FG (1998) Volunteer challenge studies. In: KG Nicholson, AJ Hay, RG Webster (eds): *Textbook of Influenza*. Blackwell Science, London, 517–537
30 Carrat F, Sahler C, Rogez S, Leruez-Ville M, Freymuth F, Le GC, Bungener M, Housset B, Nicolas M, Rouzioux C (2002) Influenza burden of illness: Estimates from a national prospective survey of household contacts in France. *Arch Intern Med* 162: 1842–1848
31 Cauchemez S, Carrat F, Viboud C, Valleron AJ, Boelle PY (2004) A Bayesian MCMC approach to study transmission of influenza: Application to household longitudinal data. *Stat Med* 23: 3469–3487
32 Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S, Burke DS (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437: 209–214
33 Fraser C, Riley S, Anderson RM, Ferguson NM (2004) Factors that make an infectious disease outbreak controllable. *Proc Natl Acad Sci USA* 101: 6146–6151
34 Istituto Nazionale di statistica (2001) 14° Censimento Generale della Popolazione e delle Abitazioni ISTAT
35 Istituto Nazionale di statistica (2006) *Statistiche del trasporto aereo – Anno 2003 ISTAT*
36 Calfee DP, Peng AW, Cass LM, Lobo M, Hayden FG (1999) Safety and efficacy of intravenous zanamivir in preventing experimental human influenza A virus infection. *Antimicrob Agents Chemother* 43: 1616–1620
37 Doyle WJ, Skoner DP, Alper CM, Allen G, Moody SA, Seroky JT, Hayden FG (1998) Effect of rimantadine treatment on clinical manifestations and otologic complications in adults experimentally infected with influenza A (H1N1) virus. *J Infect Dis* 177: 1260–1265
38 Fritz RS, Hayden FG, Calfee DP, Cass LM, Peng AW, Alvord WG, Strober W, Straus SE (1999) Nasal cytokine and chemokine responses in experimental influenza A virus infection: Results of a placebo-controlled trial of intravenous zanamivir treatment. *J Infect Dis* 180: 586–593
39 Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE (1998) Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. *J Clin Invest* 101: 643–649
40 Hayden FG, Jennings L, Robson R, Schiff G, Jackson H, Rana B, McClelland G, Ipe D, Roberts N, Ward P (2000) Oral oseltamivir in human experimental influenza B infection. *Antivir Ther* 5: 205–213

41 Hayden FG, Treanor JJ, Fritz RS, Lobo M, Betts RF, Miller M, Kinnersley N, Mills RG, Ward P, Straus SE (1999) Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: Randomized controlled trials for prevention and treatment. *JAMA* 282: 1240–1246
42 Chowell G, Hengartner NW, Castillo-Chavez C, Fenimore PW, Hyman JM (2004) The basic reproductive number of Ebola and the effects of public health measures: The cases of Congo and Uganda. *J Theor Biol* 229: 119–126
43 Flahault A, Letrait S, Blin P, Hazout S, Menares J, Valleron AJ (1988) Modelling the 1985 influenza epidemic in France. *Stat Med* 7: 1147–1155
44 Ministero della Salute (2006) *Piano Nazionale di preparazione e risposta ad una pandemia influenzale* (http://www.ministerosalute.it)
45 Colizza V, Barrat A, Barthelemy M, Vespignani A (2006) The modeling of global epidemics: Stochastic dynamics and predictability. *Bull Math Biol* 68: 1893–1921
46 Ministero della Salute (2005) *Influenza Vaccination Coverage* (http://www.ministerosalute.it/promozione/malattie/malattie.jsp)
47 Longini IM Jr, Halloran ME, Nizam A, Yang Y (2004) Containing pandemic influenza with antiviral agents. *Am J Epidemiol* 159: 623–633
48 Ciofi degli Atti ML, Rizzo C, Bella A, Massari M, Iannelli M, Lunelli A, Pugliese A, Ripoll J, Manfredi P et al (2006) Scenarios of diffusion and control of influenza pandemic in Italy. *Rapporti ISTISAN Istituto Superiore di Sanità* 33: 1–41
49 Viboud C, Boelle PY, Pakdaman K, Carrat F, Valleron AJ, Flahault A (2004) Influenza epidemics in the United States, France, and Australia, 1972–1997. *Emerg Infect Dis* 10: 32–39
50 Bella A, De Mei B, Giannitelli S, Rota MC, Salmaso S, Donatelli I, Affinito C, Fabiani C, Fiaccavento S, Frezza F et al (2005) FLU-ISS: Sistema di sorveglianza sentinella dell'influenza basata su medici di medicina generale e pediatri di libera scelta. Rapporto sulla stagione influenzale 2004–2005. *Rapporti ISTISAN Istituto Superiore di Sanità* 22: 1–79
51 Viboud C, Boelle PY, Cauchemez S, Lavenu A, Valleron AJ, Flahault A, Carrat F (2004) Risk factors of influenza transmission in households. *Br J Gen Pract* 54: 684–689
52 Daems R, Del Giudice G, Rappuoli R (2005) Anticipating crisis: Towards a pandemic flu vaccination strategy through alignment of public health and industrial policy. *Vaccine* 23: 5732–5742
53 Grais RF, Ellis JH, Glass GE (2003) Assessing the impact of airline travel on the geographic spread of pandemic influenza. *Eur J Epidemiol* 18: 1065–1072
54 Grais RF, Ellis JH, Kress A, Glass GE (2004) Modeling the spread of annual influenza epidemics in the U.S.: The potential role of air travel. *Health Care Manag Sci* 7: 127–134
55 Cooper BS, Pitman RJ, Edmunds WJ, Gay NJ (2006) Delaying the international spread of pandemic influenza. *PLoS Med* 3: e212
56 Epstein JM, Goedecke DM, Yu F, Morris RJ, Wagener DK, Bobashev GV (2007) Controlling pandemic flu: The value of international air travel restrictions. *PLoS ONE* 2: e401

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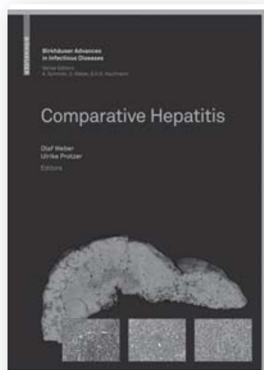

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Acute and chronic hepatitis and hepatitis-related diseases such as liver failure, liver cirrhosis and hepatocellular carcinoma are among the most important causes for disabilities and death. Hepatocellular injury is due to a variety of agents including viruses, toxins, radiation, injury and drugs but also bacteria, parasites and autoimmune reactions. This volume reviews today's knowledge about hepatitis with emphasis on comparative aspects between hepatitis in humans and animals, but also between different etiological agents. This particular viewpoint makes the book relevant for scientists from both human and veterinary medicine, gastroenterologists, pathologists, virologists and students of human and veterinary medicine.

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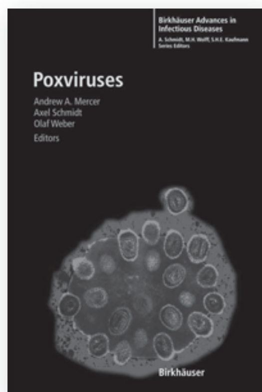

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The recent international alarm at the possible use of smallpox as weapon of bioterror has refocused the attention of the scientific community, public health policy makers and the public on poxviruses. In contrast to the threat posed by some of these viruses, other poxviruses show exciting beneficial potential including their use as valuable tools in biomedical research and in the delivery of vaccines. This monograph provides a comprehensive review of the poxvirus family with a particular emphasis on current developments. It includes the latest insights into poxviral molecular biology, diagnosis, therapy, vaccine development and the beneficial exploitation of these viruses in biomedical research. Each chapter is written by a leader in the field. The reviews present all aspects of the field, including historical perspectives, along with summaries of the latest advances. This combination makes the book relevant for graduate students, researchers new to the field and to experienced poxvirologists.

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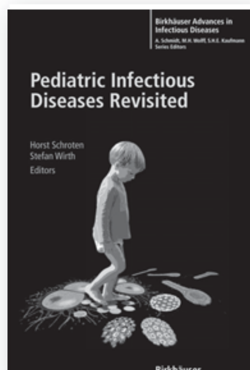

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Pediatric Infectious Diseases Revisited

Schroten, H., Universitätskinderklinik Düsseldorf, Germany / **Wirth, S.**, Helios Klinikum Wuppertal, Germany (Eds)

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The successful prevention of childhood diseases like diphtheria, tetanus and pertussis has made a major contribution to the improvement of public health, and vaccines and a variety of drugs are amongst the most fruitful applications of 20th-century research. Today pediatric infectious disease research is closely interconnected with other disciplines. Understanding the biology of the causative agents and the pathogenesis of disease is an essential step in achieving control and elimination of the diseases. Starting with historical, epidemiological and sociocultural issues, the volume presents clinical as well as molecular biological aspects of pediatric infectious diseases. New insights into the pathogenesis of infection are presented and an update on diagnostics, prevention and treatment of pediatric viral, fungal and bacterial diseases provided. The role of emerging new pathogens is also highlighted. The book aims at an interdisciplinary audience of clinicians and non-clinicians: pediatricians, infectious disease researchers, virologists, microbiologists, public health politicians and university libraries.

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