Animal Models for Evaluation of Influenza Vaccines

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Contents

Abstract Influenza viruses are emerging and re-emerging viruses that cause worldwide epidemics and pandemics. Despite substantial knowledge of the mechanisms of infection and immunity, only modest progress has been made in human influenza vaccine development. The rational basis for influenza vaccine development originates in animal models that have helped us to understand influenza species barriers, virus–host interactions, factors that affect transmission, disease pathogenesis, and disease intervention strategies. As influenza evolution can surmount species barriers and disease intervention strategies that include vaccines, our need for appropriate animal models and potentially new host species will evolve to meet these adaptive challenges. This chapter discusses animal models for evaluating vaccines and discusses the challenges and strengths of these models.

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Abbreviations

CAIV	Cold-adapted and attenuated reassortant influenza vaccine
CTL	Cytotoxic T cell
DNA	Deoxyribonucleotides
HI	Hemagglutination-inhibiting
LAIV	Live attenuated influenza virus
M1	Matrix 1 protein
M2	Matrix 2 protein
NHP	Nonhuman primate
NP	Nucleoprotein
OAS	Original antigenic sin
PR5	Puerto Rico 5
PR8-f	PR8 that had been passaged 91 times in ferrets
PR8-m	PR8 that had been passaged 332 times in mice
SAα2,3Gal	Sialic acids with an α2,3 linkage
SAα2,6Gal	Sialic acids with an α2,6 linkage
TIV	Trivalent inactivated vaccine

1 Introduction

A variety of animal models have been critical to the foundation of human influenza vaccine development. Animal models are used to characterize the host and its immune response to infection, disease course, pathogenesis, and transmission of infectious diseases, and they also enable the development of diagnostics, therapeutics, and vaccines. Indeed, diseases lacking animal models are poorly understood in comparison to those with a good animal model. Animal models also enable preclinical testing of the safety and efficacy of investigational drugs and the safety and immunogenicity of investigational vaccines. Despite the number of scientific and medical barriers that animal models have helped to overcome, there are also political and social barriers that need to be addressed for vaccine development in particular, such as age bias, vaccine supply ignorance and fear of vaccines, an emerging anti-vaccine movement, issues with social reimbursement of vaccine costs, and inadequate systems and procedures for implementing vaccination. The following sections summarize the role of animal models and their contributions to human influenza vaccine development.

1.1 Isolation of Influenza Virus

Animal models have played an important role in our understanding of the spectrum of disease caused by influenza viruses. During the early twentieth century, viruses were generally identified and isolated by inoculation and passage in experimental

animals (Eyler 2006). Likewise, the first influenza virus to be characterized (by Richard Shope in 1930; Shope 1931) was an H1N1 virus isolated from the lungs of diseased hogs, which was filtered and transferred to naïve swine, resulting in acute respiratory infection (Shope ,b). The first human influenza virus isolate, A/WS/33 (named after Wilson Smith who isolated the virus), was identified by infecting ferrets with filtered throat washings. The initial ferret infection showed that the disease could be transmitted by contact with infected animals or passaged by experimental infection with nasal washings from diseased ferrets (Smith et al. 1933). It was also shown that transmission of human influenza to ferrets was possible using sputum from patients collected during a 1934 epidemic in Puerto Rico (Francis 1934). This H1N1 influenza virus isolate, named Puerto Rico 5 (PR5), was passaged repeatedly in ferrets and was inadvertently transmitted back to a laboratory worker during the course of the animal studies (Francis 1934). Later, ferret passages of this virus were used to inoculate mice and caused variable disease; however, at the third mouse passage, the PR5 isolate was consistently lethal in mice (Francis 1934). The PR5 strain was lost, but PR8 (A/Puerto Rico/8/34) was subsequently derived (Francis 1937). By 1940, PR8 had been passaged 91 times in ferrets (PR8-f), and, after minimal passages in ferrets, 332 times in mice (PR8-m) (Horsfall et al. 1941). While the precise lineage may be uncertain, the PR8 strain of influenza (A/PR/8/34) remains a widely used laboratory strain. For the next 30 years, influenza virus was the most extensively studied viral pathogen of humans. The goal of this international effort was to develop a safe and efficacious vaccine. While some of this work was conducted in human trials, animal models were extensively used to maintain virus stocks, as well as in vaccine design, preliminary efficacy studies, and in the detection of antibodies against specific influenza viruses (Eyler 2006). By the early 1940s, World War II raised fears of a repeat of the Spanish influenza pandemic that was observed during World War I. These concerns drove the formation of the Commission on Influenza, which expanded the influenza vaccine program and focused ongoing research efforts.

2 Human Influenza Vaccines

2.1 The Early Years

The discovery of influenza A virus in 1933 (Smith 1933) and the development of an efficacious vaccine by the Commission on Influenza of the US Armed Forces Epidemiological Board during World War II marked the advent of intensive animal model studies in the development of influenza A vaccines (Francis 1953). However, once an early efficacious vaccine had been developed, limited attention was paid to additional influenza vaccine development until the 1946–1947 H1N1 influenza A epidemic in which there was lack of vaccine protection (Rasmussen et al. 1948). During the 1946–1947 H1N1 virus outbreak, it was noted that the antigenic specificity differed markedly from that of the viral antigens in the current vaccine based on

findings using hemagglutination inhibition assays with ferret antisera (Hirst 1947a). Interestingly, during this scientific investigation it was noted that the viral antigenic specificity differed between individual ferret-derived antisera; thus, chickens were intraperitoneally injected with embryonated egg-passaged virus. The viruses did not proliferate in the chickens but gave potent antibody responses that were not biased in specificity compared to the different ferret antisera. These early studies of immunologic specificity among various influenza virus strains contributed to the breakthrough discovery that there was nonrandom progressive antigenic change in influenza A virus surface proteins isolated in successive years—a feature now termed antigenic drift (Hilleman et al. 1950). Emergence of influenza drift variants continues to be an issue with influenza vaccine efficacy, as evidenced by recent vaccine failures during the 2007–2008 influenza season (Branch 2008).

2.2 Vehicles for Scientific and Biomedical Discovery

The use of multiple animal species to model human disease was highlighted during World War II as the United States prepared to deal with the potential for biological warfare. The idea that vaccine countermeasures against viruses could be tested in valid animal models was intrinsic to the military research programs at that time and continues today. The use of animals as surrogates for humans in efficacy trials came under FDA scrutiny in the late 1950s because many therapeutics that were being introduced were not effective or had serious but undiscovered side effects (Anderson and Swearengen 2006). Today the use of animal models for vaccine efficacy studies are better understood, more tightly regulated, and offer a reasonable approach to developing safe and efficacious vaccines. There is a burgeoning need for animal models to evaluate influenza vaccine safety and efficacy, particularly as vaccine is increasingly used in young children, the immune suppressed, and the elderly—groups that have traditionally not responded well to the vaccine. In addition to the use of novel and sometimes complex influenza vaccine development strategies, as well as the push toward cell-based influenza vaccine development, it is important to have ways to study influenza vaccine safety and effectiveness prior to human studies and use. As vaccine development relies heavily on appropriate animal model studies, it is becoming clearer that the translation of animal model findings to the human condition is not straightforward and has limitations.

Our understanding of the immunogenic potential of human influenza vaccines has relied on results learned from animal models. To better understand some of the mechanisms that lead to vaccine inadequacy or failure, substantial research has focused on determining the relationship between laboratory and clinical measures of protection induced by modern influenza vaccines. These studies are often specific to the type of the influenza virus vaccine e.g., inactivated vs. live attenuated. For the inactivated product, indirect methods of potency quantitation have been used for evaluation. For example, early techniques to quantitate the immunogenic potential of influenza vaccines in experimental animals included antigen extinction methods, tests based upon the intranasal vaccinating dose required to inhibit replication of

unadapted influenza viruses in the lungs of mice, and a two-step antigen extinction technique involving the intranasal instillation of pooled immune serum and virus mixtures into mice (Barry et al. 1974; Kilbourne 1976; Tannock et al. 1981). These and related methods are cumbersome, poorly reproducible, and rely excessively on the virulence of the mouse-adapted challenge virus. Current methods of evaluating the immunity induced by vaccination, particularly against a single strain, employ the analysis of antigenic differences first measured by means of red blood cell agglutination (Hirst 1943). This commonly used assay provides a qualitative view of antigenic differences, but it is considered inappropriate for quantitative analysis. Our increasing understanding of the immune response to vaccination or infection in animal models has provided important insights into other considerations that are used to assess vaccine potency and efficacy, including neutralizing antibody titers, mucosal IgA responses, original antigenic sin, and CD8 cytotoxic T cell responses important in heterotypic immunity.

3 Animal Models in Human Vaccine Development

3.1 The Ferret Model

The ferret was the first animal model used for influenza virus research and continues to have a major role in vaccine development. The concept of antigenic drift of the influenza virus was first charted in ferret studies, and early influenza vaccination studies in ferrets revealed important findings regarding vaccine efficacy. For example, the concept of original antigenic sin (OAS), defined as the tendency for antibodies produced in response to primary exposure to influenza antigens to suppress the creation of new and different antibodies to a new version of the influenza virus, was first observed in the ferret model (Webster 1966; Webster et al. 1976). The early finding of OAS highlighted the importance of developing vaccines with sufficient antigenic distance so as to broaden vaccine efficacy. This is particularly important today, as human influenza vaccine design for commercial translation to humans is done annually under considerable time constraints. The use of the ferret model in human vaccine development is based on three principal features: (1) influenza infection in ferrets emulates many features of the disease observed in humans; (2) human influenza A and B viruses infect ferrets without adaptation, and; (3) the physical features of ferrets, including their airways and sneeze response make them amenable for characterizing aspects of disease (Maher and DeStefano 2004). Ferrets and humans have similar clinical courses of disease (Leigh et al. 1995), and, similar to humans, the severity and time course of the disease can vary with virus strain, age and health of the animal. Infection with seasonal human influenza viruses is generally localized to the upper respiratory tract. Illness is usually acute, with clinical illness lasting up to a week in healthy individuals. During the peak of fever, which corresponds with peak virus shedding, both humans and ferrets transmit virus to each other. In both cases, transmission can occur by aerosol droplet and

direct or indirect contact (fomites) (Bridges et al. 2003). However, the ferret model does have caveats, including cost, housing requirements, and availability of immunological and related reagents, which limits widespread use.

Although the ferret is a small animal model (a three-month old male weighs <1 kg), the species has a long trachea which helps to separate the upper and lower respiratory tracts, a feature similar to humans (Maher and DeStefano 2004). Importantly, influenza virus susceptibility and disease patterns seen in humans are generally recapitulated in ferrets. Influenza virus attaches via the *N*-acetylneuraminic acid (sialic acid; SA) linked to galactose sugars on surface glycoproteins. It is believed that influenza viruses that infect humans preferentially bind to sialic acids with an α2,6 linkage (SAα2,6Gal), while influenza viruses that infect avian species preferentially bind to sialic acids with an α2,3 linkage (SAα2,3Gal) (Palese and Shaw 2006). SAα2,6Gal receptors are found at a high density in the human respiratory tract (Baum and Paulson 1990; Matrosovich et al. 2004). The lower respiratory tract contains predominantly SAα2,6Gal, but there are also SAα2,3Gal linkages on bronchiolar cells and type II alveolar cells (Shinya et al. 2006). The ferret has a similar density and repertoire of sialic acid receptors (Leigh et al. 1989), and therefore has a similar influenza virus susceptibility (Leigh et al. 1995; Maines et al. 2006; Matrosovich et al. 2004; Piazza et al. 1991; Tumpey et al. 2007; van Riel et al. 2007).

The sialic acid expression and virus susceptibility profiles of ferrets and humans combined with their similar physical airway features translate to similar abilities to transmit influenza viruses. Ferrets are highly susceptible to human influenza virus infection and readily transmit the virus to naïve ferrets (Herlocher et al. 2001; Maher and DeStefano 2004; Maines et al. 2006; Tumpey et al. 2007) and humans (Francis 1934; Smith and Stuart-Harris 1936). For this reason, ferrets are an excellent model to study influenza virus transmission and disease intervention strategies; however, they are also a difficult model to work with. Influenza-naïve ferrets can be difficult to acquire, particularly during the influenza season, and naïve ferrets can readily become infected through environmental exposure if appropriate barrier conditions are not maintained during shipping and housing. Importantly, unlike some animal models of influenza infection, seropositive ferrets are generally susceptible to reinfection with variant viruses (Herlocher et al. 2001), although there is evidence of limited heterosubtypic immunity as well (Yetter et al. 1980).

3.2 The Immune Response in Ferrets

The immune response to influenza virus infection in ferrets is a double-edged sword—both a strength and a weakness—in the animal model. The ferret serum antibody response to influenza virus infection or vaccination is very similar to the response seen in humans; however, there are relatively few tools available for investigating parameters of the innate or cell-mediated immune response compared to the mouse model. The first isolation of human influenza virus in 1933 demonstrated

that ferret immune serum would neutralize human influenza virus and that human immune serum would neutralize the virus during infection in ferrets (Smith et al. 1933). Years of influenza virus studies in the ferret model now predict that experimentally infected or vaccinated ferrets produce neutralizing or hemagglutination-inhibiting (HI) serum antibody responses with the same virus reactivity as would be generated in human antibody responses. For this reason, the cross-reactivity of ferret antisera to circulating human influenza virus strains is regularly used to identify strains to be included in annual formulations of the influenza virus vaccine (Jan and de Jong 2000). It is important to note that neutralizing serum antibody titers in ferrets do not correlate with prevention of upper respiratory tract infection; however, they do correlate with decreased severity of disease and prevention of lower respiratory tract infection and pneumonia. Mucosal antibody responses have also been shown to contribute to protection. The cellular immune response in ferrets has also been characterized, and similar cytotoxic T cell (CTL) responses have been noted to those of humans, indicating that CTLs play a major role in recovery from infection (Maher and DeStefano 2004). While extremely detailed studies of the immune response to influenza virus infection have been carried out in mice, these thorough studies have not been done in ferrets. This is due to a lack of immunologic reagents, including antibodies to cellular markers, cytokine reagents, and genomic tools. The absence of these tools, which are commonplace for murine studies, has limited the breadth of the ferret model. With the recent renewal of interest in influenza research and vaccine development, many of these reagents are now becoming available and will eventually eliminate this shortcoming in the ferret model.

Another related issue with the ferret model is the lack of inbred animals. Responses in ferrets are not uniform, which is both a strength and a weakness. Results may be more difficult to assess, due to variability; however, the conclusions may be more relevant to human studies for the very same reason. Several breeders are developing inbred and specific pathogen-free ferrets, which will overcome these potential hurdles, as previously noted.

Despite these issues, ferrets are currently the "gold standard" for influenza virus animal models. With concerns that H5N1 viruses might cause a pandemic, there has been a resurgence of interest in developing novel influenza vaccines, focused on H5 and a variety of platforms, including live attenuated, DNA, particle-based, inactivated, and adjuvanted vaccines. Each of these has been used in immunogenicity and challenge studies in ferrets (Subbarao and Luke 2007). These studies have presented a number of promising candidates, some of which are in clinical studies, and one of which is now licensed for use in the United States (FDA 2007). Moreover, studies comparing immunogenicity and protection in ferrets have uncovered an important issue concerning the classical correlates of protection and the actual level of protection from challenge with an H5N1 virus. Using the ferret model, it has been demonstrated that an inactivated whole-virion H5N1 vaccine could protect animals against infection with highly pathogenic H5N1 avian influenza despite inducing poor hemagglutination inhibition and virus neutralizing serum antibody titers (Lipatov et al. 2006). The disassociation of serum antibody responses from protection from challenge highlights the critical need for vaccine testing in animal models of disease.

3.3 The Murine Model

The first North American influenza isolate identified in 1934 was quickly moved from ferrets into mice and shown to cause disease in this model (Francis 1934). At the same time, researchers in Europe were demonstrating that mice were susceptible to both swine and human influenza viruses, and they showed that immune serum from immunized ferrets or horses could neutralize the infectivity of influenza virus prior to infection in mice (Andrewes et al. 1934). Since these seminal studies, mice have been widely used in all aspects of influenza virus research. The mouse model has several advantages over ferrets in that there are numerous inbred mouse strains that are commercially available, including mutant, congenic, transgenic, gene knockout, and combination mutant transgenic species. Also, the size and husbandry practices for mouse colonies make them affordable, mice have been extensively characterized, and there is an extensive array of reagents available for the study of immune responses (Novak et al. 1993). Together, these strengths allow researchers to execute in-depth studies using relatively large numbers of experimental subjects. The utility of the mouse model of influenza virus infection is reflected in the extraordinary immunologic discoveries made using this system. The study of influenza virus infection in mice has resulted in our fundamental understanding of MHC restriction, the innate immune response, immunodominance, humoral immunity, and immunologic memory.

The mouse model of influenza virus infection has notable weaknesses. First, most influenza viruses do not naturally cause disease in mice. There is no experimental evidence that human influenza viruses can be directly transmitted from humans to mice. The first successful influenza infections in mice occurred after only three passages in ferrets (Andrewes et al. 1934; Francis 1934). In later studies, human influenza A viruses were cultivated in embryonated chicken eggs prior to infection in mouse models. In these cases, the viruses replicated well but caused asymptomatic infections with little or no pathology, even when given at very high titers (Hirst 1947b; Novak et al. 1993). Murine infection with nonadapted influenza viruses has revealed that infection in mice is variable, but once established, replicating virus can be isolated from the lung, trachea, and nares for at least 5–6 days (Novak et al. 1993).

Repeated passage of human influenza viruses in mouse lungs can quickly adapt the virus to the mouse and result in virulent mouse-adapted viruses (Hirst 1947b; Novak et al. 1993; Smeenk and Brown 1994). Mouse-adapted viruses can cause severe pathology, morbidity and mortality, and lethal pneumonia caused by mouse-adapted influenza virus infection is similar to the pathology seen in human lower respiratory tract infections (Smeenk and Brown 1994). In some cases, limiting the inoculum and sedation of the mouse can limit the infection to the upper respiratory tract, resulting in apathogenic infection (Iida and Bang 1963; Novak et al. 1993). Whether infecting with wild-type or mouse-adapted influenza viruses, infected mice do not shed virus (Lowen et al. 2006). As mice can only be infected experimentally, the mouse model is not useful for transmission studies.

3.4 Vaccine Development in the Mouse Model

A substantial issue with using the mouse model for vaccine development is the relative ease in which vaccinated mice can be protected against challenge, as previously reviewed in studies of heterosubtypic immunity (Epstein 2003). In these studies, immune responses generated against conserved viral vaccine antigens, such as nucleoprotein (NP) or matrix (M1), were generally cell mediated (i.e., CTL specific for the NP or M1 proteins). However, related studies in humans have provided limited evidence that similar mechanisms of protection are efficacious (Epstein 2006; Steinhoff et al. 1993). While vaccine studies in murine models provide a wealth of information and an initial assessment of potential efficacy, there is concern that the findings will translate poorly to the clinic. Moreover, the rising concern regarding preventing transmission as a priority in vaccine development decreases the value of murine studies, as the mouse does not transmit influenza virus during infection.

3.5 Other Rodent Models

The guinea pig is a relatively new model for the study of influenza virus. Their use has been limited by the availability of the murine model; however, more recently the guinea pig has received attention as a potential model for influenza virus transmission (Lowen et al. 2006). Based upon an account of pneumonia in a laboratory guinea pig colony during the 1918 influenza epidemic, the susceptibility of the Hartley strain of guinea pigs to human influenza virus infection and their ability to transmit the virus to naïve animals was explored (Lowen et al. 2006). Wild-type, unadapted influenza virus was shown to replicate in both the upper and lower respiratory tracts of the Hartley strain guinea pigs, and to transmit to naïve animals via droplet. While high titers of virus were found in both the lungs and nasal secretions, the infection was completely asymptomatic. Interestingly, wild-type, unadapted influenza virus infection of strain 13 guinea pigs with the same virus resulted in clinical disease, although transmissibility was not addressed (Lowen et al. 2006). Similar to the ferret model, there are limited reagents available for guinea pigs. This, combined with the apparent absence of disease, reduces their value in vaccine studies; however, their size and the availability of specific pathogen-free inbred strains may make this model more appealing for prospective influenza virus transmission studies.

The cotton rat was first described as a model for influenza virus infection in 1987 (Eichelberger 2007). The cotton rat has a similar disease course to humans; however, there is no evidence of transmission. Influenza virus can be isolated from both the upper and lower respiratory tracts following intranasal infection (Ottolini et al. 2005). The cotton rat shows clinical signs of disease that include weight loss, and has pulmonary cellular infiltrates similar to humans with bronchopneumonia. A key strength of the cotton rat model is the ability to infect it with wild-type,

unadapted influenza viruses (Eichelberger 2007). Moreover, while not as expansive as the mouse model, a variety of reagents are available for characterizing the immune response. These features make the cotton rat an appealing model for vaccine and immune response studies to influenza virus infection.

Syrian hamsters have also been used as a disease model for influenza virus infection. Like the cotton rat, the hamster is susceptible to infection with unadapted human influenza viruses. In contrast, the hamster supports higher titers of virus in the lung than in the upper respiratory tract (Heath et al. 1983). Other than these defining features, the Syrian hamster has limited application as an animal model for influenza virus. The other rodent species have equivalent or better features of disease and/or a broader utility because of the availability of reagents.

3.6 Nonhuman Primate Models

Serological studies have found that many native nonhuman primate species are seropositive for human influenza viruses (Clyde 1980), suggesting that they may be a natural host for infection and a potent model to study influenza virus. As such, a variety of nonhuman primate (NHP) species have been tested for their ability to support influenza virus infection and the disease associated with infection. Rhesus macaques are susceptible to human influenza virus infection. Interestingly, intranasal instillation of influenza virus has not been successful at establishing infection, but aerosol or intratracheal delivery causes infection, clinical symptoms (in some cases), and seroconversion (Berendt 1974). Seroconversion resulted in protection against repeated challenge. Variability in clinical symptoms was suggested to be related to strain virulence.

Squirrel monkeys have also been successfully used as models for influenza virus infection. A prominent example is provided by the studies done in the late 1970s in which squirrel monkeys were inoculated intratracheally with A/New Jersey/76, a swine virus isolated at Fort Dix that threatened to become pandemic. At the time no was information available on the transmissibility or pathogenicity of A/New Jersey/76. A decision was made to develop new vaccines, and NHP disease models were needed to test the immunogenicity of these proposed vaccines. Squirrel monkeys infected with A/New Jersey/76 were shown to shed virus and to develop clinical disease (Berendt and Hall 1977). Similar results were also shown in squirrel monkeys infected with A/Aichi/2/68 virus; symptoms and virus shedding were shown to be similar to what was seen in human infections (Murphy et al. 1980, 1982a,b, 1983). The similarities in disease between humans and squirrel monkeys have elevated the squirrel monkey model as a reasonable disease model to measure influenza virus virulence.

NHP models of influenza are generally less utilized than other models because of the lack of availability of animals, difficulty in handling, and the need for special facilities and veterinary care. However, there are advantages to NHP studies, including human reagent cross-reactivity, which can be used in Old World primates such as rhesus and cynomolgus macaques. Also, the size and similar physiology of

many NHPs enable repeated sampling and monitoring of symptoms related to humans; the genetic relatedness to humans and outbred populations may enable more meaningful vaccine efficacy studies. These advantages, combined with the similarities to the disease observed in humans, make the NHP model of influenza virus infection a very powerful research tool.

3.7 Overview of Animal Models

In the 75 years since the first isolation of a human influenza virus, both ferrets and mice have continued to play a central role in our understanding of the host response to influenza virus infection, in developing correlates of protection against infection, and the development of vaccines and therapeutic drugs. Efforts towards the development of improved or even "universal" vaccines and (in the wake of drug resistance) new antiviral drugs continue. Mice and ferrets have an important role in these studies; however, there are other animal model options that can perhaps be used to better address the immunobiology of virus infection and the development of disease intervention strategies. These include other rodents (guinea pig, hedgehog, hamster, and cotton rat), birds, swine, nonhuman primates (rhesus macaque, cynomolgus macaques, squirrel monkeys, and others), and even humans. Even the observation by Frank MacFarlane Burnet that embryonated chicken eggs could support the growth of relatively pure, high-titer influenza virus stocks (Burnet 1940a,b), a critical step in influenza vaccine development (Eyler 2006), is arguably the development of a animal model. As studies continue and animal models develop, it is likely that the findings will lead to a better understanding of human influenza vaccine development, safety, and efficacy.

4 Human Vaccines: The End Game

The development of the first licensed killed influenza vaccine, led by the Commission on Influenza, relied on the cultivation and purification of the virus grown in the allantoic sac of embryonated hen's egg (Burnet 1941). This vaccine was prepared by purifying and concentrating the virus, then by absorption to and elution from red blood cells, and finally inactivation using formaldehyde (Hirst 1942). Subsequently, this crude but efficacious vaccine preparation was replaced by centrifuge-purified vaccine, which is still the basic format for much of today's influenza vaccine production (Stanley 1945). Killed influenza vaccines produced in eggs have proven to be safe, efficacious, and well tolerated, but caveats remain, such as the potential presence of residual egg proteins, the possibility that avian leukosis virus may be present in embryonated eggs used for vaccine production, and compromised production potential when highly pathogenic avian influenza virus is circulating, to name a few. To reduce some of the issues associated with killed vaccines, today's version consists of subvirion and purified surface antigen

preparations made as a trivalent inactivated vaccine (TIV). Today's TIV contains one influenza A (H3N2) virus, one influenza A (H1N1) virus, and one influenza B virus, which may change from year to year based on global influenza surveillance and the emergence of new strains.

Subunit influenza vaccines are now used widely throughout the world and are the only inactivated vaccines used in the United States. These vaccines, given as a single dose, are adequate for boosting immunologic memory, but subunit vaccines such as split vaccines are often poorly immunogenic in persons who have not been primed through previous infection or vaccination (Hilleman 1977; Parkman et al. 1977; Wareing and Tannock 2002). The focusing of recent attention on the development of a universal subunit vaccine (i.e., a conserved M2 protein vaccine) is meant to prevent loss of vaccine effectiveness through antigenic drift and shift, because the M2 protein is highly antigenically conserved and it has been shown in mice that antibody directed against it prevents infection (Fan et al. 2004; Fiers et al. 2004; Neirynck et al. 1999; Slepushkin et al. 1995; Tompkins et al. 2007). Recombinant DNA plasmid vaccines, first demonstrated to vaccinate mice for humoral and cellular immunity to HA and NP, were shown to protect against lethal challenge with virulent PR8 virus (Donnelly et al. 1994; Montgomery et al. 1993; Ulmer et al. 1994). DNA vaccine approaches are still experimental. They are readily manipulated and manufactured, and vaccination results in antigens being expressed in the cell cytosol, where they are readily loaded by both class I and II histocompatibility antigens (Dean 2005; Laddy and Weiner 2006; Webster and Robinson 1997).

Live attenuated influenza virus (LAIV) vaccines have been used for many years in Russia with success (Aleksandrova et al. 1986; Desheva Iu et al. 2002; Kendal 1997a,b; Klimov et al. 1995; Rudenko et al. 1993; Zhilova et al. 1986). Intensive research in the United States led to the development of a cold-adapted and attenuated reassortant influenza vaccine (CAIV) into which any desired HA or NA can be inserted (Block 2004; Maassab et al. 1999). LAIV vaccines use a genetic reassortment method involving a combination of six genes from a master donor strain that code for internal viral proteins and two genes from contemporary wild virus strains that code for the desired HA and NA antigens (Ambrose et al. 2006; Belshe et al. 2004; Targonski and Poland 2004). The resulting vaccine viruses are attenuated, temperature sensitive, genetically stable and nontransmissible. They offer substantial advantages over TIV or subunit vaccines as they can are administered intranasally without the use of needles, induce a broad mucosal and cellular mediated immune response, and LAIV has demonstrated broader serum antibody responses than TIV, particularly against mismatched influenza A (Ambrose et al. 2006; Glezen 2006; Lynch and Walsh 2007; Nichol 2001; Piedra et al. 2005).

Although a variety of safe and effective human vaccines and vaccine platforms are now available, there is little doubt that vaccine strategies will evolve and that appropriate animal models will play an important role in these developments. Of the plethora of animal models to choose from, reagents, rationale, cost-effectiveness, and animal welfare issues will in part dictate the models chosen. Issues remain regarding the translation of findings from one animal model to another, and from animal models to humans, but much has been learned and many of the caveats

recognized. Animal models will remain an integral part of human influenza vaccine development, safety, and efficacy studies, and can help to bridge the gaps in our understanding of the immunobiology of influenza virus infection.

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