

Influenza vaccines

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TABLE OF CONTENTS

1. Abstract
2. Introduction
 - 2.1. Mechanisms of influenza virus evolution
 - 2.2. Resurgence of influenza vaccinology
3. Current influenza vaccines
 - 3.1. Inactivated vaccines
 - 3.2. Live attenuated vaccines
 - 3.3. Limitations of current influenza vaccine strategies
4. Reverse genetics
 - 4.1. Creation of high-growth reassortant vaccine strains
 - 4.2. Other uses of reverse genetics in influenza vaccines
 - 4.3. Barriers to using reverse genetics in vaccine production
5. Cell culture vaccines
6. Antigen-sparing approaches
 - 6.1. Foreign expression of hemagglutinin
 - 6.2. DNA vaccination
7. Novel influenza vaccine targets
8. Perspectives
9. Acknowledgment
10. References

1. ABSTRACT

Influenza viruses pose a major challenge to those concerned with global public health. Not only do influenza viruses cause yearly epidemics that are associated with slight changes in viral antigenicity, but occasionally new viruses cross from animal reservoirs into humans causing major pandemics. The most effective method to lower the mortality and morbidity associated with influenza is vaccination. In this review current and pending influenza vaccine technologies will be discussed in the context of both epidemic and pandemic influenza.

2. INTRODUCTION

Influenza in humans is caused by viruses from one of three genera within the family Orthomyxoviridae: influenza A virus, influenza B virus, and influenza C virus (1). Each flu season, influenza A and B viruses combine to cause substantial human morbidity and mortality, and seasonal influenza vaccines target both strains. However, type A viruses are the ones associated with pandemic episodes and thus are most actively targeted for development of newer generation vaccines.

Influenza A viruses pose a rather challenging quandary as a target for vaccination, because the best form of immunity against these viruses is neutralizing antibodies that target the most variable viral protein, hemagglutinin (HA). Although antibodies against other viral proteins such as neuraminidase (NA) and matrix protein 2 (M2) reduce disease severity, those directed against HA are the only ones that effectively neutralize the viral infection. The difficulty lies not only in the fact that 16 subtypes of HA have been serologically defined in influenza A (2), but also in the fact that influenza viruses have evolved two mechanisms for creating antigenic diversity, antigenic drift and antigenic shift.

2.1. Mechanisms of Influenza Virus Evolution

Antigenic drift is the result of the infidelity of the virally encoded, RNA-dependent RNA polymerase. As with other polymerase enzymes using RNA as template, the lack of proofreading activity introduces a number of errors during each replication cycle. When combined with the selective pressure applied by a host population that is continually infected or vaccinated, these errors result in the rapid emergence of antigenically novel forms of the virus. Any vaccination regime based on neutralizing antibodies must therefore contend with this “moving target.” In response to the antigenic drift of influenza strains, the World Health Organization (WHO) has developed a worldwide network of more than 100 laboratories to monitor the evolution of the virus and to ensure that the most appropriate strains are included in the yearly vaccine formulations. The practical implication of influenza virus antigenic drift is that one of the vaccine components (H1N1, H3N2, or B) needs to be updated at least every other year.

Potentially of greater concern than antigenic drift is the second mechanism of influenza A virus evolution, antigenic shift, which occurs through the process of genetic reassortment. The segmented nature of the influenza virus genome allows for the mixing of gene segments in dually infected cells. This process has been responsible for the genesis of at least the last two human influenza pandemics in which circulating human viruses acquired gene segments (including HA) from animal viruses (3,4). Although humans and other mammals host limited lineages of influenza A viruses, wild aquatic bird populations serve as their major reservoir, and it is generally accepted that all influenza A viruses have ancestral links to that population (5). Although only three subtypes of influenza A virus have formed stable lineages in humans (H1, H2, and H3), viruses representing all 16 HA subtypes are harbored in aquatic birds (6). Substantial genetic and antigenic diversity exists within these aquatic bird reservoirs, and viruses of different subtypes rarely cross-react serologically (5).

2.2. Resurgence of Influenza Vaccinology

The genetic and antigenic diversity among influenza viruses presents a major challenge to preparing vaccines against them. In light of the facts that we cannot predict with any accuracy which influenza viruses pose the

greatest threat to humans and our current vaccine is unable to induce cross-protecting immunity, there is substantial room for improvement of influenza vaccination.

Although the deficiencies in influenza vaccine technologies have been long recognized, only recently has the field been substantially rejuvenated in response to contemporary outbreaks of highly pathogenic H5N1 influenza viruses in global avian populations, with sporadic spillover into the human population (7). This rejuvenation has been led by the serious public health threat posed and probably more importantly by the subsequent increase in funding. Highly pathogenic strains of influenza not only have the inherent property of variability, but also are highly virulent, which adds difficulties to preparing vaccines against these strains. With enhanced virulence comes the added challenge of enhanced biosafety-handling requirements. These requirements limit the number of academic, pharmaceutical, and government laboratories that can handle these viruses. As a result, our ability to produce vaccines against these strains is limited. In addition to the danger the highly pathogenic strains pose to poultry and humans, many kill embryonated chicken eggs, which are the backbone of current influenza vaccine strategies.

Highly pathogenic avian influenza (HPAI) viruses are not naturally present in the aquatic bird populations of the world. Instead, they evolve from less pathogenic strains that cross the species barrier into domestic poultry. After replication in these hosts and for reasons not well understood, a percentage of H5 and H7 strains accumulates additional amino acids at the HA-cleavage site (8-10). Proteolytic site-specific HA cleavage is required for all influenza virus infections, because it releases the peptide that is necessary for the fusion of viral and host membranes (*for review see* 11). The cleavage of most influenza viruses is mediated by trypsin-like proteases, which are primarily produced by Clara cells lining the respiratory tract. As a result, the growth of those viruses is limited to the respiratory tract. In contrast, the altered cleavage site of the highly pathogenic strains responds to the action of ubiquitous furin-like proteases, and these viruses can replicate outside the respiratory tract (12). Although it is unlikely that their response to the activation of the ubiquitous furin-like proteases is the sole reason for the extreme virulence of the highly pathogenic strains, removal of the additional amino acids at the cleavage site results in virus attenuation. This phenomenon has been exploited for vaccine production, as detailed below. Another aspect of H5N1 vaccines that has spurred research into adjuvants and other antigen-sparing technologies is their apparent poor immunogenicity in humans (13).

This review will focus primarily on vaccines targeting influenza A viruses, though in some cases, the approaches are also applicable to influenza B. This review is far from exhaustive, but it is designed to highlight some of the recent areas of progress in influenza vaccinology.

3. CURRENT INFLUENZA VACCINES

3.1. Inactivated Vaccines

Although live, attenuated vaccines are available in some countries (14), the vast majority of the world's supply of influenza vaccine is based on inactivated, egg-grown, intramuscularly administered vaccine, the principles of which were developed during the 1940s. In 2000, approximately 233 million doses of trivalent (H1N1, H3N2, and B strains), inactivated vaccine were distributed worldwide (15). The first trials of inactivated influenza vaccines were in the United States military during World War II, and the first commercial vaccine was available in the United States in 1945 (*see 16 for review*). During the next 30 years, relatively major changes were made to the process such as the introduction of split-virion vaccines to reduce adverse effects, or reactogenicity, (17) and high egg-growth reassortant influenza A strains to improve yields (18). The development of these high-growth strains took advantage of the capacity of the influenza genome to undergo reassortment and the finding that incorporation of HA and NA genes into the egg-adapted A/Puerto Rico/8/34 strain often led to a virus with improved growth properties in eggs. In addition to increased growth, the reassortant strains are attenuated in humans, which provides an extra level of safety in the manufacturing process (19,20). The use of reassortant strains, however, does have its problems, including the randomness of the reassortment process and the subsequent need to screen progeny viruses for the desired genotype, the need for selection methods, and the requirement that all strains are capable of egg growth. Although theoretically all but the HA and NA gene segments of the vaccine virus would be from the A/Puerto Rico/8/34 strain, in practice this is seldom the case. One has to keep in mind that the contemporary viral strains represented in the vaccine change in response to the evolution of the virus in the human population, and the production of reassortant strains is a continuing effort. Nevertheless, the increased yield offsets these limitations.

Vaccine is produced from high-growth strains by amplification in the allantoic cavity of embryonated chicken eggs. Typically, detergent is used to disrupt lipid envelopes of harvested virus. The virus is concentrated and purified via rate zonal centrifugation and inactivated with β -propiolactone or formaldehyde. Detergent is used to disrupt lipid envelopes of harvested virus. Vaccine proteins are concentrated and purified from viral constituents via rate zonal centrifugation. The vaccine is then formulated to 15- μ g HA protein per virus strain per dose. Note that high-growth reassortants are not produced against influenza B viruses. As described above, the primary mechanism of inactivated influenza vaccine is the production of antibodies targeting the HA. HA is the protein that attaches the virus to the host cell; thus, antibodies that bind HA can neutralize the virus and inhibit the infectious process. A review of efficacy data from various sources showed that in adolescents and adults (14-60 years of age), the inactivated vaccine reduced serologically confirmed influenza illness by 70% compared to controls (21). Similar analyses demonstrated a 65% efficacy of the vaccine in children older than 2 years (22) and a reduced efficacy in the elderly (23).

3.2. Live Attenuated Vaccines

In contrast to inactivated vaccines that stimulate little, if any, cellular and mucosal immunity, vaccines based on attenuated live viruses are thought to stimulate humoral and cellular immune responses (24). Another perceived benefit of the live attenuated influenza vaccines (LAIVs) is that they are administered via the intranasal route, and they do not require a needle. LAIVs are available in Russia and the United States. The basis for production of seasonal live attenuated vaccines is also the generation of reassortant strains. In these reassortant strains, the HA and NA genes of the target viruses are incorporated into the backbone of master strains attenuated by cold adaptation. Cold adaptation introduces temperature sensitivity, which limits master strain replication to the upper respiratory tract. In Russia, the cold-adapted strains are based on A/Leningrad/134/57 (H2N2) and B/USSR/60/69 (24,25); in the United States, they are based on A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 (24,26).

LAIVs are thought to induce a cellular immune response targeted toward more conserved viral proteins (e.g., nucleoprotein). Therefore, LAIVs may have an advantage over inactivated vaccine when a mismatch between the vaccine and the circulating strain occurs. This certainly appeared to be the case during the 1997-1998 influenza season, when the A/Wuhan/359/95 H3N2 vaccine component was antigenically mismatched with the circulating A/Sydney/5/97-like H3N2 viruses. During that season, the LAIV was 86% effective, despite the mismatch (27,28), and the effectiveness of the inactivated vaccine was questioned (29).

Two studies have directly compared the efficacy of LAIVs and inactivated influenza vaccines. The first by Edwards and colleagues found no differences in efficacy in adults over a 5-year period (29). In contrast and somewhat unexpectedly, a head-to-head comparison of LAIVs and inactivated vaccines in adults during the 2004-2005 season showed that the inactivated vaccine was more efficacious, when laboratory-confirmed influenza was used as a measure of vaccine efficacy (30). The 2004-2005 influenza season was also considered a mismatch season; the difference in efficacies between the two vaccines was related mainly to divergent outcomes of influenza B infection (30). Although a definitive answer will require further head-to-head trials, it appears that in adults there is little difference between the efficacies of LAIV and that of inactivated influenza vaccines against seasonal influenza. The added benefit of LAIVs might, however, manifest in young children or against pandemic influenza strains in which preexisting immunity to the vaccine strains is limited.

3.3. Limitations of Current Influenza Vaccine Strategies

The two biggest drawbacks of the current influenza vaccine strategies are their targeting of variable proteins and the time needed to produce updated vaccines. The second drawback is actually a result of the first one. Using current technologies, the production time for a seasonal influenza vaccine is approximately 6 to 7 months (31,32). This period includes the distribution and testing of

WHO-supplied seed strains and the manufacture and release of vaccine. In addition to time limitations, the current system is also limited by the reliance on eggs for vaccine virus propagation. This system is inflexible, and in some cases, viruses will not grow in this substrate. The latter problem is highlighted by a couple of recent events, i.e., the emergence of the A/Fujian/411/02-like viruses in the 2003-2004 season and the desire to produce pre-pandemic vaccines against HPAI strains.

The A/Fujian/411/02-like viruses did not replicate in embryonated chicken eggs; thus, it was very difficult to produce a matched vaccine (33-36). The A/Fujian/411/02 episode was not the first of its kind, but it does demonstrate the fragility of a system built on replication of virus on an egg substrate. The A/Fujian/411/02 virus was predicted to be the dominant strain in the 2003-2004 season, but due to the lack of an egg-grown isolate (viruses isolated on nonvalidated cell lines could not be used for production of high-growth reassortants), a matching vaccine strain could not be produced in time for the seasonal vaccine production cycle. Producing egg-grown vaccines against HPAI viruses can also be very difficult, because the viruses can cause the premature death of the developing embryo. Although this issue is very much secondary to the biosafety issues associated with bulk manufacture of HPAI viruses, it emphasizes another deficiency in the current technology.

A number of alternative strategies have been proposed for influenza vaccine design and manufacture. The present development of these strategies ranges from those in advanced clinical trial (e.g., cell culture derived vaccine) to those still in the discovery phase (e.g., virus-like particles). Perhaps the approaches that offer the most short-term hope are those aimed at improving the production of the current vaccines, particularly the incorporation of reverse genetics and cell culture systems.

4. REVERSE GENETICS

The first reverse genetics systems designed to generate replicating influenza virus were developed in the late 1980s by Palese and colleagues in New York (37). Although successful, the systems relied on purification of viral ribonucleoproteins (RNPs) and a helper virus, which meant that the desired virus had to be purified by selection. These problems were overcome in 1999, when researchers developed the first plasmid-based reverse genetics systems designed to generate influenza A virus (38). Soon thereafter, similar systems were developed that allowed the reconstitution of fully viable virus from cDNA of the viral RNA (39,40). For the first time, the genome of influenza A viruses could be manipulated with ease using the most basic of molecular biology tools.

4.1. Creation of High-Growth Reassortant Vaccine Strains

One of the first demonstrations of the power of reverse genetics was in proof-of-concept experiments in the rapid generation of high-growth reassortant vaccine strains for human (41,42) and avian (41,43) viruses. The strains

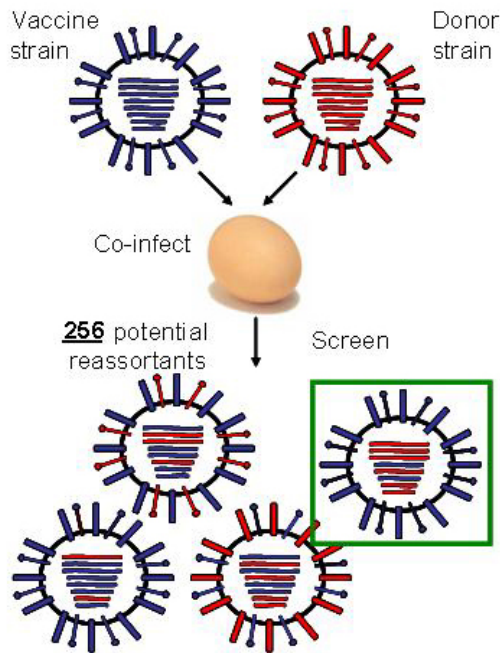
were created by transfecting cultured cells with plasmids encoding the PB2, PB1, PA, NP, M, and NS gene segments from A/Puerto Rico/8/34 and the HA and NA gene segments from the target virus (Figure 1). This method produced high-growth reassortant strains without requiring selection mechanisms and screening of progeny for the desired gene configuration; simply, what you put in is what you get out. Although these technologies are better than conventional reassortment techniques in terms of producing reference viruses for seasonal influenza vaccines, perhaps their biggest contribution is producing such viruses from HPAI isolates.

On the basis of RNP-based reverse genetics studies completed in response to the H5N1 outbreak in Hong Kong in 1997 (44,45), a number of investigators conducted proof-of-concept studies showing that A/Puerto Rico/8/34-based H5 reassortants could be easily produced using these systems (41,46,47). The basic principle underlying the methodology was as described above for non-HPAI strains, with the added step of removing from the HA cleavage site the multiple basic amino acids associated with high virulence. Although these proof-of-principle studies showed that plasmid-based reverse-genetics technologies could be exploited to produce vaccine-reference virus, it was not until 2003 that these technologies were put to the test.

In February 2003, public health officials confirmed the first cases of human infection with H5N1 HPAI virus (48) since 1997 (49,50). This news sparked fear of an impending pandemic. During the earlier 1997 H5N1 outbreak, several options for vaccine development were explored, including the attenuation of the virus by RNP reverse-genetics systems, the development of a vaccine by using an antigenically matched nonpathogenic strain, and the expression of the H5 protein by using baculovirus vectors (*for review see* 13). In 2003, the emphasis was placed on producing a matched high-growth reassortant reference strain by using plasmid-based reverse genetics techniques because a nonpathogenic, antigenically matched strain was not available, and the immunogenicity of the 1997 baculovirus-derived H5 protein was disappointing (51). The attempts at reverse genetics were very successful, and within weeks of the H5N1 virus being isolated in humans, a candidate reference virus was available for testing (35,52). Although whole vaccines derived from these 2003 reference strains have proven to be efficacious against homologous and heterologous H5N1 lethal challenge in mouse and ferret models (53,54), the demise of the 2003 threat and the emergence of an antigenically distinct virus in 2004 halted the planned production of clinical-grade vaccine.

While it was becoming obvious that the 2003 human infections of H5N1 were isolated cases, a more ominous scenario was unfolding throughout Southeast Asia. Viruses that were genetically similar but antigenically distinct to the 2003 H5N1 viruses were spreading in avian species throughout the region (55). Their spread culminated in the emergence of new human H5N1 infections in early 2004. Again, global health authorities were put on high

A. Traditional egg-based strain reassortment



B. Reverse genetics based virus reassortment

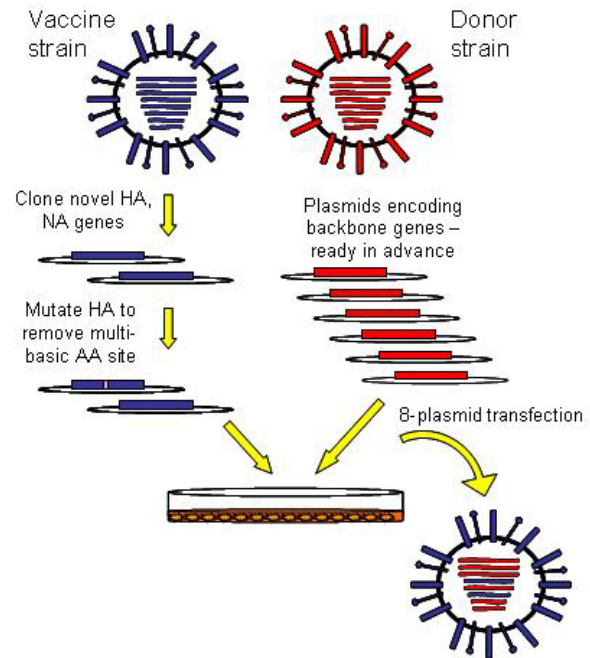


Figure 1. Traditional and reverse genetics-based reassortment of influenza strains. A) High-growth, attenuated vaccine seed viruses bearing HA and NA of seasonal influenza strains have been derived to-date by co-infection of embryonated eggs. Random reassortment of the eight gene segments potentially yields up to 256 permutations, and thus is followed by screening for proper surface proteins, as well as high growth in eggs. B) Plasmid-based reassortment of viral genes enables precision in the mixing of gene segments and versatility in the development of vaccines against highly pathogenic strains. HA and NA gene segments amplified from cDNA are cloned into plasmids. These, along with six internal gene segments cloned from the donor vaccine strain (possessing attenuated and high-growth properties), are used to co-transfect cultured cells. In the case of a highly pathogenic parent strain, nucleotides encoding the multiple basic amino acid site can be deleted from the HA plasmid via routine mutagenesis methods. Screening for desired reassortants is unnecessary, substantially reducing the biosafety requirements for deriving a reassortant vaccine.

alert, and matching vaccine strains were sought. Building on experiences and mistakes from the previous year, candidate live attenuated vaccine viruses were again produced. Plasmid-based systems accelerated the initial phase of vaccine production (i.e., reference virus creation). Unfortunately, the 2004 outbreak was not isolated, and the virus has continued to spread and cause further human infection, forcing national and international agencies to contemplate preparation of trial batches of vaccine to be tested and stockpiled.

By mid-2005, the first doses of split-virion, inactivated vaccine produced from a reverse genetics-derived strain had entered a federally funded clinical trial in the United States. Although the results of these trials were not unforeseen, they were also not encouraging. Two doses of 90 µg (six times the standard dose) were needed to elicit a detectable response in 58% of participants (56). Similarly disappointing results were reported from another trial of a split-virion vaccine formulated with aluminium hydroxide

(57). In that study, 67% of individuals seroconverted, but only after two doses of 30 µg each. Taken at face value, neither of these studies is particularly reassuring. In the event of a global pandemic, the vaccine-manufacturing resources would be under considerable strain, and the requirement for two doses of 30-µg vaccine would add even more pressure. More encouraging are studies using an aluminium hydroxide-adjuvanted whole-virus formulation. In those studies, 78% of participants seroconverted after two doses of 10 µg (58). This latter result suggests that, in the event of a pandemic, a tradeoff may need to be made between the added reactogenicity of a whole-virus formulation and its superior immunogenicity. Of course, one should be careful not to overextrapolate based on three published studies. It is also worth considering the results of preclinical studies of H5N1 vaccines in which protection from lethal disease can be observed in the absence of “protective” levels of serum antibodies (53,54,59,60). These results suggest that protection from lethal infection, which in the case of a pandemic episode might be a more

than worthy goal, can be achieved at antibody levels below what is generally considered protective. Unfortunately, this hypothesis can be tested only in humans in the event that H5N1 becomes a *bona fide* human pathogen; thus, we hope that this hypothesis will never be tested.

4.2. Other Uses of Reverse Genetics in Influenza Vaccines

The power of reverse genetics for influenza vaccine development goes well beyond the creation of high-growth or LAIV-reference strains. These systems can manipulate the viral genome, an ability that has led to a number of promising developments, many in the area of introducing attenuating mutations. For example, NS1 is a multifunction protein that antagonizes the host type I interferon response (61,62). Correspondingly, truncated NS1 can severely attenuate the growth of the virus in interferon-competent systems (Quinlivan, Zamarin *et al.*, 2005; Richt, Lekcharoensuk *et al.*, 2006; Talon, Salvatore *et al.*, 2000). The challenge for this approach will be fine-tuning the attenuation so that enough viral replication occurs in a primed individual to generate an immune response without causing disease. Another challenge for such an approach is the possibility of reversion of the vaccine strain to virulence after a reassortment event with wild-type strains. This concern has been raised often in debates on the safety of cold-adapted LAIVs, but the available data suggest that these events are unlikely and that transmission of these vaccine strains in close-contact pediatric populations is minimal (63).

In another example of harnessing reverse genetics to introduce attenuating mutations into vaccine strains, Stech and colleagues recently altered the protease requirements of a laboratory strain of influenza (64). As described above, influenza HA requires cleavage by host trypsin-like proteases to initiate fusion. In a simple but clever move, these investigators switched an amino acid at the cleavage site of A/WSN/33 from a trypsin-like protease target to a porcine elastase target. This manipulation created an attenuated virus that grows well *in vitro* in the presence of elastase but replicates only minimally (i.e., enough to induce an immune response) in mice. Although this finding is a good demonstration of the practical use of basic information on the viral-replication cycle, it would appear the ability of the attenuated strains to revert back to the pathogenic form, albeit infrequently, will likely limit the usefulness of this approach.

4.3. Barriers to Using Reverse Genetics in Vaccine Production

Although powerful, reverse genetics cannot be universally incorporated into the influenza vaccine production chain until certain legal and regulatory issues are resolved (15). One issue is that these systems require cell culture, which is not required for the development of conventional high-growth strains. Unfortunately, the number of suitable cell lines is very limited. In addition to the regulatory requirements for a well-characterized cell bank, the technology limits the choice of cell. The first-generation plasmid-based reverse-genetics systems (38-40)

use the species-specific human RNA polymerase I promoter, which limits the use of these systems to cells of primate origin. Second-generation systems utilizing avian promoters (65) are in development, and one would assume canine systems are not far behind. Among existing primate-origin cell lines, the African green monkey kidney cells, Vero cell line is likely best suited to meet technical demands and has historically been a substrate for licensed vaccines. Cultures of Vero cells are easily obtainable, but only cells from fully tested and licensed cell banks are likely to be acceptable for vaccine manufacture. Creating such cell banks requires dedicated facilities and substantial financial input. During the 2003 H5N1 outbreak, access to such cells was a significant hindrance to vaccine production, and much time was lost getting the appropriate cells to the laboratories capable of creating the vaccine strains.

In many countries, reference vaccine strains derived from reverse genetics are considered genetically modified organisms. Although classification as genetically modified does not necessarily preclude the viruses' use, it will require that manufacturers obtain additional government approval; this process is now well underway in several countries. In addition, reverse genetics technologies are covered by intellectual property patents, which again does not preclude their use, but may discourage the full involvement of commercial entities. Although these issues are potential barriers to influenza vaccine development, they can be overcome. Pandemic scares such as those sparked by the 2003 and ongoing 2004 H5N1 outbreaks are forcing commercial and regulatory parties to address these issues with some urgency.

5. CELL CULTURE VACCINES

Although the current system of influenza vaccine production in eggs works well most of the time, there is little doubt that cell culture systems have advantages and that vaccine produced in this way will be available within 5 to 10 years. A major public health advantage to using cell culture systems is that it is easier to manage an inventory of frozen cells than flocks of chickens. Therefore, cell culture systems will add substantial flexibility to the influenza vaccine production system. The end user will probably not notice any difference between the egg-grown and cell culture-grown vaccines, but in times of emergency, the speed of production in the latter system will be beneficial.

The outbreaks of H5N1 and increasing demands for seasonal influenza vaccine have driven the development of the cell culture technologies forward. Manufacturers have hesitated to invest in cell culture production, mostly due to the historically poor financial return on influenza vaccines (66). Converting from chicken egg to cell culture systems is not a simple affair. With increasing demand and increasing federal support the major influenza vaccine manufacturers are developing cell-based production systems.

At least three cell types are being explored as substrates for vaccine virus growth: Madin-Darby canine

kidney (MDCK), Vero cells, and PER-C6 cells. MDCK cells have historically been the cell type most commonly used for propagation of influenza viruses. They are susceptible to most strains of flu, and the resulting virus yields are generally good. Some anxiety accompanies the use of MDCK cells because of their tumorigenicity when injected into immunocompromised mice (67). However, licensure for an MDCK-generated influenza vaccine has been gained in The Netherlands (68), and there is an expanding safety database (69-71). After MDCK, the cell line that has been most explored for influenza vaccine production is the Vero line (67,72). The third cell line being touted as a substrate for influenza vaccine is the human embryonic retinal cell line PER-C6 (73).

6. ANTIGEN-SPARING APPROACHES

A major drawback of influenza vaccines, particularly pandemic vaccines, is their poor immunogenicity in naïve individuals. This has spurred much research into antigen-sparing approaches such as adjuvants and alternative routes of delivery. Although a number of adjuvants, many targeting mediators of the innate immune response, are in early preclinical stages of development, the two most developed for use in influenza vaccine are aluminium and MF-59. Aluminum-containing influenza vaccines have only a modest advantage over nonadjuvanted formulations, though this adjuvant is commonly used in other vaccines (74). In contrast, MF-59 (an oil-in-water emulsion) has shown encouraging results (75-81) and is licensed in seasonal influenza vaccine in parts of Europe.

The second approach that has been used to reduce the dose of inactivated influenza virus in vaccines is intradermal administration. Two trials examining the dose-sparing effect of intradermal administration of a seasonal influenza vaccine showed that reduced doses of vaccine given via this route induced equivalent or better responses than a single, intramuscular full dose in healthy adults (82,83). However, a similar study in Bangkok was not able to reproduce these results (84).

6.1. Foreign Expression of Hemagglutinin

Good immunoprotection against influenza can be generated using antibodies against the viral HA alone; thus, it is not surprising that a number of approaches have been used to create recombinant vaccines based on the expression of this protein. These approaches have the added benefit that safety concerns are alleviated when dealing with the highly pathogenic strains.

In terms of clinical development, the most advanced recombinant vaccine in production is baculovirus-expressed HA. H5 and seasonal vaccines produced using this approach have entered the clinical trial phase of development. Although the H5 trial was disappointing, in terms of immunogenicity, it did show that the baculovirus-expressed protein induced functionally relevant antibodies in humans (51). More promising results have been seen in boosting primed individuals (i.e., those who have had previous infections and/or vaccinations in a seasonal vaccine setting). In the absence of an adjuvant,

these vaccines may be better suited to the seasonal vaccine niche (85-87). Another recombinant approach is the manufacture of virus-like particles (VLPs). VLPs form spontaneously when HA, NA, and M1 proteins are expressed together. Baculovirus-expressed VLP vaccines are immunoprotective in mouse models of H9N2 (88), H3N2 (89) and H1N1 (90) infection.

In addition to recombinant HA, a number of viral vectors have been assessed for their suitability as influenza vaccines. Those in clinical development include adenovirus and alphavirus vectors. In clinical trials, nasal or epicutaneous administration of a replication-defective human Ad serotype 5-derived vector expressing an H1 HA protein was well tolerated (91). Adenovirus approaches do, however, have the limitation of vector immunity. This problem is confounded with influenza vaccination, where immunization is a yearly phenomenon. Other approaches that appear to be less limited by vector immunity include those based on alphavirus replicon systems. Both Semliki Forrest virus (92) and Venezuelan equine encephalitis virus replicon systems have shown promise as humoral and cellular immunity-stimulating influenza vaccines.

6.2. DNA vaccination

Since the 1993 report of the first experiments using influenza to demonstrate the utility of DNA vaccination against infectious diseases (93), several subsequent reports have been published. These include the demonstration of DNA vaccine efficacy against a range of influenza viral subtypes in several animal models. One attractive feature of the DNA approach is that large amounts of vaccine could theoretically be produced in a short period of time. Substantial, but not insurmountable hurdles would have to be overcome if this approach was used. For example, delivering the quantities of DNA needed during a pandemic may be difficult (94). Another advantage of this approach is the ease with which additional gene segments could be incorporated into the vaccine. A viral protein such as the nucleoprotein (NP), which contains known CD8⁺ T-cell epitopes, would be a prime example.

Although DNA vaccines in humans have yet to live up to the promise suggested by preclinical animal data, influenza DNA vaccines against pandemic and seasonal strains are in clinical trial. Data from a small Phase I study using intradermal gene gun delivery of an H3 HA-based DNA vaccine showed that the criteria for the Committee of Proprietary Medical Products (European guidelines for influenza vaccine immunogenicity) was met at a single 4- μ g dose (95). Interestingly, the kinetics of the antibody response was slower than that observed for inactivated virus vaccines (95). This DNA vaccine was given to a primed population, and for a pandemic vaccine where no preexisting immunity is present, much larger or multiple doses would almost certainly be needed.

7. NOVEL INFLUENZA VACCINE TARGETS

As discussed above, the major hurdle limiting the development of a long-lasting influenza vaccine is the fact

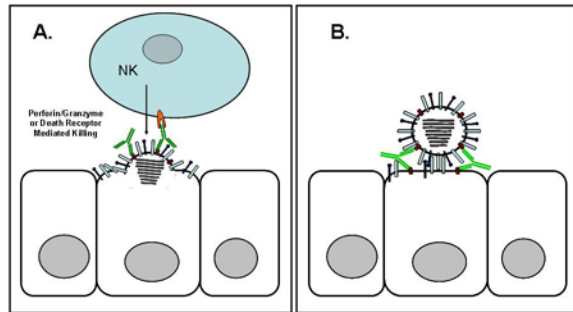


Figure 2. Potential mechanisms of M2 antibody-mediated inhibition of influenza virus. A) Immunoglobulin binding to M2 expressed on the surface of an influenza-infected cell may recruit an antibody dependent cellular cytotoxicity (ADCC) response. Binding between Fc receptors of an innate immune cell and the constant region of M2-bound antibodies might then bring the effector cell into focused contact with the infected cell and trigger its killing via perforin/granzyme-mediated or death receptor (e.g. FasL) - mediated mechanisms. B) Cross-linkage between nascent influenza virions and cell membrane by M2-specific antibodies may inhibit release and dissemination of virus to other susceptible host cells.

that the best forms of protection target the most variable viral proteins. The major surface glycoproteins HA and NA are effective immunogens in a variety of vaccine formulations; however, population-wide acquisition of immunity to HA and NA – due to infection and vaccination – drives perpetual genetic drift in these proteins, thereby forcing the constant updating of vaccine antigens. In light of this problem, much effort has been made to induce immunity against antigens derived from more conserved proteins or from highly conserved portions of HA or NA. Vaccination with such targets could protect against a wide spectrum of human influenza strains circulating from year to year and possibly provide heterosubtypic immunity against avian strains such as H5N1.

NP is conserved among influenza A viruses and elicits cytotoxic T lymphocyte (CTL) responses in infected mice. DNA vaccines based on NP genes induce CTL responses and protect against heterologous challenge in mice (96). CD4⁺ and CD8⁺ T cells help mediate protection elicited by NP DNA in this model (96). More robust protection has recently been achieved using a regimen of multiple NP DNA injections plus priming with adenovirus-vectored NP (97). The breadth and durability of this protection was demonstrated when mice were substantially protected against challenge with highly pathogenic H5N1 influenza viruses 5 months after vaccination.

Significant attention has been directed toward M2, the third transmembrane protein of influenza A viruses, as a potential vaccine target. Unlike HA and NA, M2 has an inconspicuous profile at the surface of virions or infected cells: the extracellular domain includes only the first 24 of 97 amino acids. As a result, virtually no detectable antibodies are made against this virus during a natural infection. Supporting the notion that M2 is not a

major natural target for neutralizing antibodies is its high degree of conservation. However, it has been proposed that driving an immune response against the M2 extracellular domain (M2e) via vaccination could elicit a protective antibody response. If so, this approach could potentially simplify the influenza vaccine regimen, as high cross-reactivity among influenza A strains would be predicted. Data from several studies partially support this hypothesis. An M2-specific monoclonal antibody administered to mice permitted more rapid elimination of influenza A virus from lungs during sublethal challenge (98). Protection from the challenge was observed after vaccinations with several forms of M2, including baculovirus-expressed full-length protein (99), M2 with deleted transmembrane region (100), and M2e conjugated with hepatitis B virus core (HBc), keyhole limpet hemocyanin, or *Neisseria meningitidis* outer membrane protein complex (OMPC) (101,102). Despite the apparent promise of these and other approaches, the viability of an M2-based influenza vaccination strategy still remains to be determined.

One limitation of using M2-based vaccines may be the mechanism of action of M2-based immunity, which has not been well resolved (Figure 2). The efficacy of M2-based vaccines is antibody mediated, but the antibodies are not neutralizing. Studies by Jegerlehner and colleagues consequently suggested that instead of binding to the virus, these nonneutralizing antibodies bind to the infected cells, and protection is mediated by antibody-dependent cell cytotoxicity (ADCC) of natural killer cells¹⁰³. Results of the study suggest that resistance to viral challenge was comparatively weak: M2-specific antibodies reduced disease severity but failed to prevent infection. The investigators also suggested that the mechanism of M2 action is confined largely to the lung, as opposed to upper respiratory tract tissues (103).

In the mouse model of influenza infection lungs are the primary site of influenza virus replication, but lung-localized M2 antibody action may be less relevant during human infection or the ferret model, in which upper respiratory tract virus replication is pivotal. In a ferret study, M2e-immunized animals had significantly reduced viral titers in the lung but no reduction was observed in nasal washes after influenza virus challenge, when compared to control mice (101). Another potential concern raised in an animal M2 immunization study is the possibility of exacerbated disease upon subsequent infection. Swine vaccinated with M2eHBc fusion protein or a plasmid encoding M2e and NP displayed enhanced clinical disease upon H1N1 challenge (104). Vaccination with M2e-NP DNA led to the most marked disease exacerbation. Therefore, although the idea of using M2 as a vaccine target is attractive, it remains to be seen if M2 vaccines satisfy efficacy standards for use in humans or livestock. While M2 is an attractive vaccine target, early animal studies have, not surprisingly, pointed out efficacy and safety issues for further study.

The cleavage site of HA has potential as a target for vaccines with broad efficacy. Cleavage at this site in HA₀, the precursor to HA, is an indispensable step in

maturation of virions to a form capable of fusion with host cell membranes. This helps explain the high conservation of the cleavage site and suggests that antibodies directed there could block replication by a broad spectrum of viral subtypes. One group of researchers tested a candidate vaccine in which the consensus sequence of the influenza B cleavage site was conjugated to *Neisseria meningitidis* OMPC (105). Administered to mice, this vaccine (B/HA₀-OMPC) elicited HA₀-specific antibodies and protected against lethal challenge with either of the immunologically distinct type-B lineages. Protection was mediated by antibodies, as it could be conferred by passive transfer of immune serum to naïve mice. Mice with an FcR (–) phenotype were not protected by B/HA₀-OMPC, implicating ADCC as the most likely mechanism of antibody-mediated protection. This corresponds with the above-mentioned findings for HBc-M2e-conjugate vaccine against influenza A. Unlike the M2e approaches, the B/HA₀-OMPC approach protected mice from weight loss and death. The same study reported preliminary tests of an equivalent vaccine based on the HA₀ cleavage site of influenza A. This approach provided a measure of protection against viral challenge, including heterosubtypic protection, but immunized mice were not spared from marked weight loss. Although this vaccine strategy has not been subjected to extensive testing or optimization, it holds promise for leading to valuable, broad-spectrum vaccines against influenza A or B.

7. PERSPECTIVES

Despite a substantial amount of work being done to optimize newer generation influenza vaccines, it appears that improvements in the near future will remain limited to the upgrading of current systems to cell culture systems, improving other aspects of vaccine production, and developing antigen-sparing techniques. Notwithstanding the attractiveness of a vaccine that induces immunity to more conserved viral proteins, the bottom line is that there is little data to suggest that these targets provide strong enough immunity to act as a stand-alone vaccine for seasonal influenza. Nevertheless, they may provide added benefit when used in conjunction with current influenza vaccines. This may be true in the elderly, in whom vaccines are less efficacious, or in stockpiled pandemic vaccines, when the goal of a vaccine is more directed toward reducing mortality than morbidity. HA is, and will most likely remain, the antigen of choice for influenza vaccines, despite its limitations

The production systems for current influenza vaccines need updating. A 6-month time frame for production is laudable considering the need to make new vaccine every year, but it is woefully inadequate in times of emergency. Technologies for delivery of HA antigen that decrease this down time should remain a major focus for research and development. One thing is for certain— influenza is not an eradicable disease. Therefore, there will always be a need for new and improved influenza vaccines.

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9. REFERENCES

1. ICTV. 2006. Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. Academic Press.
2. Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, & A. D. Osterhaus: Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 79, 2814-2822 (2005)
3. Kawaoka, Y., S. Krauss, & R. G. Webster: Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol* 63, 4603-4608 (1989)
4. Scholtissek, C., W. Rohde, H. von, V., & R. Rott: On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* 87, 13-20 (1978)
5. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, & Y. Kawaoka: Evolution and ecology of influenza A viruses. *Microbiol Rev* 56, 152-179 (1992)
6. Krauss, S., D. Walker, S. P. Pryor, L. Niles, L. Chenghong, V. S. Hinshaw, & R. G. Webster: Influenza A viruses of migrating wild aquatic birds in North America. *Vector Borne Zoonotic Dis* 4, 177-189 (2004)
7. The World Health Organization Global Influenza Program Surveillance Network: Evolution of H5N1 Avian Influenza Viruses in Asia. *Emerg Infect Dis* in press, (2005)
8. Bosch, F. X., M. Orlich, H. D. Klenk, & R. Rott: The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. *Virology* 95, 197-207 (1979)
9. Bosch, F. X., W. Garten, H. D. Klenk, & R. Rott: Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses. *Virology* 113, 725-735 (1981)
10. Kawaoka, Y., A. Nestorowicz, D. J. Alexander, & R. G. Webster: Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. *Virology* 158, 218-227 (1987)
11. Steinhauer, D. A.: Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 258, 1-20 (1999)
12. Klenk, H. D. and W. Garten: Host cell proteases controlling virus pathogenicity. *Trends Microbiol* 2, 39-43 (1994)
13. Wood, J. M., D. Major, R. W. Newman, U. Dunleavy, C. Nicolson, J. S. Robertson, & G. C. Schild: Preparation of vaccines against H5N1 influenza. *Vaccine* 20 Suppl 2, S84-S87 (2002)
14. Kemble, G. and H. Greenberg: Novel generations of influenza vaccines. *Vaccine* 21, 1789-1795 (2003)

15. Fedson, D. S.: Pandemic influenza and the global vaccine supply. *Clin Infect Dis* 36, 1552-1561 (2003)
16. Wood, J. M. & M. S. Williams: History of inactivated influenza vaccines. In: Textbook of Influenza. Eds: K. G. Nicholson, R. G. Webster, and A. J. Hay, Blackwell Science Ltd, Oxford (1998)
17. Davenport, F. M., A. V. Hennessy, F. M. Brandon, R. G. Webster, C. D. Barrett, Jr., & G. O. Lease: Comparisons of serologic and febrile responses in humans to vaccination with influenza A viruses or their hemagglutinins. *J Lab Clin Med* 63, 5-13 (1964)
18. Kilbourne, E. D.: Future influenza vaccines and the use of genetic recombinants. *Bull World Health Organ* 41, 643-645 (1969)
19. Beare, A. S., G. C. Schild, & J. W. Craig: Trials in man with live recombinants made from A/PR/8/34 (H0 N1) and wild H3 N2 influenza viruses. *Lancet* 2, 729-732 (1975)
20. Florent, G.: Gene constellation of live influenza A vaccines. *Arch Virol* 64, 171-173 (1980)
21. Demicheli, V., D. Rivetti, J. J. Deeks, & T. O. Jefferson: Vaccines for preventing influenza in healthy adults. *Cochrane Database Syst Rev* CD001269 (2000)
22. Jefferson, T., S. Smith, V. Demicheli, A. Harnden, A. Rivetti, & P. C. Di: Assessment of the efficacy and effectiveness of influenza vaccines in healthy children: systematic review. *Lancet* 365, 773-780 (2005)
23. Jefferson, T., D. Rivetti, A. Rivetti, M. Rudin, P. C. Di, & V. Demicheli: Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. *Lancet* 366, 1165-1174 (2005)
24. Wareing, M. D. and G. A. Tannock: Live attenuated vaccines against influenza; an historical review. *Vaccine* 19, 3320-3330 (2001)
25. Ghendon, Y. Z., F. I. Polezhaev, K. V. Lisovskaya, T. E. Medvedeva, G. I. Alexandrova, & A. I. Klimov: Recombinant cold-adapted attenuated influenza A vaccines for use in children: molecular genetic analysis of the cold-adapted donor and recombinants. *Infect Immun* 44, 730-733 (1984)
26. Maassab, H. F., C. A. Heilman, & M. L. Herlocher: Cold-adapted influenza viruses for use as live vaccines for man. *Adv Biotechnol Processes* 14, 203-242 (1990)
27. Belshe, R. B., W. C. Gruber, P. M. Mendelman, I. Cho, K. Reisinger, S. L. Block, J. Wittes, D. Iacuzio, P. Piedra, J. Treanor, J. King, K. Kotloff, D. I. Bernstein, F. G. Hayden, K. Zangwill, L. Yan, & M. Wolff: 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 (2000)
28. Nichol, K. L., P. M. Mendelman, K. P. Mallon, L. A. Jackson, G. J. Gorse, R. B. Belshe, W. P. Glezen, & J. Wittes: Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial. *JAMA* 282, 137-144 (1999)
29. Edwards, K. M., W. D. Dupont, M. K. Westrich, W. D. Plummer, Jr., P. S. Palmer, & P. F. Wright: A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J Infect Dis* 169, 68-76 (1994)
30. Ohmit, S. E., J. C. Victor, J. R. Rothhoff, E. R. Teich, R. K. Truscon, L. L. Baum, B. Rangarajan, D. W. Newton, M. L. Boulton, & A. S. Monto: Prevention of antigenically drifted influenza by inactivated and live attenuated vaccines. *N Engl J Med* 355, 2513-2522 (2006)
31. Gerdil, C.: The annual production cycle for influenza vaccine. *Vaccine* 21, 1776-1779 (2003)
32. Wood, J. M.: Developing vaccines against pandemic influenza. *Philos Trans R Soc Lond B Biol Sci* 356, 1953-1960 (2001)
33. Del, G. G., A. K. Hilbert, R. Bugarini, A. Minutello, O. Popova, D. Toneatto, I. Schoendorf, A. Borkowski, R. Rappuoli, & A. Podda: 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 (2006)
34. Lu, B., H. Zhou, D. Ye, G. Kemble, & H. Jin: Improvement of influenza A/Fujian/411/02 (H3N2) virus growth in embryonated chicken eggs by balancing the hemagglutinin and neuraminidase activities, using reverse genetics. *J Virol* 79, 6763-6771 (2005)
35. Nicolson, C., D. Major, J. M. Wood, & J. S. Robertson: Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. *Vaccine* 23, 2943-2952 (2005)
36. Widjaja, L., N. Ilyushina, R. G. Webster, & R. J. Webby: Molecular changes associated with adaptation of human influenza A virus in embryonated chicken eggs. *Virology* 350, 137-145 (2006)
37. Enami, M., W. Luytjes, M. Krystal, & P. Palese: Introduction of site-specific mutations into the genome of influenza virus. *Proc Natl Acad Sci U S A* 87, 3802-3805 (1990)
38. Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, & Y. Kawaoka: Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci U S A* 96, 9345-9350 (1999)
39. Fodor, E., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, & A. Garcia-Sastre: Rescue of influenza A virus from recombinant DNA. *J Virol* 73, 9679-9682 (1999)
40. Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, & R. G. Webster: A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci U S A* 97, 6108-6113 (2000)
41. Hoffmann, E., S. Krauss, D. Perez, R. Webby, & R. Webster: Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* 20, 3165-3170 (2002)
42. Schickli, J. H., A. Flandorfer, T. Nakaya, L. Martinez-Sobrido, A. Garcia-Sastre, & P. Palese: Plasmid-only rescue of influenza A virus vaccine candidates. *Philos Trans R Soc Lond B Biol Sci* 356, 1965-1973 (2001)
43. Ozaki, H., E. A. Govorkova, C. Li, X. Xiong, R. G. Webster, & R. J. Webby: Generation of high-yielding influenza A viruses in african green monkey kidney (vero) cells by reverse genetics. *J Virol* 78, 1851-1857 (2004)
44. Li, S., C. Liu, A. Klimov, K. Subbarao, M. L. Perdue, D. Mo, Y. Ji, L. Woods, S. Hietala, & M. Bryant: Recombinant influenza A virus vaccines for the pathogenic

- human A/Hong Kong/97 (H5N1) viruses. *J Infect Dis* 179, 1132-1138 (1999)
45. Takada, A., N. Kuboki, K. Okazaki, A. Ninomiya, H. Tanaka, H. Ozaki, S. Itamura, H. Nishimura, M. Enami, M. Tashiro, K. F. Shortridge, & H. Kida: Avirulent Avian influenza virus as a vaccine strain against a potential human pandemic. *J Virol* 73, 8303-8307 (1999)
46. Liu, M., J. M. Wood, T. Ellis, S. Krauss, P. Seiler, C. Johnson, E. Hoffmann, J. Humberd, D. Hulse, Y. Zhang, R. G. Webster, & D. R. Perez: Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. *Virology* in-press, (2003)
47. Subbarao, K., H. Chen, D. Swayne, L. Mingay, E. Fodor, G. Brownlee, X. Xu, X. Lu, J. Katz, N. Cox, & Y. Matsuoka: Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* 305, 192-200 (2003)
48. Peiris, J. S., W. C. Yu, C. W. Leung, C. Y. Cheung, W. F. Ng, J. M. Nicholls, T. K. Ng, K. H. Chan, S. T. Lai, W. L. Lim, K. Y. Yuen, & Y. Guan: Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* 363, 617-619 (2004)
49. Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. de Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, & R. G. Webster: Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351, 472-477 (1998)
50. Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, & N. Cox: Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279, 393-396 (1998)
51. Treanor, J. J., B. E. Wilkinson, F. Masseoud, J. Hu-Primmer, R. Battaglia, D. O'Brien, M. Wolff, G. Rabinovich, W. Blackwelder, & J. M. Katz: Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. *Vaccine* 19, 1732-1737 (2001)
52. Webby, R. J., D. R. Perez, J. S. Coleman, Y. Guan, J. H. Knight, E. A. Govorkova, L. R. McClain-Moss, J. S. Peiris, J. E. Rehg, E. I. Tuomanen, & R. G. Webster: Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet* 363, 1099-1103 (2004)
53. Govorkova, E. A., R. J. Webby, J. Humberd, J. P. Seiler, & R. G. Webster: Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. *J Infect Dis* 194, 159-167 (2006)
54. Lipatov, A. S., R. J. Webby, E. A. Govorkova, S. Krauss, & R. G. Webster: Efficacy of H5 influenza vaccines produced by reverse genetics in a lethal mouse model. *J Infect Dis* 191, 1216-1220 (2005)
55. Li, K. S., Y. Guan, J. Wang, G. J. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auevarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, & J. S. Peiris: Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 430, 209-213 (2004)
56. Treanor, J. J., J. D. Campbell, K. M. Zangwill, T. Rowe, & M. Wolff: Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* 354, 1343-1351 (2006)
57. Bresson, J. L., C. Perronne, O. Launay, C. Gerdil, M. Saville, J. Wood, K. Hoschler, & M. C. Zambon: Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. *Lancet* 367, 1657-1664 (2006)
58. Lin, J., J. Zhang, X. Dong, H. Fang, J. Chen, N. Su, Q. Gao, Z. Zhang, Y. Liu, Z. Wang, M. Yang, R. Sun, C. Li, S. Lin, M. Ji, Y. Liu, X. Wang, J. Wood, Z. Feng, Y. Wang, & W. Yin: Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. *Lancet* 368, 991-997 (2006)
59. Lipatov, A. S., E. Hoffmann, R. Salomon, H. L. Yen, & R. G. Webster: 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 (2006)
60. Suguitan, A. L., Jr., J. McAuliffe, K. L. Mills, H. Jin, G. Duke, B. Lu, C. J. Luke, B. Murphy, D. E. Swayne, G. Kemble, & K. Subbarao: Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med* 3, e360 (2006)
61. Krug, R. M., W. Yuan, D. L. Noah, & A. G. Latham: Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* 309, 181-189 (2003)
62. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, & Reis e Sousa: RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314, 997-1001 (2006)
63. Vesikari, T., A. Karvonen, T. Korhonen, K. Edelman, R. Vainionpaa, A. Salmi, M. K. Saville, I. Cho, A. Razmpour, R. Rappaport, R. O'Neill, A. Georgiu, W. Gruber, P. M. Mendelman, & B. Forrest: 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 (2006)
64. Stech, J., H. Garn, M. Wegmann, R. Wagner, & H. D. Klenk: A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. *Nat Med* 11, 683-689 (2005)
65. Massin, P., P. Rodrigues, M. Marasescu, W. S. van der, & N. Naffakh: Cloning of the chicken RNA polymerase I promoter and use for reverse genetics of influenza A viruses in avian cells. *J Virol* 79, 13811-13816 (2005)
66. Ulmer, J. B., U. Valley, & R. Rappuoli: Vaccine manufacturing: challenges and solutions. *Nat Biotechnol* 24, 1377-1383 (2006)
67. Govorkova, E. A., G. Murti, B. Meignier, T. C. de, & R. G. Webster: African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. *J Virol* 70, 5519-5524 (1996)
68. Brands, R., J. Visser, J. Medema, A. M. Palache, & G. J. van Scharrenburg: Influvac: a safe Madin Darby Canine Kidney (MDCK) cell culture-based influenza vaccine. *Dev Biol Stand* 98, 93-100 (1999)

69. Halperin, S. A., B. Smith, T. Mabrouk, M. Germain, P. Trepanier, T. Hassell, J. Treanor, R. Gauthier, & E. L. Mills: Safety and immunogenicity of a trivalent, inactivated, mammalian cell culture-derived influenza vaccine in healthy adults, seniors, and children. *Vaccine* 20, 1240-1247 (2002)
70. Medema, J. K., J. Meijer, A. J. Kersten, & R. Horton: Safety assessment of Madin Darby canine kidney cells as vaccine substrate. *Dev Biol (Basel)* 123, 243-250 (2006)
71. Percheson, P. B., P. Trepanier, R. Dugre, & T. Mabrouk: A Phase I, randomized controlled clinical trial to study the reactogenicity and immunogenicity of a new split influenza vaccine derived from a non-tumorigenic cell line. *Dev Biol Stand* 98, 127-132 (1999)
72. Kistner, O., P. N. Barrett, W. Mundt, M. Reiter, S. Schober-Bendixen, & F. Dorner: Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. *Vaccine* 16, 960-968 (1998)
73. Pau, M. G., C. Ophorst, M. H. Koldijk, G. Schouten, M. Mehtali, & F. UytdeHaag: The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. *Vaccine* 19, 2716-2721 (2001)
74. Nichol, K. L. and J. J. Treanor: Vaccines for seasonal and pandemic influenza. *J Infect Dis* 194 Suppl 2, S111-S118 (2006)
75. Atmar, R. L., W. A. Keitel, S. M. Patel, J. M. Katz, D. She, S. H. El, J. Pompey, T. R. Cate, & R. B. Couch: Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. *Clin Infect Dis* 43, 1135-1142 (2006)
76. Baldo, V., T. Menegon, C. Bonello, A. Floreani, & R. Trivello: Comparison of three different influenza vaccines in institutionalised elderly. *Vaccine* 19, 3472-3475 (2001)
77. Baldo, V., T. Baldovin, A. Floreani, M. Minuzzo, & R. Trivello: Response to influenza vaccine in people with non-protective HI antibody titers. *Eur J Epidemiol* 21, 843-845 (2006)
78. Gasparini, R., T. Pozzi, E. Montomoli, E. Fragapane, F. Senatore, M. Minutello, & A. Podda: Increased immunogenicity of the MF59-adjuvanted influenza vaccine compared to a conventional subunit vaccine in elderly subjects. *Eur J Epidemiol* 17, 135-140 (2001)
79. Menegon, T., V. Baldo, C. Bonello, C. D. Dalla, T. A. Di, & R. Trivello: Influenza vaccines: antibody responses to split virus and MF59-adjuvanted subunit virus in an adult population. *Eur J Epidemiol* 15, 573-576 (1999)
80. Nicholson, K. G., A. E. Colegate, A. Podda, I. Stephenson, J. Wood, E. Ypma, & M. C. Zambon: 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 (2001)
81. Stephenson, I., R. Bugarini, K. G. Nicholson, A. Podda, J. M. Wood, M. C. Zambon, & J. M. Katz: 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 (2005)
82. Belshe, R. B., F. K. Newman, J. Cannon, C. Duane, J. Treanor, H. C. Van, B. J. Howe, & G. Dubin: Serum antibody responses after intradermal vaccination against influenza. *N Engl J Med* 351, 2286-2294 (2004)
83. Kenney, R. T., S. A. Frech, L. R. Muenz, C. P. Villar, & G. M. Glenn: Dose sparing with intradermal injection of influenza vaccine. *N Engl J Med* 351, 2295-2301 (2004)
84. Auewarakul, P., U. Kositanont, P. Sornsathapornkul, P. Tothong, R. Kanyok, & P. Thongcharoen: Antibody responses after dose-sparing intradermal influenza vaccination. *Vaccine* 25, 659-663 (2007)
85. Lakey, D. L., J. J. Treanor, R. F. Betts, G. E. Smith, J. Thompson, E. Sannella, G. Reed, B. E. Wilkinson, & P. F. Wright: Recombinant baculovirus influenza A hemagglutinin vaccines are well tolerated and immunogenic in healthy adults. *J Infect Dis* 174, 838-841 (1996)
86. Treanor, J. J., R. F. Betts, G. E. Smith, E. L. Anderson, C. S. Hackett, B. E. Wilkinson, R. B. Belshe, & D. C. Powers: Evaluation of a recombinant hemagglutinin expressed in insect cells as an influenza vaccine in young and elderly adults. *J Infect Dis* 173, 1467-1470 (1996)
87. Treanor, J. J., G. M. Schiff, R. B. Couch, T. R. Cate, R. C. Brady, C. M. Hay, M. Wolff, D. She, & M. M. Cox: Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza-virus hemagglutinin vaccine in elderly adults. *J Infect Dis* 193, 1223-1228 (2006)
88. Pushko, P., T. M. Tumpey, F. Bu, J. Knell, R. Robinson, & G. Smith: 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 (2005)
89. Galarza, J. M., T. Latham, & A. Cupo: Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge. *Viral Immunol* 18, 365-372 (2005)
90. Quan, F. S., C. Huang, R. W. Compans, & S. M. Kang: Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. *J Virol* (2007)
91. Van Kampen, K. R., Z. Shi, P. Gao, J. Zhang, K. W. Foster, D. T. Chen, D. Marks, C. A. Elmets, & D. C. Tang: Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. *Vaccine* 23, 1029-1036 (2005)
92. Berglund, P., M. N. Fleeton, C. Smerdou, & P. Liljestrom: Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice. *Vaccine* 17, 497-507 (1999)
93. Robinson, H. L., L. A. Hunt, & R. G. Webster: Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine* 11, 957-960 (1993)
94. Hoare, M., M. S. Levy, D. G. Bracewell, S. D. Doig, S. Kong, N. Titchener-Hooker, J. M. Ward, & P. Dunnill: Bioprocess engineering issues that would be faced in producing a DNA vaccine at up to 100 m3 fermentation scale for an influenza pandemic. *Biotechnol Prog* 21, 1577-1592 (2005)
95. Drape, R. J., M. D. Macklin, L. J. Barr, S. Jones, J. R. Haynes, & H. J. Dean: Epidermal DNA vaccine for influenza is immunogenic in humans. *Vaccine* 24, 4475-4481 (2006)

96. Ulmer, J. B., T. M. Fu, R. R. Deck, A. Friedman, L. Guan, C. DeWitt, X. Liu, S. Wang, M. A. Liu, J. J. Donnelly, & M. J. Caulfield: Protective CD4⁺ and CD8⁺ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J Virol* 72, 5648-5653 (1998)
97. Epstein, S. L., W. P. Kong, J. A. Misplon, C. Y. Lo, T. M. Tumpey, L. Xu, & G. J. Nabel: Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine* 23, 5404-5410 (2005)
98. Treanor, J. J., E. L. Tierney, S. L. Zebede, R. A. Lamb, & B. R. Murphy: Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. *J Virol* 64, 1375-1377 (1990)
99. Slepishkin, V. A., J. M. Katz, R. A. Black, W. C. Gamble, P. A. Rota, & N. J. Cox: Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine* 13, 1399-1402 (1995)
100. Frace, A. M., A. I. Klimov, T. Rowe, R. A. Black, & J. M. Katz: Modified M2 proteins produce heterotypic immunity against influenza A virus. *Vaccine* 17, 2237-2244 (1999)
101. Fan, J., X. Liang, M. S. Horton, H. C. Perry, M. P. Citron, G. J. Heidecker, T. M. Fu, J. Joyce, C. T. Przysiecki, P. M. Keller, V. M. Garsky, R. Ionescu, Y. Rippeon, L. Shi, M. A. Chastain, J. H. Condra, M. E. Davies, J. Liao, E. A. Emini, & J. W. Shiver: Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. *Vaccine* 22, 2993-3003 (2004)
102. Neirynck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W. M. Jou, & W. Fiers: A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med* 5, 1157-1163 (1999)
103. Jegerlehner, A., N. Schmitz, T. Storni, & M. F. Bachmann: Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *J Immunol* 172, 5598-5605 (2004)
104. Heinen, P. P., F. A. Rijsewijk, E. A. de Boer-Luijtz, & A. T. Bianchi: Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. *J Gen Virol* 83, 1851-1859 (2002)
105. Bianchi, E., X. Liang, P. Ingallinella, M. Finotto, M. A. Chastain, J. Fan, T. M. Fu, H. C. Song, M. S. Horton, D. C. Freed, W. Manger, E. Wen, L. Shi, R. Ionescu, C. Price, M. Wenger, E. A. Emini, R. Cortese, G. Ciliberto, J. W. Shiver, & A. Pessi: Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *J Virol* 79, 7380-7388 (2005)

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