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Traditional and New Influenza Vaccines

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SUMMARY

The challenges in successful vaccination against influenza using conventional approaches lie in their variable efficacy in different age populations, the antigenic variability of the circulating virus, and the production and manufacturing limitations to ensure safe, timely, and adequate supply of vaccine. The conventional influenza vaccine platform is based on stimulating immunity against the major neutralizing antibody target, hemagglutinin (HA), by virus attenuation or inactivation. Improvements to this conventional system have focused primarily on improving production and immunogenicity. Cell culture, reverse genetics, and baculovirus expression technology allow for safe and scalable production, while adjuvants, dose variation, and alternate routes of delivery aim to improve vaccine immunogenicity. Fundamentally different approaches that are currently under development hope to signal new generations of influenza vaccines. Such approaches target nonvariable regions of antigenic proteins, with the idea of stimulating cross-protective antibodies and thus creating a "universal" influenza vaccine. While such approaches have obvious benefits, there are many hurdles yet to clear. Here, we discuss the process and challenges of the current influenza vaccine platform as well as new approaches that are being investigated based on the same antigenic target and newer technologies based on different antigenic targets.

INTRODUCTION

Influenza virus is a successful human pathogen: its persistence in the human population and ability to cause sporadic pandemics make it a continuous public health threat. Seasonal influenza causes approximately 500,000 deaths worldwide and an estimated 16.7 deaths per 100,000 persons in the United States (1, 2). The burden of disease is especially high in populations with less robust immunity, such as children, the elderly, and chronically ill patients (2). It was estimated that in 2008, seasonal influenza viruses caused 90 million new infections worldwide in children younger than 5 years of age and were responsible for up to 20% of all pediatric acute lower respiratory infections (3). In the elderly, an aging immune system is thought to impair the ability to control infections, leading to increased morbidity and mortality (reviewed in reference 4). However, the impact of the disease on other age groups can be significantly greater during a pandemic.

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The 1918 Spanish flu pandemic was the most catastrophic, causing more than 50 million deaths worldwide, with a case fatality rate of 2.5% (5). The 2009 H1N1 pandemic virus was estimated to have caused more than 200,000 deaths during the first 12 months of its circulation (6). In addition, outbreaks of the highly pathogenic H5N1 avian influenza virus strains (with a case fatality rate of over 50% in zoonotic infections) are continuing throughout Asia and Africa, maintaining the global threat of influenza (7).

The three major genera of influenza virus, types A, B, and C, are all capable of causing human infections. Influenza A viruses are found in animal and human populations, whereas humans are considered the reservoir hosts of influenza B and C viruses. Types A and B are largely responsible for the annual incidences of human disease, whereas influenza C viruses cause sporadic, mild upper respiratory infections in children (8). Human seasonal influenza is typically mild, manifesting as fever, myalgia, and respiratory symptoms such as cough, sore throat, and rhinitis. More severe symptoms may include lower respiratory tract indications such as bronchitis and pneumonia, and patients may have an increased risk of secondary bacterial infections. Severe influenza infection can also lead to cardiovascular complications (reviewed in reference 9). Ninety percent of influenza-related deaths have been attributed to individuals older than 65 years of age (2).

Highly pathogenic strains of influenza virus, such as some of the avian H5 subtypes, on the other hand, can cause severe respiratory distress and multiorgan failure in infected humans (10). Atypical presentations of influenza can include gastrointestinal and neurological symptoms, both of which were observed frequently in individuals infected with the 2009 pandemic H1N1 strain (11, 12). Given their disease-causing potential, vaccination against influenza A and B viruses is a high public health priority.

Variability of Influenza Virus

The influenza A and B virus genome is composed of eight negative-sense RNA segments. The virus particle consists of a host-derived lipid envelope embedded with 3 or 4 glycoproteins surrounding the ribonucleoprotein complex (RNP) and the polymerase (Pol) proteins (PB1, PB2, and PA). The major influenza virus surface glycoproteins are hemagglutinin (HA), neuraminidase (NA), and, in smaller proportions, matrix protein 2 (M2). Influenza B virus has an additional NB (neuraminidase gene region B) protein. Matrix protein 1 (M1) and nucleoprotein (NP) are associated with viral RNA and are important for viral assembly as well as budding, while the polymerase proteins are important for viral genome replication (13). HA and NA are the major antigenic proteins and are used to further classify influenza A viruses into multiple subtypes. Sixteen HA (H1 to H16) and 9 NA (N1 to N9) influenza A virus subtypes that are antigenically distinct have been identified, with the possible addition of a new subtype, H17, identified recently (13, 14). Currently, lineages of H1 and H3 viruses circulate endemically in humans. Only a single subtype of influenza B virus has been identified, although two distinct antigenic lineages (named B/Victoria and B/Yamagata) have evolved over time and cocirculate at variable levels in humans.

Influenza viruses have two major mechanisms of antigenic evolution: antigenic drift and antigenic shift. Antigenic drift occurs when the virus accumulates mutations at antigenic sites during replication through the actions of the inherently error-prone RNA polymerase, producing variant viruses that can escape existing immunity. This phenomenon is common to both influenza A and B viruses. More dramatically, antigenic shift occurs when a virus acquires an antigenically novel HA through reassortment, a property made possible due to the segmented nature of the viral genome. Although both influenza A and B viruses undergo reassortment, antigenic shift is a feature of influenza A viruses only, due to the animal sources of antigenically distinct HA genes.

Animal Reservoirs

Of the influenza viruses, only influenza A viruses have animal reservoirs. The natural reservoirs of influenza A viruses are wild aquatic birds, but a wide range of animal hosts can be infected. Apart from the recognized reservoir animal hosts (avian, swine, and equine), influenza viruses have also been isolated from cats, dogs, seals, and cheetahs, and most recently, viral RNA from a potentially novel H17 virus was detected in bats (13, 14). As infection can occur in a wide range of animal hosts, the potential for viral spread and reassortment in new hosts is high, necessitating active and constant surveillance to monitor the presence and spread of the virus. Furthermore, some of these animals, for example, the avian and swine hosts, have significant interactions at the animal-human interface, increasing the risk of zoonotic transmission and the possibility of subsequent human-to-human spread and pandemic emergence. Direct transmission of avian- or swine-origin influenza viruses can have grave implications; human infections by the highly pathogenic avian H5N1 subtype result from direct exposure to infected birds. While evidence of human-to-human transmission of the H5 virus is limited (15, 16), the threat of the strain acquiring airborne transmissibility in a mammalian host is real (17, 18). In addition, all human pandemics in the last century have occurred due to the introduction of either avian or swine influenza viruses or virus genes (19) into the human population.

Immunological Response to Influenza Virus Infection

A challenge to the development of an effective immune response to influenza virus is the acute nature of the infection. Meta-analyses of human challenge studies with seasonal influenza viruses have shown that typical clinical symptoms peak on day 2 postinfection and resolve within 10 days, while viral shedding lasts an average of 4.8 days (20). The short infection period means that the local innate immune response is critical for restricting and clearing the infection. In humans, innate immunity to influenza virus is mediated by several mechanisms. Within infected cells, the viral RNA is recognized by pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (TLR-3 and TLR-7), retinoic acid-inducible gene 1 (RIG-1), and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR). Activation of these immune pathways leads to induction of interferon-mediated antiviral responses and secretion of proinflammatory cytokines, which helps to restrict virus replication. In addition, alveolar macrophages and natural killer (NK) cells and dendritic cells (DCs) all play a role by clearing virus-infected cells through phagocytosis and induction of apoptosis (reviewed in references 21 and 22).

The cellular arm of the adaptive immune response, mediated by $CD4^+$ T lymphocytes and $CD8^+$ cytotoxic T lymphocytes (CTLs), has been shown in murine models to aid in virus clearance, suggesting that the same mechanism is probably important in human infections, although proof for the latter is still accumulating (23).

The $CD8^+$ CTLs recognize a broad range of influenza virus antigens, including the more conserved internal viral proteins that are presented in the context of HLA class molecules (24, 25). The ability of the cellular immune response to recognize conserved influenza virus proteins means that it is able to mount a cross-reactive response to various influenza virus strains (26–28). Conversely, the humoral arm of the immune response mediates it effector functions primarily by generating antibodies that target the virion surface-exposed viral proteins HA, NA, and, to a lesser extent, M2 (which is less abundant on the viral surface). HA, the major antigenic protein of influenza virus, functions as the viral attachment and membrane fusion protein. Thus, antibodies that target HA can neutralize the virus and prevent infection. The functional HA unit is a homotrimer, with each monomer comprised of 2 domains, HA1 and HA2, linked by a disulfide bond. HA2 forms the membrane anchor and the long alpha-helix "stem," and HA1 forms the distal globular head that contains the receptor-binding sites and the majority of the antigenic sites. As discussed above, antigenic drifts in circulating strains are caused by changes to the antigenic sites in the HA1 globular head. It was predicted that the human seasonal H3 and H1 viruses have undergone between 2.1% and 3% amino acid changes per drift variant from 1999 to 2010. In contrast, the HA2 region is remarkably conserved, with only 3 amino acid changes noted for both H1 and H3 in the same time period (29). More importantly, HA2 is also immunogenic. Antibodies that target this region have been found in humans after infection and vaccination although at markedly reduced levels compared to those targeting the globular head (30, 31).

Influenza virus NA, the second major surface glycoprotein, is an enzyme that cleaves sialic acid residues on the cell surface to facilitate virus release. Antibodies to NA therefore do not prevent virus infection but instead will limit virus spread. Similarly, M2 and NP are both viral proteins that are important during the virus replication cycle. Antibodies that target these two proteins will not neutralize the virus but have been shown to restrict virus replication in mice (32).

Induction of HA-specific serum antibodies, as typically measured by a hemagglutination inhibition or neutralization assay, is used as an indicator of recent exposure or vaccination. Based on reported vaccine and clinical studies, a titer of $\geq$40 may be considered to be an immune correlate for protection for inactivated influenza vaccines in adults. Immune correlates of protection for other vaccine platforms are, however, lacking. This has been identified as an important aspect hampering the development of novel influenza vaccination approaches (33). Direct measurement of vaccine protective efficacy, which refers to the ability of the vaccine to prevent infection in a field setting, is difficult to assess empirically, as, among many obstacles, it requires a large study cohort and the ability to monitor the participants over at least one influenza season (34). With these points in mind, development of a clinically proven influenza vaccine is a lengthy and costly endeavor.

CONVENTIONAL APPROACHES TO INFLUENZA VACCINES

Influenza Vaccination

In humans, the seasonal influenza vaccine is supposed to protect against endemic H1N1, H3N2, and B strains that circulate in humans globally. While efforts have been made to prepare stockpile vaccine against zoonotic animal strains, the following descriptions relate primarily to seasonal human vaccines, unless otherwise stated. The goals of influenza vaccination can be broadly defined on 2 levels: (i) protection against infection and disease and (ii) induction of herd immunity to restrict virus transmission within the population (35). Major events in the development of influenza vaccines are presented in Fig. 1. Although early vaccine studies in the 1930s and 1940s that used crude preparations of live and inactivated influenza A virus were not consistently successful in reducing the incidence of febrile illnesses, they did demonstrate the importance of antigen potency and matching vaccine strains (36). Subsequent multicenter studies commissioned by the U.S. Armed Forces Epidemiological Board used inactivated, concentrated virus stocks incorporating multiple virus strains and found prophylactic protection and a much lower incidence of febrile illnesses in vaccinated groups than in controls, laying the foundations for influenza vaccination programs (37). Current recommendations for influenza vaccination vary among countries. In the United States, annual influenza vaccination has been recommended for high-risk groups since the 1960s, but it was only in 2010 that the Advisory Committee on Immunization Practices (ACIP) made the first recommendation for a national influenza vaccination to include individuals aged 6 months to 18 years (38). In Europe, most countries still generally recommend vaccination only for the elderly, the chronically ill, and residents of health care facilities (39). Because influenza vaccines do not induce long-lasting antibody titers, annual influenza vaccination is recommended prior to the start of the winter seasons, a fact that in itself poses difficulties for tropical countries, where the peak influenza seasons are less well delineated (40, 41).

Trivalent inactivated vaccine. The most widely used seasonal influenza vaccine is the trivalent inactivated vaccine (TIV). The conventional vaccine is composed of the 3 currently circulating seasonal influenza virus strains: two influenza A virus types (H3N2 and H1N1) and a B type. The vaccines come in three major formulations: inactivated whole-virus, "detergent"-split, or subunit vaccines. Traditional licensed influenza vaccines are prepared from embryonated chicken eggs, inoculated individually with each virus type. Whole-virus vaccines are prepared from harvested allantoic fluid, chemically inactivated with formalin or β-propiolactone, and subsequently concentrated and purified to remove nonviral protein contaminants. The split-virus vaccine has an additional treatment step with detergent to dissociate the viral lipid envelope, exposing all viral proteins and subviral elements (42, 43). In subunit vaccines, the HA protein is further enriched through additional purification steps (44–46). Because the split-virus and subunit vaccines had comparable immunogenicity in primed populations but reduced reactogenicity compared to the whole-virus vaccine preparations, most contemporary vaccines since the 1970s have been split-virus or subunit formulations. In unprimed populations, such as young children, split-virus and subunit vaccines are less immunogenic, and two doses are required to achieve the 1:40 titer (47–49). TIVs provide immunity primarily by inducing antibodies that target the protective epitopes on HA. Some formulations may also induce NA-specific antibodies that do not protect from infection but may modulate the resulting disease.

A standard dose of TIV contains 15 μg of HA per strain (total HA concentration of 45 μg) and is administered as a single dose to those aged >9 years. Younger children (between 6 months and 8 years of

FIG 1 Timeline of major events in influenza vaccine development. Influenza pandemics and major H5N1 outbreaks are indicated in red, while major breakthroughs in influenza vaccine development are highlighted in blue. Superscript numbers indicate references. *, see the FDA website (http://www.fda.gov/); ^, see the European Medicines Agency website (http://www.ema.europa.eu); #, see the CDC website (http://www.cdc.gov/vaccines/acip/index.html).

age) require two doses administered 4 weeks apart, if they have not been vaccinated in previous influenza seasons (for complete recommendations, see Table 1 and reference 38). The standard TIV is typically delivered as an intramuscular (i.m.) injection (although intradermal [i.d.] formulations are also available) and remains the most popular vaccine, primarily because of its widespread availability and relatively low cost. Recently, the U.S. Food and Drug Administration (FDA) has approved the quadrivalent version of Fluarix, a split-virion vaccine manufactured by GlaxoSmithKline Biologics that includes an additional type B strain to represent both antigenic lineages. By including an additional type B strain, it has been argued that the chance for a vaccine mismatch is reduced (50, 51). The quadrivalent inactivated vaccine (QIV) formulations appear to have immunogenicity and a safety profile comparable to those of the standard TIV (52; http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM220624.pdf).

Live attenuated influenza virus. The rationale for developing the live attenuated influenza vaccine (LAIV) was to create a vaccine that mimics natural infection and, in doing so, theoretically induces both cellular and humoral immunity. Attenuated influenza viruses were first developed in the 1960s by serial passage of the virus in eggs under suboptimal conditions (53). The resulting attenuated viruses had temperature-sensitive phenotypes. These viruses were adapted to grow at 25°C (cold adapted [*ca*]), which is the normal temperature of the nasal passage, but not at temperatures higher than 35°C (temperature sensitive [*ts*]), which is the temperature of the respiratory tract. Because this restricts virus replication to the nasal passage, LAIV was expected to stimulate local humoral and cell-mediated immunity. These attenuated viruses were stable, immunogenic, and nontransmissible and were thus subsequently developed into "master donor" strains (54). Master donor strains contribute their internal genes (all genes except the HA and NA genes) to generate vaccine strains with the desired HA and NA of the circulating strains. This is done either by classical reassortment in eggs or, when the technology became available, by reverse genetics. Since then, however, other strategies to generate attenuated viruses have been developed, mostly through molecular manipulations of internal genes. Several mutations in the PB2 and NS1 genes, for example, could also attenuate the virus and confer the temperature sensitivity phenotype to the virus (55–58). In contrast to TIV, LAIV is delivered intranasally (i.n.), induces a longer-lasting antibody titer, and is efficacious in children 2 to 7 years old (59, 60). However, because of the inherent risk of immunizing with live viruses, the LAIV is not recommended for immunocompromised individuals or individuals in close contact with these vulnerable populations. This vaccine platform may also have its drawbacks for development of vaccines against avian zoonotic strains, since they typically do not replicate in the human upper respiratory tract (61, 62), leading to disappointing results in some early clinical trials (63). A commercial LAIV produced by MedImmune was approved in 2003 by the FDA under the trade name FluMist. As of 2012, the FDA also approved the use of the quadrivalent formulation of FluMist, which includes an additional strain of the circulating influenza virus B type. LAIVs have been developed and used in Russia since the 1980s (64, 65).

Vaccine Strain Selection

For conventional influenza vaccines to be maximally effective, the vaccine viruses have to be antigenically matched to the influenza viruses circulating in humans. The most appropriate vaccine strains are identified via an extensive and complex global surveil-

TABLE 1 Summary of FDA-approved seasonal influenza vaccines as of 2013[a]

Vaccine (yr licensed)	Manufacturer	Composition(s), delivery method	HA dose	Production source	Age(s) of target population(s)	Dose
Afluria (2007)	CSL, Australia	Trivalent, split virion, intramuscular	45 μg (15 μg HA/strain)	Egg	5–8 yr (unprimed) 5–8 yr (primed), ≥9 yr	Two 0.5-ml doses, 4 wk apart 0.5-ml single dose
FluLaval (2006)	ID Biomedical Corporation, Quebec, Canada	Trivalent, split virion, intramuscular	45 μg (15 μg HA/strain)	Egg	≥18 yr	0.5-ml single dose
Fluarix (2012)	GlaxoSmithKline Biologics	Quadrivalent, split virion, intramuscular	60 μg (15 μg HA/strain)	Egg	3–8 yr (unprimed) 3–8 yr (primed), ≥9 yr	Two 0.5-ml doses, 4 wk apart 0.5 ml single dose
Fluvirin (1998)	Novartis Vaccines and Diagnostics, Inc.	Trivalent, subunit, intramuscular	45 μg (15 μg HA/strain)	Egg	4–8 yr (unprimed) 4–8 yr (primed), ≥9 yr	Two 0.5-ml doses, 4 wk apart 0.5-ml single dose
Agriflu (2009)	Novartis Vaccines and Diagnostics, Inc.	Trivalent, subunit, intramuscular	45 μg (15 μg HA/strain)	Egg	≥18 yr	0.5-ml single dose
Flucelvax (2012)	Novartis Vaccines and Diagnostics, Inc.	Trivalent, subunit, intramuscular	45 μg (15 μg HA/strain)	MDCK cell derived	≥18 yr	0.5-ml single dose
Fluzone (1980)	Sanofi Pasteur, Inc.	Trivalent, split virion, intramuscular	45 μg (15 μg HA/strain)	Egg	6 mo–3 yr (unprimed) 6 mo–3 yr (primed) 3–8 yr (unprimed) 3–8 yr (primed), ≥9 yr	Two 0.25-ml doses, 4 wk apart 0.25-ml single dose Two 0.5-ml doses, 4 wk apart 0.5-ml single dose
Fluzone High-Dose (2009)	Sanofi Pasteur, Inc.	Trivalent, split virion, intramuscular	180 μg (60 μg HA/strain)	Egg	≥65 yr	0.5-ml single dose
Fluzone-Intradermal (2011)	Sanofi Pasteur, Inc.	Trivalent, split virion, intradermal	27 μg (9 μg HA/strain)	Egg	18–64 yr	0.1-ml single dose
FluBlok (2013)	Protein Sciences	Trivalent, intramuscular	135 μg each	ExpresSF+ (proprietary insect cells) using baculovirus expression system	18–49 yr	0.5-ml single dose
FluMist (2003)	MedImmune	Live attenuated, trivalent, intranasal	$10^{6.5}$-$10^{7.5}$ FFU[b] virus/strain	Egg	2–8 yr (unprimed) 2–8 yr, (primed) 9–49 yr	Two 0.2-ml doses, 4 wk apart 0.2-ml single dose
FluMist Quadrivalent (2012)	MedImmune	Live attenuated, quadrivalent, intranasal	$10^{6.5}$-$10^{7.5}$ FFU virus/strain	Egg	2–8 yr (unprimed) 2–8 yr (primed), 9–49 yr	Two 0.2-ml doses, 4 wk apart 0.2-ml single dose

[a] Information was compiled from the FDA website (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm094045.htm).
[b] FFU, fluorescent focus units.

lance effort, coordinated by the World Health Organization (WHO) through its Global Influenza Surveillance and Response System (GISRS) network. The network comprises 6 collaborating centers (CCs), 4 essential regulatory laboratories, and 137 WHO-recognized national influenza centers (NICs) in 107 of its member states. More than 500,000 virological samples are screened at these NICs every year, of which approximately 8,000 are then sent to regional CCs for detailed genetic and antigenic analysis (66). Three types of data are generated and subsequently used to assess the relationship of circulating strains to the vaccine components and hence inform vaccine strain selection. The data types are antigenic data collected primarily through hemagglutinin inhibition assays using postinfection ferret antisera, and human serologic analyses using pre- and postvaccination serum samples and also viral HA sequence data. These data are presented during strain selection meetings held twice a year before the start of the winter seasons for the northern (February) and southern (September) hemispheres and form the basis for the annual recommendation for vaccine strains. It is worth noting that these recommendations are essentially made at least 6 months prior to the season in which the corresponding vaccine formulations will be used. Since 2004, the system has also provided additional guidance on representative zoonotic viruses for those countries and other entities interested in producing vaccine for clinical trials and/or national stockpiles.

Timeline Challenges in Conventional Vaccine Manufacturing

The influenza vaccine pipeline faces a continual battle between the desire to delay vaccine strain selection to gather data on the most recently circulating viruses and the desire to make the selection earlier to allow for potential delays in vaccine manufacture. It is this battle of competing elements that is perhaps the single greatest challenge to the production of current influenza vaccines. The actual decision of vaccine strains to be used in a specific country is decided by national regulatory bodies, although these decisions typically mirror those recommended by the WHO. Selection of the appropriate strains begins the manufacturing stage of the influenza vaccine pipeline. The influenza A viruses that are used in vaccine manufacture are not the wild-type viruses themselves but rather high-growth reassortants that are hybrid viruses containing at least the HA and NA gene segments from the target strains and other gene segments from the master strain A/Puerto Rico/8/1934 (PR8), which has properties of high growth in eggs and also a favorable safety profile. Reassortant viruses are made by coinfection of eggs with PR8 and target strains, followed by selection of appropriate seed viruses by amplification in the presence of antibodies against the HA and NA of PR8. The resulting viruses are cloned and then sequenced for confirmation. The influenza B vaccine viruses are typically wild-type viruses, although type B reassortants have been utilized more recently when viral growth properties of the wild-type viruses have been suboptimal. In practice, the availability of high-growth influenza A virus reassortants is factored into WHO vaccine strain recommendations, as the process of their production can take weeks, and not all viruses generate reassortants with desirable growth and antigenic properties. Selection of a vaccine virus with poor growth properties would have major implications for the prompt delivery of vaccine, as the multivalent nature of the vaccine means that the worst-growing strain dictates the production time frame. Once appropriate high-growth reassortants are available, vaccine manufacturers then operate in a strict timeline to produce the vaccine before the start of the influenza seasons. As discussed above, the time from strain selection to distribution is at least 6 months in an ideal setting (http://www.who.int/csr/disease/swineflu/notes/h1n1_vaccine_20090806/en/index.html). However, the manufacturing process is susceptible to potential setbacks and requirements, such as a continuous egg supply, growth of virus, and meeting of regulatory requirements (67). Furthermore, manufacturers have to ensure strict conditions of biosafety and sterility. Because of these tight timelines, some manufacturers opt to start making vaccine from at least one of the three components "at risk" before the official strain decisions are made in order to allow for unexpected setbacks in production. These decisions are based on summaries from the WHO about influenza activity leading up to the vaccine composition meetings. Another time-critical step in this process is the development of potency reagents that are used to assess the amount of antigen in the final bulk vaccine. This activity is coordinated by the essential regulatory agencies and relies on the production of bulk antigen of the selected strain (HA) and raising of sheep antiserum against it, both activities which take time and can encounter technical difficulties.

Achilles' Heels of the Current Influenza Vaccines

The success of current influenza vaccination campaigns depends heavily on extensive surveillance and manufacturing resources to ensure timely vaccine delivery. Given that each component of the vaccine is updated every 2 to 3 years on average, it is not too surprising that there are occasional problems. Some of the Achilles' heels of the current influenza vaccine pipelines and the strategies needed to address them are summarized as follows.

1. Variable efficacy in specific populations. Influenza vaccines are relatively poorly immunogenic and do not induce long-lasting serum antibody titers. In the elderly, waning immunity (immunosenescence) also means poor responsiveness to vaccines. Therefore, strategies are required to improve the immune response to vaccine, especially in various at-risk target populations.
2. Variable virus. Antigenic matching between vaccine strains and circulating strains is critical. Current strategies to address this include improvement of global surveillance and the development of new-generation vaccines that target conserved regions of the virus.
3. Production time frame and manufacturing limitations. The time frame between vaccine strain selection and vaccine delivery is short and allows for few unanticipated delays. The requirement for large quantities of vaccine also places a production burden on the manufacturing process. Strategies are required to improve the growth of conventional vaccine viruses, reduce the production time, improve manufacturing processes, and increase the immunogenicity of vaccine (dose sparing).
4. Limited vaccine availability in resource-limited countries. Although not specific for influenza vaccines, there is great disparity in vaccine availability in countries throughout the world. More scalable platforms and cheaper vaccines that induce longer-lasting immunity are needed to address this need.

VARIATION ON THE CONVENTIONAL

With the well-accepted limitations and deficiencies in the current influenza vaccine system, substantial resources have been spent trying to improve it. The improvements that are most advanced, many of which are already in use, are those that contribute incremental, but important, advances to the current TIV and LAIV platforms.

Egg versus Cell Culture

Egg-based vaccine production, although time tested, is a resource- and time-intensive process that is critically dependent on a continuous supply of eggs and the ability of viruses to grow on this substrate. The risk of contamination by avian pathogens in the egg supply or microbial contaminants during processing has previously jeopardized vaccine supplies (68; http://www.nature.com/news/2004/041004/full/news041004-8.html). Furthermore, egg supply could be limited in the event of a pandemic, and egg-based vaccines may still pose a theoretical risk of anaphylactic responses in egg-allergic individuals. The ACIP recommends that TIVs be used under medical supervision for individuals with a severe allergy to egg proteins (69).

One strategy to complement the egg-based process is the use of a cell culture platform. The cell culture platform provides the theoretical advantages of scalability, availability, and ease of manipulation from a manufacturing perspective and host compatibility from a biological perspective. Growth of human influenza viruses in cultured cells typically results in fewer adaptive mutations, some of which can change virus antigenicity, than are seen after growth in eggs (70, 71). In addition, cell culture, unlike embryonated eggs, can be cryopreserved, reconstituted, and scaled up at any time. By process improvements such as cell adaptation and the use of bioreactors, vaccine strains can be successfully grown to high yields, although it is not clear if this is universally the case (72, 73). To date, the continuous mammalian cell lines Vero (monkey kidney cells) and MDCK (canine kidney cells) and human-derived PER.C6 cells have been used successfully to prepare seasonal and prepandemic influenza vaccines (74, 75). The MDCK-derived influenza vaccine (Optaflu) is currently licensed in Europe and was shown to be comparable with egg-derived TIV in terms of safety, immunogenicity, and efficacy in children and adults up to 50 years of age (76–79). The U.S. FDA also recently approved Novartis' MDCK-derived vaccine Flucelvax for adults 18 years of age and older (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm328629.htm).

The future of cell culture platforms for influenza vaccines relies heavily on reproducibly providing vaccine yields at an acceptable cost; without this, the commercial incentives to switch production systems are minimal.

Reverse Genetics

A second variation of conventional influenza vaccine production is the implementation of reverse genetics, an approach that utilizes molecular techniques to generate a specific virus phenotype. As transcription of the negative-sense viral RNA is mediated by the viral RNA polymerase complex, any *in vitro* reconstruction strategies for influenza viruses require the presence of active viral polymerase to initiate transcription. In the early systems, this was achieved by transfection of the active ribonucleoprotein complex (RNP) or helper viruses (80), but the development of influenza virus plasmid-based reverse genetic systems in the late 1990s allowed for the generation of influenza viruses from cloned cDNA (15). To generate cloned reverse genetic viruses, the viral RNA segments are first amplified by PCR and cloned into expression plasmids containing RNA polymerase I and/or II sequence, which drives the transcription and protein expression of the viral cDNA. There are variations of the plasmid-based systems: for example, the 12-plasmid system is comprised of 4 protein expression plasmids to produce the polymerase complex (PB2, PB1, PA, and NP) and 8 plasmids to generate the negative-sense viral RNA segments separately, while the 8-plasmid system has both RNA polymerases I and II in the same plasmid vectors, and it is thus able to produce both viral proteins and RNA simultaneously (81, 82). These plasmids are then transfected into cells, and viable virus can be recovered from the culture supernatant. Other recent modifications to the system include incorporation of multiple transcription cassettes into a single plasmid to improve transfection efficiency and, consequently, virus yield (83, 84). An early proof-of-principle use for the technology was the development of high-growth vaccine seed strains. Strains with the desired genotype could be produced easily without the need for selection systems. Although theoretically useful for production of conventional TIV seed strains, in practice, it has not been possible to accurately predict which gene segments from the master PR8 strain are required for high-level growth of different vaccine viruses, and as such, conventional reassortment procedures have remained preferred (they allow for a number of different gene combinations to be generated). There are, however, two areas where the reverse genetics technologies have proved invaluable. The first area is in the development of reassortant strains for some of the LAIVs. In this case, as the seed viruses are required to contain 6 gene segments from the master strain, which encode the attenuating mutations, and the HA and NA from the target virus, reverse genetics has streamlined the process of seed virus development. The second area of use is where specific mutations have had to be introduced into the virus. For example, reverse genetics was used to produce an attenuated H5N1 vaccine strain that lacks the HA cleavage sequence associated with high-level virulence in avian and mammalian hosts (although proof of its role in humans is lacking) (85). To date, reverse genetics has remained the only working method to produce safe, matching H5 vaccine seed strains.

One limitation to the plasmid-based reverse genetic systems, however, is the host cell specificity of the RNA Pol I promoter, which is used to produce the negative-sense viral RNA in transfected cells. The promoter is relatively specific to RNA polymerases of human or primate origin, limiting the practical application of the system to human/primate cell lines. The approved cell lines for human vaccine production that are compatible for influenza virus growth include MDCK cells, Vero cells, and chicken embryonic fibroblasts (CEFs), but these cells do not have high transfection efficiency. Thus far, the original plasmid system has been applied to generate H5 vaccine strains primarily in Vero cells. Alternative reverse genetics systems using canine- and avian-derived Pol I promoter sequences have been cloned to enable the generation of recombinant viruses in MDCK and CEF cells (86, 87). As these modifications develop and as we learn more about specific gene mutations and compatibilities that control virus growth, we expect the use of reverse genetics to become more widespread in the development of influenza vaccines.

Role of Adjuvants

As discussed above, the relatively poor immunogenicity of split or subunit TIV formulations is of some concern and has been a target for improvement. One way to improve the immunogenicity of influenza vaccines, especially in vulnerable populations, is via adjuvants, which are compounds that can enhance the immune response elicited by an antigen. Generally, adjuvants exert their effect by improving antigen delivery or targeting specific immune pathways to improve the immunogenicity of vaccines (88). Currently licensed adjuvants for vaccine usage include aluminum salt (alum) and the squalene oil-in-water emulsion systems MF59 (Novartis) and AS03 (GlaxoSmithKline). MF59 has been licensed for use with seasonal vaccines in the elderly in some countries, while ASO3 has been used in conjunction with monovalent preparations of inactivated 2009 pandemic H1N1 and prepandemic H5N1 virus vaccines (http://www.ema.europa.eu/).

Although alum has been successfully used as an adjuvant in many other vaccines, alum-adjuvanted influenza virus H5N1 vaccines have shown either no improvement or only marginal improvement over nonadjuvanted formulations (89, 90). Oil-in-water-adjuvanted vaccines may be more promising, given that the MF59-adjuvanted pandemic H1N1 and H5N1 virus vaccines were able to induce higher antibody titers than nonadjuvanted formulations in naive populations (90–92). Besides boosting antibody titers, oil-in-water adjuvants have also been shown to broaden the immune recognition repertoire in H5 (92–94) and seasonal TIV vaccinees (95–97), generating cross-reactive antibodies as well as stimulating both cellular and humoral immunities. Importantly, adjuvanted vaccines induce a stronger immune response in the elderly (>65 years old) (98–100) and have double the efficacy of unadjuvanted formulations in young children (101). Although adjuvanted vaccines can be associated with more severe reactogenicity, the symptoms are still considered mild and transient (102). The MF59-adjuvanted pandemic vaccine Focetria, marketed by Novartis, was licensed for use in Europe during the 2009 pandemic, and postmarketing data confirmed its safety and tolerability (103, 104). Recent data also indicate that Focetria is safe, well tolerated, and immunogenic in infants between 6 and 23 months of age (105). However, several countries in Europe reported incidences of narcolepsy associated with the AS03-adjuvanted pandemic vaccine Pandemrix (GlaxoSmithKline), but it is not clear whether this effect is due to the vaccine itself or infection with the pandemic strain (106, 107).

Other adjuvants in experimental stages that are being tested with the aim of improving antigen delivery, processing, and recognition are compounds that target the pattern recognition receptors of immune cells (108). These adjuvants can be immunostimulatory DNA sequences (109–111) or bacterium-derived components (112, 113) and are expected to stimulate both arms of the innate and adaptive immune response. For example, Taylor et al. (112) advanced a fusion protein comprising the immunogenic domain of HA (HA1) and the bacterial protein flagellin as a vaccine to a phase II clinical study. Their vaccine was well tolerated and immunogenic in subjects >65 years old, even with just a single 5-μg dose.

Route of Vaccine Delivery

Another approach being explored to increase the immunogenicity of influenza vaccines is to differentially stimulate the immune system through alternative delivery routes. Such an approach in terms of influenza vaccine delivery is intradermal (i.d.) inoculation, with the rationale of engaging the abundant pool of professional antigen-presenting cells (APCs) in the skin. APCs are efficient at capturing and processing antigens for subsequent presentation in the lymphoid organs, resulting in stimulation of both innate and adaptive immunity (114). In some cases, i.d. vaccines performed better than the conventional intramuscular or subcutaneous vaccines in stimulating a robust immune response (reviewed in reference 115). This approach is especially relevant for the elderly, since in two head-to-head studies, i.d. vaccines were superior to i.m. vaccines in terms of immunogenicity in the elderly (>60 years old) (116, 117). Initial reports on the i.d. delivery of the influenza vaccine in healthy adults showed that it was as efficacious as i.m. vaccines in stimulating an antibody response, even with only half the standard dose of HA (118, 119). A systematic review of clinical studies with i.d. vaccines showed that most studies reported comparable immunogenicity between reduced doses of i.d. vaccines and standard i.m. vaccines (120), suggesting that i.d. vaccination could provide a dose-sparing strategy.

Traditional i.d. delivery requires skill and experience to deliver the vaccine to the right layer of the skin (dermis or epidermis). Recent developments included the use of microneedles for i.d. delivery. Microneedles are submicrometer-length structures that pierce the skin superficially for antigen delivery. There are several types of microneedle delivery systems, including array-type microneedles, coated with antigen, or a single hollow microneedle attached to an injector device that dispenses liquid antigen (121). Microneedles have shown promise in the delivery of vaccines and are reportedly easy to use, painless, and effective, even improving the immunogenicity of DNA vaccines (122). Intanza (or Fluzone-Intradermal in the United States), marketed by Sanofi Pasteur, which uses the hollow-type microneedles attached to a microinjection system with a lower dose of HA, has been licensed for use in the elderly in several countries (123–125) and in the United States since 2011 (Table 1).

High Dose of Hemagglutinin

Given the poor performance of TIVs in the elderly, Fluzone High-Dose, a new formulation of TIV that contains four times the standard dose of HA, was approved for use in the elderly in the United States in 2010. This vaccine is administered in a single dose and can induce antibody titers equal to or better than those induced by the standard TIV (126). High-dose HA was also shown to improve immunogenicity in HIV-immunocompromised patients, despite their low overall CD4 counts (127), indicating that increasing the antigenic dose can result in a correspondingly increased immune response (128). The high dose, however, may present a manufacturing burden, especially during a pandemic, when demand is anticipated to be high, and, although effective, may prove impractical outside high-risk groups.

SAME TARGET, DIFFERENT APPROACH

While the discussion above has focused on improving the influenza vaccine pipelines currently in use, such approaches are still limited by factors intrinsic to the TIV and LAIV platforms themselves. As such, it is likely that major improvements to influenza vaccines will come about through development of distinct vaccination platforms. Correspondingly, new vaccine delivery and manufacturing technologies are being investigated for use in pro-

duction of influenza vaccines. These vaccine approaches include the use of vectored or expressed HA antigens and/or targeting of different viral antigens.

Baculovirus Expression Systems

A relatively advanced technology used to manufacture influenza vaccine is the baculovirus expression vector system (BEVS). Baculoviruses are DNA viruses that infect insects, specifically of the lepidopteran species. During infection, baculoviruses make large amounts of a viral protein, polyhedrin, late in the infection cycle. As this protein is not essential for viral replication, it has been widely utilized as a foreign protein production vehicle. The BEVS relies on the generation of a recombinant baculovirus, in which the polyhedrin gene is replaced with the foreign gene of interest (for more detailed information, see reference 129). Subsequent purification by filtration and chromatographic separation can be used to isolate the protein of interest (130). One caveat to the BEVS is that the use of insect cell lines may not posttranslationally modify the expressed proteins with the same accuracy as mammalian cells, which may affect the function of the expressed protein.

The BEVS was successfully used to produce the licensed human papillomavirus vaccine (131), demonstrating its safety and feasibility for commercial vaccine production. FluBlok (Protein Sciences), a recombinant HA trivalent influenza vaccine derived from the company's proprietary insect SF9 cells (ExpresSF+), has also recently been approved by the FDA for use in persons between 18 and 49 years of age. Because it is produced in insect cells, recombinant HA is not cleaved into its two subunits (HA1 and HA2) and remains as the full-length uncleaved precursor (HA0). Since HA0 retains all the antigenic domains, it is expected that the immunization mechanism will result in immunity comparable to that induced by TIV. FluBlok, however, contains a higher dose of HA (about 3 times more) than the licensed TIV. The major advantages of the baculovirus system are the scalability, quick production, and suitability for production of vaccines against highly pathogenic influenza viruses. Clinical studies in healthy adults showed that FluBlok was well tolerated and induced better antibody responses (albeit at 3 times the antigen dose) than a licensed TIV in adults between 50 and 64 years of age (132, 133) but was not as efficacious in children younger than 5 years of age (134).

Virus-Like Particles

Virus-like particles (VLPs) are essentially "hollow-core" virus particles formed by the expression and self-assembly of only the viral structural proteins. VLPs retain the morphology and antigenicity of the whole virus, but they are replication incompetent, as they do not have a genomic component. VLPs can also activate innate immunity via pathogen recognition receptors, perhaps because they retain the structural components of the virus (135). In the case of influenza virus, VLPs are most often produced by coexpression of HA, NA, M1, and, sometimes, M2 (136, 137), although HA and NA alone may be sufficient for VLP production (138). Influenza VLPs have been produced from various expression systems, including baculovirus, vaccinia virus, DNA transfection in insects, and even plant-derived systems (reviewed in reference 139). A baculovirus-derived VLP vaccine encoding HA, NA, and M1 induced a broader immune response than a corresponding whole-virion vaccine and had a more dominant Th1 response than a recombinant HA vaccine in mice and ferrets (140).

The ease of manipulation of the VLP composition is a major advantage of the platform. Pushko et al. recently constructed multitypic VLPs incorporating 3 different subtypes. Both the pandemic-subtype VLP (incorporating H5N1, H7N2, and H2N3) and seasonal VLP (incorporating circulating strains) induced neutralizing antibodies and protected ferrets from lethal challenge (141). VLPs based on non-influenza virus proteins but incorporating influenza virus peptides have also been developed (142). VLP vaccines, although promising, are still restricted mostly to animal studies, and only two vaccines against the pandemic H1N1 and H5N1 viruses have been tested in humans. From a manufacturing perspective, VLP-based vaccines can be produced rapidly and are cost-effective, safe, and scalable. In a proof-of-concept study, Lopez-Macias described the production of a baculovirus-expressed VLP vaccine to be tested in the field within 12 weeks of the start of the 2009 pandemic (143). A phase II study in 18- to 60-year-olds showed that the vaccine induced protective titers and seroconversion at a single HA dose of 15 μg in most study participants (144). Since there was no head-to-head comparison in this particular study, further work must be conducted to assess the induced immunity compared to that induced by the standard TIV.

Virus Vector Approaches

As the protective antigens of influenza virus are well described, generation of influenza vaccines has been explored in almost all vectored viral systems. Adenovirus, poxvirus, parainfluenza virus, and alphavirus systems are all being developed as influenza vaccine vectors (145–148), although most have not advanced past preclinical studies. A major perceived advantage of some of these viral vectors is direct delivery to the mucosal site, which mimics the process of natural influenza virus infection without the presence of the actual virus. Furthermore, it is a safe approach to engineer vaccines for the highly pathogenic subtypes. In one of the few phase I studies reported, the adenovirus-vectored H5 vaccine was also immunogenic in humans when delivered by the epicutaneous route, although the potency was found to be inferior to that of intranasal delivery (149). Following on this, a larger dose-escalation phase I clinical study with intranasal delivery was initiated, but the outcome of that study has not yet been reported (http://clinicaltrials.gov/ct2/show/record/NCT00755703).

Although most of the approaches to date have used expressed HA as the primary antigen, it is possible that virus-vectored vaccines may be particularly well suited to target some of the other viral targets under development (150). Indeed, as discussed in the section on CTL vaccines, the poxvirus vector was used successfully as the delivery vehicle to induce a T-cell response to NP and M in vaccinated subjects (151). Since there are limited data on their efficacy in humans, it is difficult to ascertain if these vectored approaches would fare better than current vaccines.

DNA Vaccines

Although by no means a new technology, DNA vaccines are also being developed for the control of influenza. Many of the theoretical benefits of nucleic acid-based vaccines, such as rapid production and ease of immunogen exchange, are appealing in terms of influenza vaccination. As a consequence, many DNA vaccine formulation and delivery systems have been applied to preclinical influenza vaccines. In one example of many, mice immunized with linear expression cassettes encoding the M and NP antigens

via electroporation survived lethal challenge with a heterologous H5 strain better than did i.d. vaccinated mice (152).

Influenza virus DNA vaccines have also advanced to clinical trials. In small phase I studies, an H5 HA-based DNA vaccine was assessed in terms of immunogenicity after vaccination by different delivery routes (intramuscular and intradermal) (153). All 3 doses of the vaccine were well tolerated and immunogenic, with only minor differences between routes. In another study, H5 DNA vaccine primed for a superior response when boosted by the monovalent H5 vaccine (MIV), compared to the MIV-MIV prime-boost approach (154). Such a priming strategy appears to be a particularly strong niche for DNA vaccines and may counteract some of the issues associated with their intrinsic poor immunogenicity as a stand-alone vaccine platform. Others have looked to adjuvanted DNA vaccines to overcome immunogenicity problems. In a double-blind, placebo-controlled, phase I trial, Smith and colleagues showed that a Vaxfectin (a cationic lipid-based adjuvant)-formulated H5 HA DNA vaccine was well tolerated and provided protective responses in up to 67% of recipients, findings comparable with responses typically seen with MIV recipients (155). Promising results from clinical studies with a different adjuvanted DNA vaccine combined with a human challenge component (156) suggest that influenza virus DNA vaccines are very much still worthy of further investment and evaluation.

The most recent variation to the theme of nucleic acid-based vaccines is the mRNA-based vaccine. Compared to DNA vaccines, RNA vaccines do not require translocation to the nucleus to achieve protein expression, which in theory should increase the efficiency of antigen expression. In addition, RNA vaccines would be degraded more rapidly than DNA vaccines and, as such, pose no risk of chromosomal integration with the host genome. Petsch and colleagues showed that a stabilized mRNA vaccine formulation, delivered intradermally, was immunogenic and protective in various animal models (157).

DIFFERENT TARGET, DIFFERENT APPROACH

While many of the newer approaches for delivering influenza virus antigens can accommodate any viral protein or peptide, most proof-of-concept studies have been conducted with HA. While HA-based immunity is potent and well described, and improving its delivery an important task, a true overhaul of influenza vaccination requires a search for more conserved antigens. The discovery of a protective "universal" influenza vaccine would essentially remove many of the major issues associated with influenza vaccination. As the vaccine would not need to be updated, the vaccine could be produced year round with a timetable set by manufacturers and not the virus itself. While the search for a universal influenza vaccine is far from over, some more conserved viral epitopes have been evaluated as vaccine targets.

M2e-Based Vaccines

Perhaps the most explored universal influenza vaccine target is the extracellular domain of the M2 protein (M2e). M2e has a number of very attractive traits, including its relative conservation across viral strains and its linear peptide nature. The M2 protein is a tetrameric integral membrane protein that forms a minor component of the virus envelope. M2e is exposed on the virion surface, although its virion-associated form is poorly immunogenic (158). Slepushkin et al. (159) provided the earliest support for the protective nature of the anti-M2 antibody, showing that mice vaccinated with baculovirus-derived M2 were protected from lethal challenge with H1N1 and H3N2 subtype viruses. To improve immunogenicity, subsequent modifications to the M2-based vaccines have included addition of adjuvants and genes that target and improve immune function and expression on VLP or liposomal platforms (reviewed in reference 139). Although the exact mechanism of M2e-specific immunity is not entirely clear, M2 antibodies do not neutralize the virus, and there is some evidence that they instead function though an antibody-dependent cell cytotoxicity-dependent and/or Fc-macrophage interaction process (160, 161). While there are ample proof-of-concept studies for a protective effect of anti-M2e immunity in mice, extension of these studies to other animals, including ferrets and primates, has been disappointing (162). Nevertheless, a small phase I trial of an M2e-flagellin-conjugated vaccine has been conducted in healthy adults, with a 4-fold increase in M2e titers observed (163). A major challenge for future clinical evaluation of M2e platforms will be the determination of immune correlates of protection.

Cytotoxic T-Lymphocyte-Inducing Vaccines

As cytotoxic T lymphocytes (CTLs) have a tendency to target more conserved influenza virus proteins, there have been several approaches to increase their numbers through vaccination. Unlike antibody-mediated responses, CTL responses to influenza virus infection are heterotypic, since they are induced mainly by the internal viral proteins (26, 164, 165). Like M2 antibodies, CTL responses do not prevent initial infection but, once primed, exert their effector function on infected cells to aid in recovery and restrict disease progression. In serologically naive humans, a robust CTL response is crucial for early clearance of the virus (166). CTLs are believed to clear the virus mainly through $CD8^+$ T-cell-granule-mediated cytotoxicity (167), but $CD4^+$ T cells are important in the subsequent generation and maintenance of memory T cells (168, 169). The current rationale for using CTL vaccines is to provide baseline immunity in the population which may be protective against a newly emerging pandemic virus. While CTL immunity on its own is unlikely to prevent infection and perhaps disease, it could substantially reduce mortality. A key issue with CTL vaccines, however, is that they need to incorporate an epitope that can be recognized universally by all major histocompatibility complex (MHC) subclasses. Assarsson et al. showed that although epitope recognition varied across individuals, it is possible to identify epitope regions that are recognized by all 6 HLA supertypes, which should cover most of the major global ethnicities (170). Their study and a study by Lee et al. (171) identified highly conserved epitopes in the M1, NP, and PB1 proteins from more than 17 strains across 6 different subtypes that are targeted by $CD4^+$ and $CD8^+$ T cells.

Another factor that can affect the performance of CTL vaccines is epitope immunodominance, in which the CTL response is focused on one or a select few epitopes (172). CTLs recognize epitopes in a hierarchical manner, which is dependent on many factors (reviewed in reference 173). If the CTL vaccine epitope is immunodominant, it is likely to reduce the breadth of response in primed (i.e., vaccinated) individuals. The choice of epitope can also alter the outcome of the response, depending on the binding avidity of the T-cell antigen receptors (TCRs) with the MHC peptide (174). The mode of delivery for CTL-based vaccines must also be considered. To induce a strong $CD8^+$ T-cell response, the antigen must be processed through the MHC class I processing path-

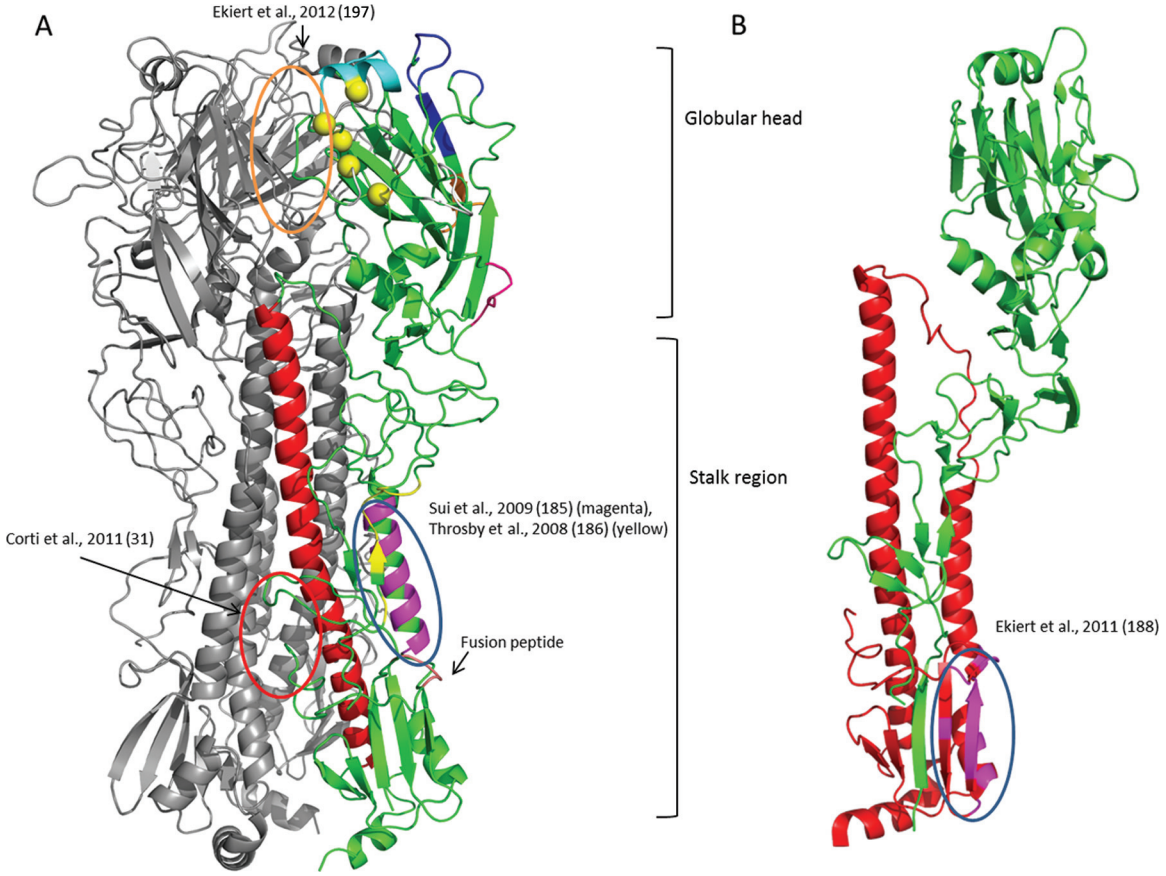

FIG 2 (A) Crystal structure of the hemagglutinin (HA) trimer. Shown in this figure is a group 1 HA (H1 subtype) (Protein Data Bank [PDB] accession number 3LGZ), and only one monomer is colored for clarity. The receptor-binding domain (yellow spheres) and the antigenic sites (for H1N1, Sa [blue], Sb [cyan], Cb [magenta[, Ca2 [white[, and Ca1 [orange]) are all located within the globular head of the protein. The stalk region, consisting of long alpha-helix loops (in red) and the fusion peptide, is more conserved across the different subtypes and plays an important role in the fusion process during virus uncoating. Epitopes/regions in the stalk region that induce broadly cross-reactive antibodies to group 1 (circled in blue) and both group 1 and 2 (circled in red) have been identified (as indicated). (B) A group 2 HA monomer (H3 subtype) (modified from data reported under PDB accession number 3DSY), with the group-reactive epitope highlighted in magenta. References are indicated in parentheses.

way of dendritic cells (DCs). During infection, the viral antigen is loaded onto DCs by direct entry of the virus or through uptake of infected cells undergoing apoptosis (175). Hence, CTL responses during vaccination differ: LAIV induces a strong $CD8^+$ T-cell response, whereas the conventional subunit TIV is less effective than the whole-virus vaccine at inducing CTLs (166). Therefore, the design and execution of a successful CTL-based vaccination approach are crucial and should incorporate all of these considerations. Strategies that have been used for antigen delivery include the use of viral vectors (176, 177), liposomes, and virosomes (178) as well as the lipid/adjuvant-immunostimulatory complex (ISCOM)-based particulate delivery platform (179, 180). Most CTL vaccines are currently designed to target conserved T-cell epitopes in the M or NP protein, and two CTL vaccines have already reached clinical testing in humans (150, 181). Preliminary data from a phase II trial based on a vaccinia virus-vectored (poxvirus) vaccine suggest higher levels of stimulation of T-cell responses and protection in vaccinees than in controls (151). Perhaps, the greatest potential for CTL-inducing influenza vaccines is as a complement to antibody-producing approaches.

Cross-Reactive HA Stalk Antibodies

The newest class of "universal" influenza vaccine in development is the cross-reactive HA stalk antibodies. The 16 subtypes of HA are classified into 2 major phylogenetic groups, group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16) and group 2 (H3, H4, H7, H10, H14, and H15), with less than 40% amino acid similarity between the groups (182–184). While it has long been known that cross-reactive antibodies that target the HA stalk can be induced, developments and the production of human monoclonal antibodies (MAbs) have rejuvenated this area. Broadly neutralizing antibodies (bnAbs) that could neutralize group 1 viruses were detected in a human antibody library (185), in immune memory B cells of vaccinees (186), as well as in survivors of H5N1 infections (187). These MAbs bound to the conserved region in the HA stalk that is crucial for membrane fusion and less sensitive to immune pressures (185). Subsequent studies identified similar bnAbs for group 2 viruses (188) and eventually both group 1 and 2 viruses (189) binding to similar but distinct domains in the stalk region (summarized in Fig. 2). It is interesting to note that these bnAbs have been identified under different circumstances: naturally oc-

curring (although with low prevalence) (190, 191), induced because of "original antigenic sin" (a phenomenon whereby the immune system preferentially reacts to epitopes that it has previously encountered at the expense of those on the immunizing antigen) (192), or after vaccination (193).

The identification of these antibodies, which are produced in only small amounts after infection, has led to attempts to induce them to protective levels through vaccination strategies. Steel et al. made a VLP-based "headless" stalk construct (194), and Bommakanti et al. made an *Escherichia coli*-expressed truncated HA2 protein (195). Both constructs protected mice from homologous lethal challenge, with the VLP-based construct showing improved breadth of reactivity against multiple group 1 subtypes (194). Stalk-specific synthetic peptides spanning the long alpha-helix region (residues 76 to 130, based on H3 numbering) also protected mice from H1, H3, and H5 virus challenge, even in passive-transfer experiments (196).

It is of note, however, that more recent publications have found cross-reactive epitopes outside the stalk region. Cross-reactive neutralizing antibodies that recognize the globular head (191) as well the receptor-binding pocket (197) have been identified, suggesting that there are regions on the HA1 domain that can be targeted for a universal vaccine. The identification of conserved domains across all influenza virus subtypes provides an exciting prospect for the development of a universal vaccine; the challenge remains in designing a vaccine that can induce these broadly reactive antibodies to sufficiently protective levels in humans.

PERSPECTIVES

Although recent meta-analyses have questioned the efficacy of the current TIV in some populations and influenza seasons (60), the collaborative mechanism that is required for its yearly production has been refined over decades. Future improvement will require novel approaches to both vaccine formulation and manufacturing processes. Although fostering of such approaches is a worthy public health endeavor, transformative changes to the system and tipping of the virus-vaccine battle firmly in favor of the vaccine will require the identification of more conserved and protective vaccine antigens. The most promising approaches currently in development are arguably those targeting conserved HA epitopes. The unknowns in this approach are whether the antibodies under investigation will be potent enough for a seasonal vaccine and, even if so, whether their levels can be induced to substantial degrees. The coming years should answer these questions and see other novel approaches trialed, with outcomes that can hopefully improve on the current influenza vaccine platform.

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