Toward a Universal Influenza Virus Vaccine: Prospects and Challenges

Natalie Pica[1] and Peter Palese[1,2]

[1]Department of Microbiology and [2]Department of Medicine, Mount Sinai School of Medicine, New York, New York 10029; email: peter.palese@mssm.edu

Annu. Rev. Med. 2013. 64:189–202

The *Annual Review of Medicine* is online at med.annualreviews.org

This article's doi: 10.1146/annurev-med-120611-145115

Keywords

hemagglutinin, stalk antibodies, chimeric influenza viruses, DNA vaccines, heterologous protection

Abstract

Current influenza virus vaccines are annually reformulated to elicit protection by generating an immune response toward the virus strains that are predicted to circulate in the upcoming influenza season. These vaccines provide limited protection in cases of antigenic mismatch, when the vaccine and the circulating viral strains differ. The emergence of unexpected pandemic viruses presents an additional challenge to vaccine production. To increase influenza virus preparedness, much work has been dedicated to the development of a universal vaccine. Focusing on regions of viral proteins that are highly conserved across virus subtypes, vaccine strategies involving the matrix 2 protein, stalk domain of the hemagglutinin, and multivalent approaches have provided broad-based protection in animal models and show much promise. This review summarizes the most encouraging advances in the field with a focus on novel vaccine designs that have yielded promising preclinical and clinical data.

INTRODUCTION

Hemagglutinin (HA): a glycoprotein that mediates virus attachment and entry into host cells. There are 17 HA subtypes

Influenza viruses are members of the family *Orthomyxoviridae* and can be classified as A, B, or C viruses (1). Influenza A viruses can be further divided into two main phylogenetic groups based on hemagglutinin (HA) subtype: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, and H17) and group 2 (H3, H4, H7, H10, H14, and H15). Circulating influenza B viruses are classified as either Yamagata-like or Victoria-like (**Figure 1**) (1).

Influenza Vaccines: Current Standards and Challenges

At present, influenza A viruses of the H1 or H3 subtype or influenza B viruses can cause epidemic infections in the human population. In addition, these viral strains can also cocirculate (2). As such, trivalent vaccines are annually reformulated to protect against these influenza viruses. Two types of trivalent vaccines are approved for use in the United States: a killed "split" vaccine (known as a TIV, for trivalent inactivated vaccine) and a live attenuated virus vaccine. Despite much advancement in the field of influenza virology, vaccine production in the United States relies on rather antiquated techniques that involve the growth of high-yield reassortants for influenza A strains and the passage of viral stocks in eggs (3, 4). Following propagation, viruses are inactivated and then split using nonionic detergents (4). This manufacturing process can take several months (5) and leaves the population susceptible to a pandemic when the production of new vaccine doses lags behind the spread of a virus (6). The current system also relies on the availability of eggs for expedient vaccine

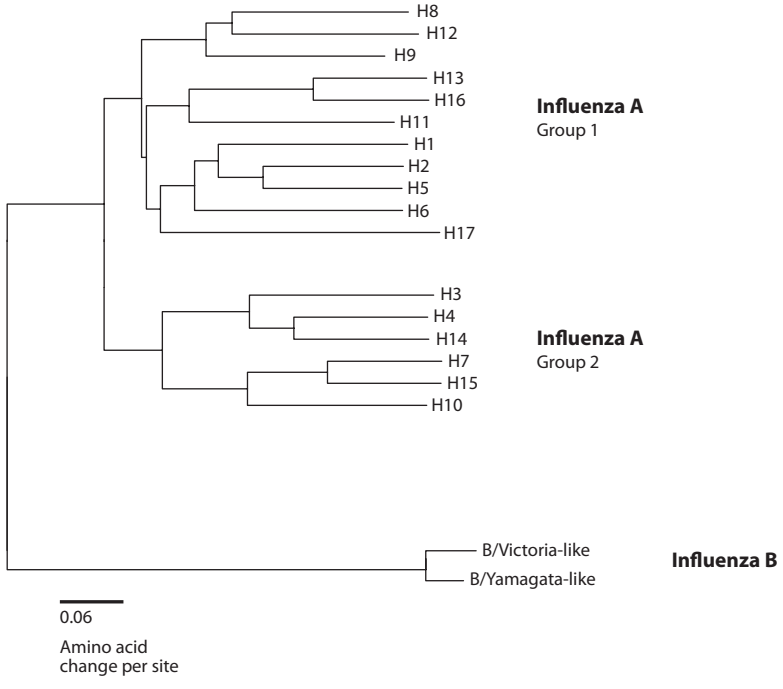

Figure 1

Phylogeny of hemagglutinin from influenza A and B viruses. Rooted phylogenetic tree is based on amino acid sequences of hemagglutinin segments from influenza A and B viruses. Representative viruses were selected from GenBank (available by request) and then aligned using ClustalW. Phylogenetic trees were constructed using FigTree software and labeled to distinguish group 1 and group 2 influenza A viruses, as well as influenza B viruses.

production and excludes the vaccination of those with egg-related allergies. Because avian virus strains can be "egg lethal," avian influenza viruses represent a challenge to current manufacturing techniques.

Commercial vaccines are designed primarily to elicit an immune response to the main antigenic driver of influenza virus, HA, a surface glycoprotein that mediates virus binding and entry into host cells (1). Antigenic areas that map near to the receptor binding site in the membrane-distal "head" portion of HA have been well defined; antibodies directed toward these areas limit the ability of the virus to bind host substrates and enter the cell. The current vaccines are designed to elicit such antibodies, whereby vaccine efficacy is often quantified through hemagglutination inhibition (HI) assays (1).

The production of a protective immune response, however, cuts both ways. Although these antibodies provide protection from infection, they also place selective pressure on the virus, driving it to evolve, a process known as antigenic drift. This mechanism, as well as antigenic shift, enables new influenza virus strains to emerge in the human population and predicates the need to design new influenza virus vaccines for each influenza season (1).

The Federal Drug Administration (FDA) specifies which strains should be incorporated into the annual vaccines for the United States (4). Although surveillance data help in selecting these viruses, it remains difficult to predict which strains will in fact circulate in the upcoming year. Rates of morbidity and mortality increase in years when the virus and the vaccine are "mismatched" (7–9) or when a pandemic virus emerges. In particular, influenza B viruses are often poorly predicted, and as such, a quadrivalent vaccine that includes viruses from the two different phylogenetic influenza B lineages has been suggested as a way to improve protection (10, 11). Overall, the efficacy of commercially available vaccines has recently become a topic of debate (12). There is thus a need for next-generation influenza virus vaccines that robustly protect against a broad spectrum of influenza virus isolates and leave us less vulnerable to the threats of epidemic and pandemic infection.

Universal Vaccination Strategies

The segmented genome of influenza virus can encode 11 viral proteins (**Table 1**) (1), providing several possible targets against which influenza vaccines could be employed. Because it is argued that the glycoproteins on the surface of the influenza virion can be readily recognized by the immune system and are critical to the virus life cycle, it is not surprising that substantial efforts have been made to target these proteins. Multivalent approaches have also shown promise in the development of universal influenza virus vaccines. In general, vaccination modalities heavily rely on the use of conserved epitopes that exist across influenza virus subtypes. Although there are many examples in the literature of heterologous or heterosubtypic protection, with the inclusion of adjuvants for example, this review focuses on rational universal vaccine design. Here we review the most promising preclinical studies as well as approaches that are in clinical trials in humans.

M2e-BASED VACCINES

The Matrix (M) gene encodes two influenza virus proteins, M1 and M2 (**Table 1**). The M2 protein of influenza A virus is a transmembrane, homotetrameric proton ion channel involved in virus uncoating following entry (1). An 18–24-amino-acid region of the protein that extends outwardly from the surface of the virion is known to be highly conserved among human influenza viruses (13, 14), and as such has been targeted in universal vaccine design. The potential of M2-based protection was first demonstrated using 14C2, a monoclonal antibody (mAb) directed against the extracellular domain of the protein (M2e). Although 14C2 is non-neutralizing and only reduces plaque size formation in vitro (15), passive transfer of this antibody in an ascites preparation

Hemagglutination inhibition (HI) assay: measures the degree to which monoclonal antibodies or polyclonal sera can inhibit hemagglutinin binding to host sialylated receptors

Antigenic drift: changes in influenza A and B viruses that involve the accumulation of point mutations within the antibody-binding sites of the hemagglutinin (and, to a lesser degree, neuraminidase). The resulting viruses can escape antibody inhibition, allowing them to cause disease and spread within the population

Antigenic shift: occurs when the genomes of two or more viruses recombine to create a new virus, often resulting in the expression of a hemagglutinin (and neuraminidase) to which the human population is immunologically naïve

Table 1 Influenza A and B viral proteins and their functions

Influenza A viral gene	Protein function	Influenza B viral gene	Protein function
Hemagglutinin (HA)	Surface glycoprotein that mediates receptor binding and fusion activity	Hemagglutinin (HA)	Surface glycoprotein that mediates receptor binding and fusion activity
Matrix (M)	Matrix protein 1 (M1) is involved in viral budding M2, expressed via alternative splicing of the viral RNA, is a protein ion channel	Matrix (M)	M1 is involved in viral budding BM2 is a protein ion channel involved in assembly that is encoded by an alternate reading frame
Neuraminidase (NA)	Surface glycoprotein with neuraminidase activity—releases progeny viruses from cell surface	Neuraminidase (NA)	NA is a surface glycoprotein with neuraminidase activity—releases progeny viruses from cell surface NB is also expressed on the surface of the virus and is produced from an alternate reading frame
Nucleoprotein (NP)	RNA binding protein that binds virus genome; involved in RNA synthesis and RNA nuclear export	Nucleoprotein (NP)	RNA binding protein that binds virus genome; involved in RNA synthesis
Nonstructural (NS)	NS1 is an interferon antagonist with a variety of described functions Splice variant NEP is involved in the nuclear export of the viral genome	Nonstructural (NS)	NS1 is an interferon antagonist with a variety of described functions Splice variant NEP is involved in the nuclear export of the viral genome
Polymerase acidic (PA)	Component of the viral polymerase with endonuclease activity	Polymerase acidic (PA)	Component of the viral polymerase with endonuclease activity
Polymerase basic 1 (PB1)	Component of the viral polymerase involved in elongation PB1-F2 protein, produced by an alternate reading frame, is an interferon antagonist that has proapoptotic function	Polymerase basic 1 (PB1)	Component of the viral polymerase involved in elongation
Polymerase basic 2 (PB2)	Component of RNA polymerase that can recognize the 5′ cap from host mRNA	Polymerase basic 2 (PB2)	Component of RNA polymerase that can recognize the 5′ cap from host mRNA

reduced viral replication in the lungs of mice by 100-fold compared to controls following challenge (16). Because of the highly conserved sequence of M2e across influenza A subtypes, it was reasoned that a vaccine designed to boost immunity to this epitope could function as a universal influenza virus vaccine (14).

In first experiments, M2e from A/Puerto Rico/8/1934 virus (PR8) or a consensus M2e sequence was fused to the N terminus of hepatitis B core (HBc) antigen, a protein that had been previously used as an efficient carrier in various vaccine strategies. Mice immunized three times intraperitoneally with adjuvant or intranasally had a boost in M2 titer with each immunization and were protected from lethal challenge following infection with PR8 virus, despite significant morbidity (14). Survival was thought to be antibody dependent because naïve animals survived challenge following passive transfer of sera from vaccinated mice (14). Further experiments highlighted the role of alveolar macrophage clearance via an Fc receptor–based mechanism in the protection elicited by this vaccine protocol (17, 18).

Various strategies have been used to increase the potency of M2e-based vaccines, yielding mixed results depending on the animal model system (13, 18, 19). In phase I clinical trials, M2e-based vaccines have been shown to be safe

and immunogenic in humans (18), although efficacy has yet to be determined.

It is possible that vaccination schemes that involve M2 could also be used to increase the breadth of influenza virus vaccination. For example, a single intranasal administration of 10^{10} recombinant adenovirus particles expressing a consensus M2 sequence with nucleoprotein (NP) completely protected mice from death following lethal challenge with H1, H3, and H5 viruses (20). A study in mice has shown that M2-based baculovirus-expressed virus-like particles, when administered with inactivated whole virus vaccines, induces an immune response that protects against heterosubtypic viral challenge (21).

Despite the potential of an M2-based vaccine strategy, the use of M2e as a universal influenza virus vaccine antigen is likely specific for influenza A viruses. The ectodomain of the influenza B virus M2 protein (BM2) is structurally different from that of influenza A M2 proteins, with a small ectodomain (5–6 amino acids) that may be difficult for immune cells to access (22).

HEMAGGLUTININ-BASED VACCINES

The HA molecule is an attractive target for a universal influenza virus vaccine. Relative to the other glycoproteins on the surface of the virus, HA is large and readily accessible, mediating virus binding to host targets as well as entry. During viral replication, HA is transcribed and translated, and then undergoes post-translational modifications in the endoplasmic reticulum (ER) and Golgi. Once it is exported to the surface of the cell, it is cleaved from HA0 into HA1 and HA2, which are linked by a disulfide bond. These modifications are required for proper HA activity (1). Functionally, HA can be divided into a head domain, composed mainly of HA1, and a stalk domain, composed of a portion of HA1 and all of HA2 (**Figure 2**). Unlike the head, the stalk has been shown to have a high degree of sequence conservation (23–25). Various strategies have taken advantage of this feature, driving the immune response toward this conserved region in the development of a universal influenza vaccine. Indeed, the identification of broadly reactive stalk-specific mAbs that are prophylactically and therapeutically effective (26–33) predicts the efficacy of such strategies.

LD90: the amount of virus that causes death in 90% of infected animals

Protection Based on Single Epitopes

Because the HA0 cleavage site is highly conserved among group 1 and group 2 influenza A viruses, and among both antigenic lineages of influenza B viruses (22), it was reasoned that an immunogenic response to this region of HA could provide universal influenza virus protection. As a proof of principle, a consensus sequence of 19 amino acids was generated based on Yamagata-like and Victoria-like influenza B viruses. This peptide was linked to the outer membrane protein (OMP) of *Neisseria meningitides* and this complex was administered thrice with adjuvant. Although morbidity was seen, vaccinated mice were completely protected from death following challenge with 1 LD90 of mouse-adapted B/Ann Arbor/4/1955, B/Hong Kong/330/2001, and B/Yamanashi/166/1998 viruses. Passive transfer experiments protected naïve mice from lethal challenge, implicating a humoral response in the protection seen. Further experiments in Fcγ-receptor knockout mice suggested that antibody-dependent cell cytotoxicity or macrophage phagocytosis might be involved in viral clearance. Impressively, when an H3 consensus HA0 sequence was linked to an OMPC carrier and delivered with adjuvant, mice were partially protected from challenge with group 1 and group 2 influenza A viruses, as well as lethal influenza B virus infection (22).

Although vaccination based on the HA0 sequence has shown impressive results, follow-up studies are warranted. HA0 cleavage of avian influenza viruses occurs in the Golgi, so it is possible that this vaccination strategy would not protect against avian strains, as HA would already exist in its cleaved form on the surface of

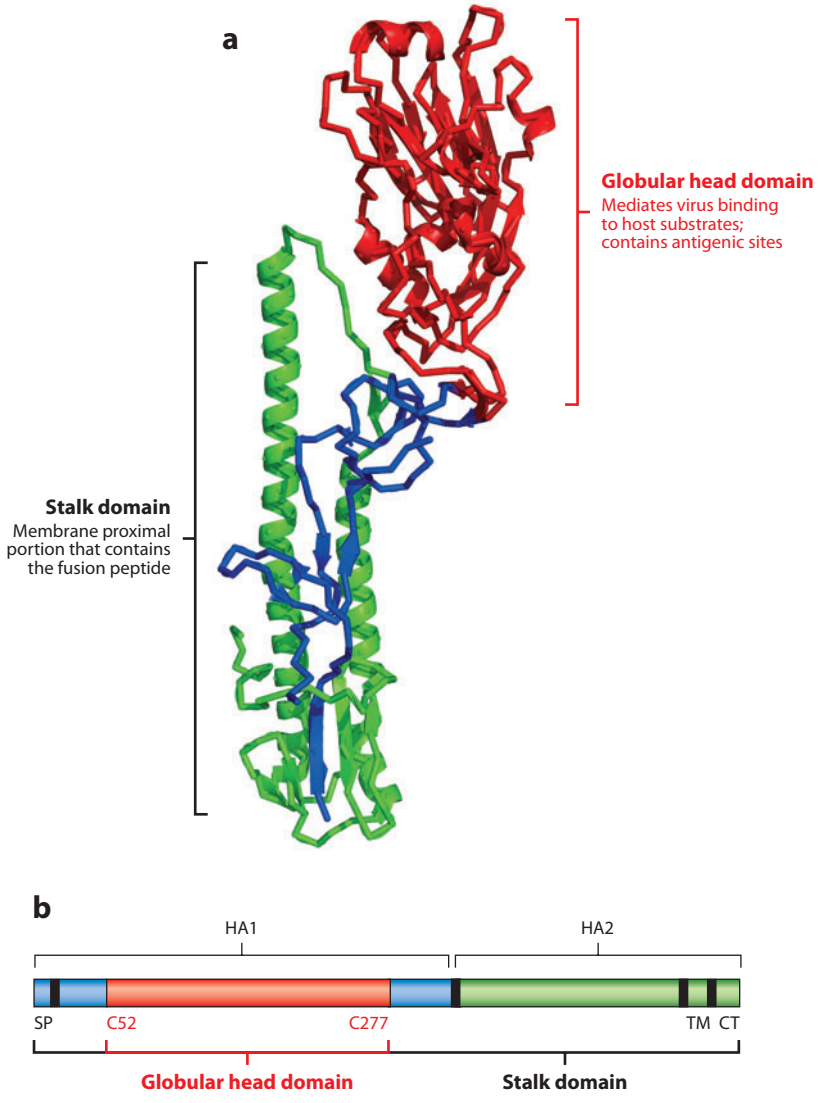

Figure 2

Ribbon diagram and schematic representation of hemagglutinin monomer (H1 subtype). (*a*) The HA1 subunit is depicted in red (globular head domain) and blue (stalk domain), and the HA2 subunit in green. The head domain (red) contains the receptor-binding site and the five predicted antigenic sites; the fusion peptide is located within the HA2 portion of the stalk (green). The HA structure was downloaded from the Protein Database [A/Puerto Rico/8/34 (H1N1) HA (PDB ID 1RVX)], and the final image was generated with PyMol (Delano Scientific), courtesy of Irina Margine. (*b*) Schematic of the hemagglutinin monomer. The globular head domain is shown to be the intervening sequence from cysteine 52 to cysteine 277 (H3 numbering), with the rest of HA1 and HA2 comprising the stalk domain. SP, signal peptide; TM, transmembrane domain; CT, cytoplasmic domain.

the infected cell (34). It is also possible that once immune pressure is placed on the virus at this site, mutations could arise that allow the virus to evade this vaccination scheme. This vulnerability could be avoided by designing immunogens based on multiple epitopes.

Protection Based on Conserved Domains

Several universal vaccine approaches involve large portions of the HA molecule rather than a single epitope. These strategies have been driven, at least in part, by the identification of neutralizing HA antibodies that are reactive to multiple subtypes. With few exceptions, many of the mAbs that have been identified to date react to epitopes within the stalk domain of the HA molecule (26–33). As such, various strategies to elicit antibodies with similar specificities have been employed.

Wang et al. have demonstrated a proof of concept of such an approach. The binding specificity of a mAb reactive to group 2 HAs, 12D1 (27), was localized to a portion of the long alpha helix (LAH) of the HA molecule. It was therefore hypothesized that vaccination with the entirety of the LAH would elicit an antibody response that would be reactive across HA subtypes. Mice primed and boosted with an LAH construct from A/Hong Kong/1/1968 (H3N2) virus linked to the carrier protein keyhole limpet hemocyanin in the presence of adjuvant demonstrated partial protection when challenged with an H5 virus. Although animals were not protected from H1 challenge, death was delayed (35). Bommakanti and colleagues have generated immunogens based on the HA2 portion of the viral HA that protect mice from homologous and heterologous challenges (36).

In the hopes of broadening the response to vaccination, attempts have been made to generate vaccine responses to the entire HA stalk. It is hypothesized that the immunodominance of the head of the HA molecule limits the potential efficacy of such a technique, and "headless" HA constructs have been developed. In a study by Sagawa et al., mice were vaccinated with cells transfected either with a full-length HA from A/Okuda/1957 (H2N2) virus or with a mutant that did not express a large portion of the globular head domain. Following a boost, animals were challenged with A/Fort Monmouth/1/1947 (H1N1) virus. Only one animal vaccinated with transfected cells expressing the full-length HA survived challenge; in contrast, 70% survival was seen in animals vaccinated with the headless construct. The mechanism of protection was not definitively demonstrated, but survival decreased to 30% when animals were vaccinated with a heat-treated version of headless transfected cells, suggesting that conformational epitopes are likely important for protection (37).

Steel et al. (38) designed a different headless construct. They selected a disulfide bond at C52 and C277 (see **Figure 2*b***) as the demarcation point between the head and stalk domains of HA—the intervening sequence that encodes the head domain was replaced with a flexible glycine linker. Mice were primed and boosted with DNA encoding the headless construct, and then boosted again with gag-driven virus-like particles expressing the headless HA with Freund's adjuvant. Mice were protected from homologous challenge compared to controls and generated antibody responses that were cross reactive to various HA subtypes within group 1.

Another approach attempts to immunize with the entire HA molecule but uses a "centralized" or consensus sequence that mitigates some of the sequence diversity between strains, particularly in the globular head domain. When this sequence was administered using an adenovirus vector, mice were completely protected from stringent PR8 virus challenge and partially protected from FM1 or mouse-adapted A/California/04/2009 virus challenge (39). A similar vaccine strategy has been employed for H5 subtype viruses (40). Although this approach might be applicable for protection against intrasubtype viruses, the high degree of sequence divergence across subtypes might be too great to overcome. A centralized sequence that reflects H1, H2, H3, H4, and H5

sequences, for example, was not protective in the context of H1N1 challenge (39).

Impressive preclinical results have been demonstrated by Wei et al., showing that the combination of DNA primes followed by a protein boost can elicit broad humoral immune responses to a variety of HA subtypes (41). In this study, mice were vaccinated three times with plasmids expressing A/New Caledonia/200/1999 and then boosted with a TIV preparation that contains the same HA, or vaccinated with DNA or TIV alone, and then challenged with drifted virus A/Puerto Rico/8/1934. Vaccination with DNA or TIV alone did not provide protection from challenge; in contrast, animals primed with DNA and boosted with TIV had an overall survival rate of 80%, although they lost ~15% of their body weight. Using a similar immunization strategy, the authors showed decreased nasal wash titers in ferrets on day 5 when challenged with PR8 or A/Brisbane/59/2007 virus, compared to animals vaccinated with control DNA. The authors extended their work by vaccinating nonhuman primates and demonstrated the broadly reactive nature of their sera against a variety of pseudotyped viruses (41).

Clinical trials to assess the safety and immunogenicity of a DNA/protein boost strategy have been conducted in a human cohort. Subjects were immunized with one or two doses of DNA encoding an H5 HA followed by administration of a monovalent inactivated H5 vaccine. This immunization strategy was safe and in some individuals produced serum antibody responses that neutralized H5 and H9 pseudoparticles (42).

Novel Reagents to Directly Detect Stalk-specific Antibodies in Polyclonal Serum

Wei et al. (41) suggested that a stalk-directed antibody response accounts for the protection elicited by their vaccination strategy using ΔStem HA. This mutant encodes two additional glycosylation sites that abrogate binding of well-defined stalk-specific mAbs. By pseudoparticle assay and by ELISA, the authors demonstrated a loss of binding to diverse HA subtypes when sera from mice, ferrets, and monkeys are preabsorbed with wildtype HA but not when the ΔStem HA mutant is used (41). Other groups have used similar techniques, including competition ELISAs using broadly reactive mAbs, in order to indirectly assess the proportion of stalk antibodies in polyclonal sera (43). In the clinical trial described above, the stalk-specific response of vaccinees was assessed by both methods (42).

A more specific technique has been developed to directly assess stalk-specific antibodies in sera. Building on earlier observations that antigenic sites can be exchanged between virus subtypes (44), Hai and colleagues engineered chimeric hemagglutinin (cHA) constructs in which the entire head domain is replaced with that from another virus subtype (23, 45). By creating cHAs that express exotic globular head domains—heads from viruses that the human population has not been, on the whole, previously exposed to—it is possible to directly assess the presence of antibodies specific to the HA stalk.

For this purpose, cHAs that expresses the head of an H9 (cH9/1) or an H6 (cH6/1) virus atop the stalk of an H1 virus were generated. Using these reagents, it was demonstrated that a cohort of individuals who had been infected with the 2009 pandemic H1N1 (pH1N1) swine virus had higher titers of stalk-specific antibodies compared to noninfected adult and pediatric controls (all individual sera were HI negative to H9 and H6 HAs) (45). These antibodies were neutralizing, as demonstrated by plaque reduction assay using a virus expressing the cH9/1 HA with an N3 neuraminidase. Unlike previously published techniques (41–43), assays involving cHAs enable direct detection and quantification of stalk-reactive antibodies with neutralizing capabilities in polyclonal sera and are therefore an important contribution to the development and assessment of any stalk-centric vaccination technique.

Induction of Stalk Antibodies Using Chimeric Hemagglutinins

The introduction of a new pandemic strain to which humans are immunologically naïve can result in rapid transmission of the virus, as well as higher rates of morbidity and mortality compared to those seen during annual epidemics caused by seasonal influenza viruses (2). Interestingly, the introduction of a pandemic virus also coincides with the disappearance of the previously circulating seasonal influenza strain from the human reservoir. We have hypothesized that exposure to the new pandemic virus contributes to the production of antihemagglutinin stalk antibodies, and, on the population level, results in the extinction of the seasonal strains (46). In support of this hypothesis, antibodies with stalk specificities have been isolated from individuals infected with the 2009 pandemic influenza virus strain (47). In addition, stalk-specific antibodies have been detected in the polyclonal serum of pH1N1 infected patients (45). cHA constructs, those with exotic heads atop stalks from viruses that circulate in the human population, could therefore be an invaluable tool for the induction of HA stalk antibodies, mimicking what occurs when pandemic viruses are introduced into humans. In mice, we have shown that immunization with different cHA constructs elicits a protective stalk-specific antibody response (N. Pica, F. Krammer, P. Palese, unpublished data). Further development and evaluation of these constructs as vaccines could therefore be a monumental step toward universal influenza vaccine development.

NEURAMINIDASE-BASED VACCINES: UNTAPPED POTENTIAL?

Although not neutralizing, passive transfer of neuraminidase (NA)-specific antibodies has a protective effect in humans and in animal models of influenza virus infection. In human studies, serum NA antibody titers have been inversely correlated with severity of infection with H3N2 virus in subjects seronegative for H3-specific antibodies but previously exposed to N2 viruses. NA antibody titer was also inversely correlated with degree of viral shedding (48). Findings were similar in a mouse study that demonstrated the protective nature of NAs of the same subtype in reducing lung titers following challenge (49). Passive transfer of NA antibodies has also been shown to reduce lung virus titers in mice following infection (50, 51).

Like M2 and HA, antibodies that bind to conserved regions of NA have been produced, although by an alternative method. Using alignments of multiple NA sequences, it was hypothesized that antibodies directed toward regions with high amino acid conservation would be cross reactive to NAs of various subtypes. Antibodies raised against synthesized peptides based on two such regions were shown to be cross reactive by Western blot to representative NAs from the nine subtypes (52). Although the in vivo efficacy of such antibodies has not yet been investigated, it is possible that NA-based universal vaccine approaches have potential.

Neuraminidase (NA): a glycoprotein that mediates the release of newly formed virion from the cell surface. There are nine well-defined NA subtypes

T CELL–BASED VACCINES AND OTHER APPROACHES

Following infection, influenza virus–specific CD4 T helper cells as well as CD8 cytotoxic T cells (CTLs) are activated, specifically through the recognition of epitopes that are highly conserved across influenza virus subtypes such as those encoded by NP and M1 (53, 54). Stimulation of T cell–based immunity has been demonstrated in a variety of different contexts, including natural infection as well as DNA and vectored vaccines expressing conserved epitopes (55). Experimentally, CD8 and CD4 T cells have been shown to mediate heterosubtypic protection in animal models (well reviewed in Reference 55). In addition, CD8 T cells have been shown to contribute to the clearance of influenza virus in infected individuals (56). Thus, the stimulation of T cell–based immunity has been another strategy deployed in the fight against emerging influenza virus strains. Although the diverse expression and prevalence of HLA class I and II

HLA supertype: an organization or clustering of human leukocyte antigen (HLA) molecules with overlapping binding specificities

molecules in the human population present an obstacle for the feasibility of such a technique, the identification of conserved peptide regions that bind to multiple HLA supertypes makes the strategy theoretically feasible (57, 58).

Recent clinical trials using the replication-defective vaccinia Ankara strain expressing M1 and NP from A/Panama/2007/1999 virus (H3N2) (MVA-NP+M1) have suggested the possible efficacy of such a vaccine strategy. Following preclinical studies that demonstrated the safety and immunogenicity of such a vaccine (59), individuals were immunized with MVA-NP+M1 or unimmunized prior to challenge with A/Wisconsin/67/2005 virus (H3N2). Vaccinated individuals demonstrated increased influenza-specific CTL responses compared to controls. Of the people who were infected following challenge, those vaccinated with MVA-NP+M1 shed virus for fewer days than controls who did not receive a vaccine. In this study, few subjects were enrolled and even fewer in both the vaccinated and control groups became infected following challenge, rendering it difficult to assess the ability of the vaccine to prevent infection (60). Further validation with a larger number of subjects is warranted in order to assess the efficacy of this vaccine.

Other groups have developed vaccines that seek to broaden the protection elicited through vaccination using both B and T cell epitopes. Earlier work had shown that using both types of epitopes confers greater protection than either epitope administered singly (61), so conserved regions from HA and NP were recombinantly expressed linked to flagellin and administered intranasally to humanized mice. The epitopes used were restricted to HLA subtypes that are prevalent in the human population in an attempt to gauge the feasibility of such a vaccine strategy in humans. Compared to animals receiving flagellin alone, vaccinated mice had reduced viral lung titers following challenge with sublethal doses of influenza virus. All immunized mice survived lethal challenge with A/Texas/1/1977 (H1N1) virus, in contrast to 50% of those that received flagellin alone (62). It is of note that animals were challenged seven days after vaccination in this experiment, and as such, innate responses could be contributing to the protection seen here, as demonstrated by the moderate protection levels seen in the flagellin-only control group. A similar experiment was performed by immunizing animals three times with a mixture of six influenza virus peptides linked to flagellin, either intranasally or intramuscularly. Four weeks after the final immunization, animals were challenged with an H5N1 virus. Although statistically different survival rates were seen in treatment groups compared to phosphate-buffered saline-administered controls, the virus challenge was relatively weak ($<1LD_{90}$) (63).

This vaccine approach has also been tested in humans in a single-blinded clinical study. Using purified preparations from a bacterial expression system, nine highly conserved, linear B and T cell epitopes from HA, NP, and M1 were synthesized as a single recombinant protein. When Multimeric-001 was administered twice intramuscularly to individuals aged 18–49, patients experienced only mild side effects to vaccination with or without adjuvant. Correlates of protection such as seroconversion, complement-mediated cell lysis, and T cell proliferation assays demonstrated the immunogenicity of the vaccine; however, more robust responses were typically appreciated when the vaccine was adjuvanted (64). Continued work will be necessary to fully evaluate the protective efficacy of such a vaccine.

Other NP-based approaches, including those that aim to stimulate humoral responses to NP (65–70), have been efficacious in reducing the burden of heterologous or heterosubtypic influenza virus infection in preclinical animal models, although the applicability of such techniques to human vaccination has not yet been determined.

CONCLUSIONS

Despite our arsenal of influenza virus vaccines and antivirals, morbidity and mortality caused by influenza virus remain high (71, 72). There is therefore a need to engineer next-generation

vaccines that provide broad-based, universal protection against influenza virus infection. Much progress has been made toward the development of such vaccines, but further work in animal models, as well as subsequent validation in humans, is warranted. Encouraging results with vaccination strategies that boost regions conserved across influenza virus strains, such as the HA stalk, suggest that universal influenza virus vaccination may be within our reach.

DISCLOSURE STATEMENT

Mount Sinai School of Medicine has filed patents for the use of hemagglutinin-based constructs as universal influenza virus vaccines. P. Palese currently receives grant and financial support to study universal influenza virus vaccines from the NIH and PATH (Seattle).

ACKNOWLEDGMENTS

Partial support for this work was provided by PATH and by the National Institute for Allergy and Infectious Diseases–funded Center for Research on Influenza Pathogenesis (CRIP) (HHSN266200700010C).

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