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Novel vaccines against influenza viruses

Sang-Moo Kang^{1,*}, Jae-Min Song², and Richard W. Compans^{2,*}

¹Center for Inflammation, Immunity & Infection, and Department of Biology, Georgia State University, Atlanta, Georgia 30303

²Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia, 30322

Abstract

Killed and live attenuated influenza virus vaccines are effective in preventing and curbing the spread of influenza epidemics when the strains present in the vaccines are closely matched with the predicted epidemic strains. These vaccines are primarily targeted to induce immunity to the variable major target antigen, hemagglutinin (HA) of influenza virus. However, current vaccines are not effective in preventing the emergence of new pandemic or highly virulent viruses. New approaches are being investigated to develop universal influenza virus vaccines as well as to apply more effective vaccine delivery methods. Conserved vaccine targets including the influenza M2 ion channel protein and HA stalk domains are being developed using recombinant technologies to improve the level of cross protection. In addition, recent studies provide evidence that vaccine supplements can provide avenues to further improve current vaccination.

1. Introduction

Epidemics and pandemics of respiratory disease have been recorded since the 16th century. Isolation of the first human influenza A virus in 1933 resulted in the identification of this virus as the cause of previous epidemics and pandemics of respiratory disease, as well as the development of influenza vaccines in the 1930s and 1940s (Davenport, 1962; Stokes et al., 1937). Influenza virus infections can occur in all age groups during seasonal epidemics. The resulting illness substantially contributes to losses in work and school time, increases in influenza-related hospitalizations, and deaths (Glaser et al., 2002; Poehling et al., 2006; Thompson et al., 2004; Thompson et al., 2003). In a typical year, influenza virus afflicts 10%–20% of the United States (US) population, causing an average of 200,000 hospitalizations and 40,000 deaths (Thompson et al., 2004). The greatest disease severity occurs in the elderly, infants, and young children, and persons with underlying chronic diseases.

Influenza is a lipid-enveloped virus with a segmented negative sense RNA genome, which belongs to the family *Orthomyxoviridae*. The envelope of the virion contains two types of surface glycoproteins, which play essential roles in viral infection. The hemagglutinin (HA)

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*Corresponding authors: Center for Inflammation, Immunity & Infection (S.M.K.), Department of Biology, Georgia State University, 100 Piedmont Avenue, 7th Floor 718, Atlanta, Georgia 30303. Phone: 404-413-3588. Fax: 404-413-3580; skang24@gsu.edu. Department of Microbiology and Immunology (R.W.C), Emory University School of Medicine, 1510 Clifton Rd, Atlanta, GA 30322. Phone: 404-727-2015; Fax, 404-727-3295; rcompan@emory.edu.

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is responsible for attachment of the virus to sialic acid-containing receptors and viral entry by membrane fusion, whereas the neuraminidase (NA) is a receptor-destroying enzyme which plays important roles in viral release and cell-to-cell spread (Matrosovich et al., 2004; Palese and Compans, 1976). There are three distinct antigenic types of virus, designated A, B, and C, with types A and B playing the major role in human infection. Influenza A viruses occur in birds, humans, horses and other species, whereas types B and C are primarily found in man. Human influenza viruses are continuously evolving, resulting in variants with surface glycoproteins that have distinct antigenic properties. Most commonly, these changes result from point mutations in the viral genome RNA, and are responsible for emergence of new strains responsible for seasonal epidemics that occur with both influenza A and B viruses. Less frequently, influenza A viruses appear with novel HA proteins that are completely unrelated to pre-existing human strains with respect to antigenic properties, as a result of introduction of new HA and/or NA genes from other species. These “major antigenic shifts” result in novel antigenic subtypes of the HA and/or NA glycoproteins that had not previously infected most of the human population, and therefore can spread rapidly causing global disease pandemics. Three global pandemics of influenza occurred during the 20th century, and were caused by H1N1 subtype viruses in 1918, H2N2 viruses in 1957, and H3N2 viruses in 1968. In addition to the circulating human influenza subtypes, other avian origin influenza viruses including H5N1, H7N2, H7N3, H7N7 and H9N2 subtypes have been shown to cause human infections on multiple occasions (Cheung et al., 2006; de Jong et al., 2005; Fouchier et al., 2004; Le et al., 2005; Peiris et al., 1999; Wong and Yuen, 2006). The emergence or re-emergence of highly pathogenic avian influenza H5N1 viruses in domestic poultry and the increasing numbers of direct transmission of avian viruses to humans underscore a persistent threat to public health (Claas et al., 1998; Subbarao et al., 1998). Most recently, the 2009 outbreak of a new H1N1 virus illustrates how fast a new pandemic virus can spread in the human population once it acquires the ability to transmit among humans (Nava et al., 2009; Soloviyov et al., 2009). Indeed, the recent experience with the 2009 H1N1 virus demonstrates the need to develop novel vaccines as well as improved methods of immunization as the presently used vaccination programs showed a significant delay in controlling the spread of new pandemics. In this review, we briefly describe current influenza vaccines and then discuss novel approaches to develop improved cross protection by employing conserved targets such as the M2 and HA stalk domains as well as supplemented vaccination.

2. Influenza vaccines

The first efforts to develop influenza vaccines were initiated soon after influenza A and B viruses were identified as the etiologic agents of clinical influenza. The first commercial vaccines using whole-inactivated influenza virus were approved for use in the United States in 1945 (Francis et al., 1946; Salk et al., 1945). Because of the devastation caused in both military and civilian populations by the 1918–1919 influenza pandemic, development of an influenza vaccine was a high priority to the U.S. military during World War II.

Inactivated influenza A and B virus vaccines have been extensively used in humans. The vaccines consist of purified virus that has been chemically inactivated with formalin or β -propiolactone, and in most vaccines the virus is also detergent-treated to produce soluble forms of the viral surface antigens. Influenza epidemics in human population currently contain two influenza A subtypes (H1N1 and H3N2) and one variant of influenza B virus. Thus, the composition of the trivalent vaccine is determined based on the strains of virus that are expected to be circulating in the human population. The development of ‘high-growth’ influenza A viruses suitable for maximal replication in eggs has helped to increase vaccine production. The influenza A subtypes are adapted to growth in embryonated eggs, or may be reassortant viruses containing HA and neuraminidase (NA) of strains needed for

vaccination and other remaining genes (PB1, PB2, PA, NP, M1-M2, NS) which encode the internal proteins from A/Puerto Rico/8/34 (PR8) (H1N1) virus which confer high growth capacity in eggs (Robertson et al., 1992). Influenza virus A/PR/8/34 (PR8) has been used to develop influenza A virus reassortants containing the HA and NA from wild-type virus strains selected for vaccine production (Baez et al., 1980; Kilbourne and Murphy, 1960). The growth characteristics of such reassortants vary because the HA and NA also affect the adaptation and replication capabilities of the viruses in growth substrates. Therefore, there might be instances in which it is difficult to obtain high growth reassortants using classical methods to reassort wild-type viruses with PR8 due to unknown effects of HA and NA.

Since the recognition that dissolution of the lipid envelope allows retention of immunogenicity with reduction in reactogenicity, detergent mediated disruption ('splitting') of influenza viruses to produce subvirion preparations has been most commonly used in recent vaccines. Although whole-virus vaccines are still in use in some countries and are highly effective, most vaccines manufactured since the 1970s have been 'split' preparations. An intact viral membrane is essential for infectivity of enveloped viruses. Therefore, disruption of the viral envelope adds further assurance of viral inactivation. These split vaccines retain the immunogenic properties of the viral proteins but they have lower reactogenicity than whole-virion vaccines (al-Mazrou et al., 1991; Cate et al., 1977; Quinnan et al., 1983; Wright et al., 1983). Additional purification steps can be taken to reduce the amounts of viral non-membrane proteins, resulting in vaccines referred to as subunit or purified surface antigen preparations (Bautista Rentero et al., 1995; Jennings et al., 1984; Klenk and Stickl, 1981).

Annual surveillance data of epidemics in worldwide populations are used to recommend the strains for vaccine production in the coming winter season. Several factors can affect the efficacy of influenza vaccines. One major factor is the antigenic similarity between circulating strains and vaccine strains. In years with a suboptimal match, vaccine benefit is likely to be lower. The immune responses to the HA protein determine the efficacy of the inactivated vaccines. A dose of 15 µg of HA is generally sufficient to induce an acceptable level of antibody in adults who were previously exposed to an influenza virus within the same subtype. In contrast, higher doses of vaccine are needed to produce a protective level of antibody in unprimed individuals, and two doses have been used for primary immunization of children (Mostow et al., 1970; Palache et al., 1993). The levels of antibody induced by the inactivated influenza vaccine have been observed to decrease by 75% over an 8-month period (Ohmit et al., 2008; Wright and Webster, 2001).

The variable efficacy of the inactivated vaccine, short duration of protective immunity, and failure to induce local or cellular immunity have stimulated additional research. As an alternative approach to influenza immunization, live attenuated influenza virus (LAIV) vaccines administered by nasal spray (FluMist®) have been successfully developed. The vaccine is trivalent, containing influenza virus reassortants of the strains recommended for the current season. These strains are cold-adapted (*ca*) (i.e., they replicate efficiently at 25°C, a temperature that is restrictive for replication of most wild-type viruses); temperature-sensitive (*ts*) (i.e., they are restricted in replication at 37°C or 39°C, temperatures at which many wild-type influenza viruses grow efficiently); and are attenuated (*att*) so as not to produce influenza-like illness. Each of the three strains is a reassortant of internal proteins of a master donor virus (MDV) and surface proteins (HA, NA) of a wild-type influenza virus. The MDVs (A/Ann Arbor/6/60 and B/Ann Arbor/1/66) were developed by serial passage at sequentially lower temperatures (Murphy and Coelingh, 2002). During this process, the MDVs acquired the *ca*, *ts* and *att* phenotypes as a result of multiple mutations in the gene segments that encode internal viral proteins. LAIV has the potential to significantly contribute to the control of influenza and influenza-associated

illnesses. The efficacy of LAIV is relatively high in children compared to the inactivated vaccines (Belshe et al., 2008; Harper et al., 2004). Intranasal delivery of LAIV elicits both serum IgG and mucosal IgA antibodies (Belshe et al., 2000). Protection would be synergic since both types of antibodies are protective. In addition, cellular immune responses induced by replicating vaccine virus may also contribute to protection against clinical symptoms (Forrest et al., 2008). However, it is less effective in adults, and is not approved for use in persons over the age of 50 (Belshe et al., 2008; Block et al., 2008).

Subunit vaccines alone may have some limitation in providing effective immunity. They are less immunogenic compared to the particulate vaccines such as whole inactivated virus, and thus require adjuvants or high vaccine doses. Supplementing a subunit vaccine by an immunogenic component might offer an attractive approach in improving the efficacy of subunit based vaccines.

3. Development of Universal Influenza Vaccines

A limitation of current vaccines is that the antigenic regions of HA are highly susceptible to continuous mutation in circulating epidemic virus strains (Bush et al., 1999; Plotkin and Dushoff, 2003). Most common changes result from point mutations in the viral genome RNA, and are responsible for seasonal epidemics. Less frequently, influenza A viruses occur with novel HA proteins that are completely unrelated to pre-existing human strains with respect to antigenic properties. These “major antigenic shifts” result in new antigenic subtypes of the HA and/or NA glycoproteins that had not previously infected most of the human population, and therefore can spread rapidly causing global disease pandemics. Thus, the currently available influenza vaccines need to be updated every year to match the antigenicity of the virus strains that are predicted to circulate in the next season. However, current vaccines would not be effective in preventing the spread of a new pandemic strain containing a substantially different HA protein. Therefore, new approaches are being investigated to develop broadly cross-protective vaccines, focused primarily on type A influenza viruses. Two such approaches are targeted to conserved regions of the viral M2 protein and the HA protein.

3.1 Recombinant vaccines based on M2

M2 is a tetrameric membrane protein with pH-controlled, passive proton-selective channel activity (Schnell and Chou, 2008; Stouffer et al., 2008). This activity of M2 promotes acidification of the virion interior after endocytosis, allowing the release of the viral core constituents into the cytosol. The extracellular domain of M2, designated M2e, is known to be involved in its incorporation into newly formed virions (Iwatsuki-Horimoto et al., 2006; McCown and Pekosz, 2005). The 54-amino acid residue cytoplasmic tail of M2 is suggested to play a role in assembly or release of virus particles by interacting with M1 (Chen et al., 2008; McCown and Pekosz, 2006). M2 is translated from a spliced variant of the mRNA coding for the matrix protein M1, which is a further restriction on its sequence diversity. The extracellular domain of M2 (M2e) is highly conserved among multiple influenza A viruses, indicating that M2 is an attractive antigenic target for developing a universal influenza vaccine.

Previous studies provided evidence that anti-M2 immunity would confer protective effects against influenza virus infection. Monoclonal antibodies directed against M2 could reduce the plaque size of some influenza A virus strains (Zebedee and Lamb, 1988). Passive administration of M2 monoclonal antibodies reduced lung virus titers in mice infected with influenza A virus (Treanor et al., 1990; Wang et al., 2008). The phenotype of plaque size reduction of influenza virus growth might be due to blocking a late stage of replication by M2 antibodies as similarly observed by treatment with antibodies specific to neuraminidase

(Hay et al., 1985). Although the mechanism of M2 antibody mediated inhibition of influenza virus growth is not clear, its putative interactions with other viral proteins, the mobility of integral membrane proteins, and the assembly and budding at the plasma membrane might explain the observed phenomenon of reduction in plaque size. While M2e antibody therapies can be an antiviral strategy, induction of adaptive anti-M2 immunity would be far more cost effective and practical for controlling influenza epidemics or pandemics.

M2-based vaccine studies are summarized in Table 1. Slepushkin et al (1995) first reported cross protection by vaccination of mice with the full-length M2 protein as a vaccine antigen in combination with incomplete Freund's adjuvant (IFA). This M2 vaccine consisted of a partially purified M2-containing membrane fraction derived from the recombinant baculovirus insect-cell expression system. Mice that were vaccinated with M2 vaccines exhibited reduced morbidity and virus titers in lungs, and showed a survival advantage against lethal challenge infection (Slepushkin et al., 1995). DNA and recombinant adenoviral vectors expressing full-length M2-protein have also been used to vaccinate mice or ferrets (Jimenez et al., 2007; Lalor et al., 2008; Tompkins et al., 2007). The prime-boost vaccination regimen induced both humoral and cellular immune responses to M2 and conferred survival after lethal challenge infection but significant morbidity was still observed. Although DNA and viral vector vaccines may avoid the process of purifying membrane-anchored M2 for use as a vaccine antigen, there are concerns on using live viral vectors and a problem with pre-existing immunity to adenovirus in the population. In a recent study, a full-length of M2 was presented on recombinant virus-like particles (M2 VLPs) also containing the influenza M1 matrix protein, produced using the baculovirus-insect cell expression system (Song et al., 2011b). Mice vaccinated with M2 VLP vaccines were protected against lethal infection with different subtypes of influenza A viruses (Song et al., 2011b).

Previous studies also reported protection of mice against lethal challenge after vaccination of mice with M2e peptide in the presence of incomplete Freund's and alum adjuvants (Wu et al., 2007; Wu et al., 2009b). However, a drawback of M2e is its small size and low immunogenicity. Therefore, most studies have focused on M2e fusion constructs using a variety of carrier molecules (Table 1): hepatitis B virus core (HBc) particles (De Filette et al., 2006b; Fan et al., 2004; Neirynck et al., 1999), human papilloma virus L protein (Ionescu et al., 2006), keyhole limpet hemocyanin (Tompkins et al., 2007), bacterial outer membrane complex (Fan et al., 2004; Fu et al., 2009b), liposomes (Ernst et al., 2006), and flagellin (Huleatt et al., 2008). Vaccination with these M2e fusion protein antigens in the presence of diverse adjuvants was demonstrated to provide protection against lethal challenge with H1N1, H3N2, and H5N1 viruses (Table 1). The protection was observed after vaccination via intranasal, intraperitoneal, or intramuscular routes. The induction of anti-M2e IgG antibodies was shown to be responsible for the protection observed although cellular immune responses might have played a role in some M2e vaccine platforms such as M2 VLPs. In particular, M2e HBVc VLP vaccines were demonstrated to induce high levels of anti-M2e antibody responses (De Filette et al., 2006a; De Filette et al., 2005; Fiers et al., 2004; Heinen et al., 2002; Neirynck et al., 1999). VLPs as M2e antigen carriers present M2e epitopes in an ordered array, enabling a strong immune response as well as increasing their stability and immunogenicity (Bachmann et al., 1993; De Filette et al., 2005).

Chemical or genetic conjugation of M2e may not present M2e in its tetrameric native form. In a previous study, M2 antibodies induced by vaccination with M2e-HBVc conjugates did not efficiently bind to the free virus particles (Jegerlehner et al., 2004). As a vaccine antigen, use of the wild type M2 protein would be advantageous since it is likely to present M2 in a membrane-anchored native conformation. Vaccination with M2 VLPs without adjuvants was found to induce antibodies recognizing cell-surface expressed native M2 proteins and

influenza viral particles of different subtypes, as well as survival protection against lethal challenge infection (Song et al., 2011b). An alternative strategy to express M2e in a tetrameric form is to link M2e to a form of the leucine zipper of the yeast transcription factor GCN4 (M2e-tGCN4). De Filette and coworkers found that M2e-tGCN4 chimeric proteins mimic the quaternary structure of the ectodomain of the natural M2 protein (De Filette et al., 2008). Antibodies raised by M2e-tGCN4 immunization bound to the surface of influenza virus-infected cells and to an M2-expressing cell line, providing evidence that M2e-tGCN4 induces antibodies that are specific for the native tetrameric M2 ectodomain. In support of this idea, it was demonstrated that M2 monoclonal antibodies which preferentially bind to M2 multimeric forms but not the monomeric form were more protective, and that this was independent of natural killer cell mediated effector functions (Fu et al., 2009a). In a previous study, M2 antibodies induced by vaccination with M2e conjugates did not efficiently bind to the free virus particles (Jegerlehner et al., 2004). Therefore, new vaccine designs that mimic natural conformation of M2 would be desirable for inducing reactive M2 antibodies.

Although anti-M2 antibodies do not directly neutralize the virus (Jegerlehner et al., 2004; Song et al., 2011a; Zebedee and Lamb, 1988), influenza viruses bound to M2 antibodies are likely to be recognized and removed by opsonophagocytosis by macrophages. It was demonstrated that non-neutralizing anti-influenza humoral immunity was dependent on opsonophagocytosis of influenza virions by macrophages (Huber et al., 2001; Huber et al., 2006; Jayasekera et al., 2007; Mozdzanowska et al., 2006). IgG2a antibody is an isotype known to interact efficiently with complement and Fc receptors (Gessner et al., 1998; Heusser et al., 1977; Huber et al., 2001; Neuberger and Rajewsky, 1981). M2 VLP vaccination induced IgG2a antibody as the predominant isotype as well as IgG1 at lower levels (Song et al., 2011b), which is similar to those induced by HA based VLP vaccination (Bright et al., 2007). Furthermore, this protection by M2 immune sera might be mediated by dendritic and macrophage cells as shown by depletion experiments using clodronate-liposomes (Song et al., 2011b). Therefore, we suggest that induction of virion-binding antibodies by M2 VLP vaccination contributes to viral clearance, possibly via opsonizing virus by macrophages and dendritic cells. Elimination of virus-infected cells might also be mediated by complement, natural killer cells or phagocytosis by macrophages.

It was demonstrated that intranasal immunization of mice with M2 vaccines provided better protection despite the induction of lower levels of serum IgG specific for native M2 compared with parenteral immunization (Mozdzanowska et al., 2007). Most likely, local airway-associated immunity, involving mucosal anti-M2e IgA and activated M2e-specific B and T cells, is induced efficiently after intranasal vaccination (Song et al., 2011b). Furthermore, mucosal M2e-specific immunity in the upper respiratory tract may operate effectively against nasal infection. Further research is needed to better identify the mechanism of anti-M2e immunity, since this information will be essential for understanding true correlates of protection and important to develop assays for predicting the efficacy of M2 vaccines.

The candidate universal vaccines studied thus far in animal models have not achieved the same level of protection provided by current inactivated vaccines. M2e based immunity is infection-permissive and can reduce but not eliminate disease symptoms. A realistic goal for an appropriate vaccine for pandemic influenza will be to diminish morbidity and mortality, even without obviating all disease symptoms caused by infection. Alternatively, it is suggested to use such vaccines as adjunct to current vaccines to provide increased resistance in case of the unanticipated emergence of a major drift variant or new subtype. In this regard, supplementation of inactivated influenza vaccine with M2e-based vaccines could significantly improve the cross protective efficacy of vaccination. It was reported that the addition of M2e peptide significantly enhanced the cross-protection provided in mice by

intraperitoneal vaccination with aluminum-adjuvanted split H3N2 virus but not with oil-in-water-emulsion adjuvanted split H3N2 virus vaccine (Wu et al., 2009a). However, the addition of the M2e peptide did not prevent weight loss after heterosubtypic challenge infection. Therefore, an M2e peptide vaccine may have some limitations for improving the efficacy of cross protection.

Recently, our laboratory demonstrated that an inactivated influenza vaccine supplemented with M2 VLPs prevents disease symptoms with no observed weight loss, and confers complete cross protection against lethal challenge with heterologous influenza A viruses including the 2009 H1N1 pandemic virus as well as heterosubtypic H3N2 and H5N1 influenza viruses (Song et al., 2011a). Immune sera from mice immunized with the M2 VLP-supplemented vaccine were shown to transfer cross protection to naïve mice (Song et al., 2011a), indicating an important role of humoral immunity even in the absence of significant cross reactive hemagglutination-inhibition activity. Therefore, supplementation of seasonal influenza vaccines with M2 VLPs provides a promising approach for overcoming the limitation of strain-specific protection by current vaccines and developing an effective universal influenza A vaccine. However, it remains to be determined whether conventional intramuscular vaccination with split vaccines would be similarly effective in combination with M2 VLP vaccines. Ultimately, the efficacy of M2 and/or other potential universal vaccines has to be determined by clinical trials, which are required to determine whether it is possible to develop an effective universal influenza vaccine for human use.

3.2 Vaccines targeted to conserved hemagglutinin stalk domains

Another potential target for a broadly cross-protective influenza vaccine is the HA protein. HA is a homotrimeric molecule consisting of disulfide-linked glycoproteins, a globular head of HA1 and a stem domain composed of part of HA1 and all of HA2 (Wilson et al., 1981). The globular head contains the receptor-binding pocket surrounded by variable antigenic sites (Laver et al., 1980; Laver et al., 1979). The protease mediated cleavage between the HA1 and HA2 domains is required for fusion and productive replication of influenza viruses. Although HA is known to undergo extensive sequence and antigenic variation in its receptor-binding subunit, it also possesses conserved structural features in the HA2 segment involved in anchoring to the viral membrane. It has recently been recognized that this segment, termed the stalk, is a potential target for inducing broadly cross-reactive immunity.

The fusion peptide is known to be the most highly conserved region of all influenza HA proteins (Chun et al., 2008; Li et al., 2010). The HA cleavage site forms an extended, highly exposed loop structure on the surface and is highly conserved in most influenza A viruses. Particularly the sequence of the HA2 N-terminal 11 amino acids is invariant among most influenza A virus strains and differs only by 1 or 2 conservative amino acid replacements in influenza B virus. It is likely that this invariant domain needs to be maintained because of functional constraints for being a suitable substrate for host-encoded proteases and to retain a functional domain in mediating virus entry by membrane fusion. This cleavage domain is located in a loop of the HA precursor, and thus is accessible to antibody on the uncleaved HA precursor expressed on the plasma membrane of infected host cells. As described in Table 2, studies targeting the fusion peptides or HA2 stalk domain are summarized. The protective potential of antibodies directed to the HA1/HA2 joining region has been explored in two studies using synthetic peptides (Bianchi et al., 2005; Horvath et al., 1998). Both studies demonstrated that mice vaccinated with a peptide (or peptide conjugates) spanning the HA1/HA2 joining region displayed less illness and lower rates of death upon virus challenge (Bianchi et al., 2005; Horvath et al., 1998). Antibodies binding to the vaccine peptide were found to play a major role in conferring the observed protection (Bianchi et al., 2005). Importantly, antibodies targeting the fusion peptide or part of this fusion peptide could protect from challenge by a broad spectrum of influenza virus strains (Ekiert et al.,

2009; Prabhu et al., 2009; Sui et al., 2009). Immunization with fusion peptides chemically modified and then conjugated to keyhole limpet hemocyanin raised antibodies reactive to HA proteins from different subtypes by specifically recognizing the fusion peptide sequence (Li et al., 2010). Therefore, the development of a universal vaccine strategy targeting the fusion peptide would be of further interest.

The HA2 subunit of the influenza virus hemagglutinin is relatively well conserved compared to HA1 globular head domains (Krystal et al., 1982). The HA2 domain has the up to 85% sequence homology among different subtypes and 95% homology within strains of the same subtype (Fouchier et al., 2005). However, during natural infection or vaccination with conventional influenza vaccines, immune responses to the HA2 domain are weak and not commonly reported, probably due to the presence of the bulky and highly immunogenic globular HA1 head domain. In a study using a modified vaccine, immunization of rabbits or mice with subviral particles after chemically removing the HA1 domain induced a humoral response against HA2, but this response did not provide good protection against influenza viral challenge (Graves et al., 1983). However, other recent studies reported successful vaccines designed to induce immunity to HA2. A truncated hemagglutinin molecule lacking a significant portion of the globular head conferred protection to mice upon subsequent challenge with influenza virus (Sagawa et al., 1996). This same study also reported that a monoclonal antibody (C179) which recognizes a conformational epitope in the middle of the stem region of HA and neutralized all H1 and H2 subtypes (Sagawa et al., 1996). Since then, there have been reports of other monoclonal antibodies that were isolated and characterized, which bind the HA stalk domain and were demonstrated to have broadly cross-reactive neutralizing activity (Kashyap et al., 2008; Okuno et al., 1993; Wrammert et al., 2011) (Corti et al., 2011; Ekiert et al., 2011). Structural and functional analysis of such a broadly neutralizing antibody indicated that this antibody blocks infection by inserting its heavy chain into a conserved pocket in the stem region, thus probably preventing the membrane fusion step in viral entry (Ekiert et al., 2009; Sui et al., 2009).

Recent studies demonstrated that vaccination with recombinant and/or genetically modified immunogens can elicit the production of antibodies to conserved epitopes. Prime-boost vaccination with plasmid DNA and recombinant adenovirus vector vaccines expressing H1 HA induced antibodies recognizing the conserved stem region and thus exhibited broadly neutralizing activity (Wei et al., 2010). Antibodies cross-reactive to multiple subtypes of influenza virus were raised by 3 vaccinations of mice with DNA and VLP vaccines having a modified HA molecule which lacks the globular head domain but maintains the integrity of the stalk region, including both the HA1 and HA2 portions (Steel et al., 2010). A monoclonal antibody (12D1) with broadly neutralizing activity within H3 subtypes was identified to recognize an α -helical region of the HA2 protein corresponding to the amino acids 76-106 of HA2 (Wang et al., 2010b). Based on the binding site of the 12D1 antibody, a synthetic peptide containing amino acids 76-130 of the HA2 was designed, coupled to keyhole limpet hemocyanin carrier protein, and then used to elicit antibodies against the stalk of the influenza virus HA protein (Wang et al., 2010a). This vaccine had protective activity against antigenically divergent virus subtypes (Wang et al., 2010a). Taken together, these studies provide evidence supporting a new concept for a broadly protective influenza vaccine based on the relatively conserved stalk domain of HA.

4. Conclusions

During the last decades, many approaches to develop new vaccines targeting the relatively conserved ectodomain of M2 and HA fusion peptide and stalk domains have provided a proof-of-concept that development of a vaccine inducing cross protection will be feasible. The efficacy of cross protection may be improved by approaches to design M2e vaccine

antigens for induction of antibodies that react with the native, tetrameric membrane-anchored form of M2. Still, a caveat is that although the M2e sequence is highly conserved among human influenza A strains, there are differences in amino acids in the ectodomain of M2 in swine and avian influenza viruses. Vaccines targeting the whole HA2 domain or the helical region of HA are likely to show subtype-specific cross protection reflecting the degree of sequence homology among different subtypes (Steel et al., 2010; Wang et al., 2010a).

There are several important considerations for developing effective universal influenza vaccines. 1) M2 or other conserved influenza vaccines have to be produced in a highly efficient microbial expression system and, thus can be made very cheaply. 2) Unlike commercial vaccines that are strain-specific, universal vaccines would not have time constraints for administration. That is, universal influenza vaccines can be made, distributed, and administered long before emergence of new epidemic or pandemic strains. 3) It will be important to place more focus on designing and testing of improved universal vaccines that mimic the native conformation of target antigens, so that immune responses effectively recognize infected cells and viruses.

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References

- al-Mazrou A, Scheifele DW, Soong T, Bjornson G. Comparison of adverse reactions to whole-virion and split-virion influenza vaccines in hospital personnel. *Cmaj*. 1991; 145(3):213–8. [PubMed: 2070311]
- Bachmann MF, Kundig TM, Kalberer CP, Hengartner H, Zinkernagel RM. Formalin inactivation of vesicular stomatitis virus impairs T-cell- but not T-help-independent B-cell responses. *J Virol*. 1993; 67(7):3917–22. [PubMed: 8389912]
- Baez M, Palese P, Kilbourne ED. Gene composition of high-yielding influenza vaccine strains obtained by recombination. *J Infect Dis*. 1980; 141(3):362–5. [PubMed: 7365284]
- Bautista Rentero D, Tenias Burillo JM, Sabater Pons A, Perez Bermudez B, Zanon Viguer V. The comparison of adverse reactions to anti-influenza vaccines of split-vision and surface antigens. *Rev Esp Salud Publica*. 1995; 69(6):463–8. [PubMed: 8789358]
- Belshe RB, Ambrose CS, Yi T. Safety and efficacy of live attenuated influenza vaccine in children 2–7 years of age. *Vaccine*. 2008; 26(Suppl 4):D10–6. [PubMed: 18611422]
- Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K, Treanor J, Zangwill K, Hayden FG, Bernstein DI, Kotloff K, King J, Piedra PA, Block SL, Yan L, Wolff M. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. *J Infect Dis*. 2000; 181(3):1133–7. [PubMed: 10720541]
- Bianchi E, Liang X, Ingallinella P, Finotto M, Chastain MA, Fan J, Fu TM, Song HC, Horton MS, Freed DC, Manger W, Wen E, Shi L, Ionescu R, Price C, Wenger M, Emini EA, Cortese R, Ciliberto G, Shiver JW, Pessi A. Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *J Virol*. 2005; 79(12):7380–8. [PubMed: 15919893]
- Block SL, Yogev R, Hayden FG, Ambrose CS, Zeng W, Walker RE. Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5–49 years of age. *Vaccine*. 2008; 26(38):4940–6. [PubMed: 18662737]
- Bright RA, Carter DM, Daniluk S, Toapanta FR, Ahmad A, Gavrillo V, Massare M, Pushko P, Mytle N, Rowe T, Smith G, Ross TM. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine*. 2007; 25(19):3871–8. [PubMed: 17337102]

- Bush RM, Bender CA, Subbarao K, Cox NJ, Fitch WM. Predicting the evolution of human influenza A. *Science*. 1999; 286(5446):1921–5. [PubMed: 10583948]
- Cate TR, Couch RB, Kasel JA, Six HR. Clinical trials of monovalent influenza A/New Jersey/76 virus vaccines in adults: reactogenicity, antibody response, and antibody persistence. *J Infect Dis*. 1977; 136(Suppl):S450–5. [PubMed: 342620]
- Chen BJ, Leser GP, Jackson D, Lamb RA. The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding. *J Virol*. 2008; 82(20):10059–70. [PubMed: 18701586]
- Cheung CL, Rayner JM, Smith GJ, Wang P, Naipospos TS, Zhang J, Yuen KY, Webster RG, Peiris JS, Guan Y, Chen H. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. *J Infect Dis*. 2006; 193(12):1626–9. [PubMed: 16703504]
- Chun S, Li C, Van Domselaar G, Wang J, Farnsworth A, Cui X, Rode H, Cyr TD, He R, Li X. Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. *Vaccine*. 2008; 26(48):6068–76. [PubMed: 19007587]
- Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet*. 1998; 351(9101):472–7. [PubMed: 9482438]
- Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, Silacci C, Fernandez-Rodriguez BM, Agatic G, Bianchi S, Giacchetto-Sasselli I, Calder L, Sallusto F, Collins P, Haire LF, Temperton N, Langedijk JP, Skehel JJ, Lanzavecchia A. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*. 2011; 333(6044):850–6. [PubMed: 21798894]
- Davenport FM. Current knowledge of influenza vaccine. *Jama*. 1962; 182:11–3. [PubMed: 13883724]
- De Filette M, Fiers W, Martens W, Birkett A, Ramne A, Lowenadler B, Lycke N, Jou WM, Saelens X. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine*. 2006a; 24(44–46):6597–601. [PubMed: 16814430]
- De Filette M, Martens W, Roose K, Deroo T, Vervalle F, Bentahir M, Vandekerckhove J, Fiers W, Saelens X. An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. *J Biol Chem*. 2008; 283(17):11382–7. [PubMed: 18252707]
- De Filette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, Resch S, Fiers W. Universal influenza A vaccine: optimization of M2-based constructs. *Virology*. 2005; 337(1):149–61. [PubMed: 15914228]
- De Filette M, Ramne A, Birkett A, Lycke N, Lowenadler B, Min Jou W, Saelens X, Fiers W. The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. *Vaccine*. 2006b; 24(5):544–51. [PubMed: 16169634]
- de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JS, Tran TH, Farrar J. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N Engl J Med*. 2005; 353(25):2667–72. [PubMed: 16371632]
- Denis J, Acosta-Ramirez E, Zhao Y, Hamelin ME, Koukavica I, Baz M, Abed Y, Savard C, Pare C, Lopez Macias C, Boivin G, Leclerc D. Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. *Vaccine*. 2008; 26(27–28):3395–403. [PubMed: 18511159]
- Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. *Science*. 2009; 324(5924):246–51. [PubMed: 19251591]
- Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science*. 2011; 333(6044):843–50. [PubMed: 21737702]
- Eliasson DG, Bakkouri KE, Schon K, Ramne A, Festjens E, Lowenadler B, Fiers W, Saelens X, Lycke N. CTA1-M2e-DD: A novel mucosal adjuvant targeted influenza vaccine. *Vaccine*. 2008; 26(9):1243–52. [PubMed: 18243429]

- Ernst WA, Kim HJ, Tumpey TM, Jansen AD, Tai W, Cramer DV, Adler-Moore JP, Fujii G. Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. *Vaccine*. 2006; 24(24):5158–68. [PubMed: 16713037]
- Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, Fu TM, Joyce J, Przysiecki CT, Keller PM, Garsky VM, Ionescu R, Rippeon Y, Shi L, Chastain MA, Condra JH, Davies ME, Liao J, Emini EA, Shiver JW. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. *Vaccine*. 2004; 22(23–24):2993–3003. [PubMed: 15297047]
- Fiers W, De Filette M, Birkett A, Neiryneck S, Min Jou W. A “universal” human influenza A vaccine. *Virus Res*. 2004; 103(1–2):173–6. [PubMed: 15163506]
- Forrest BD, Pride MW, Dunning AJ, Capeding MR, Chotpitayasunondh T, Tam JS, Rappaport R, Eldridge JH, Gruber WC. Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. *Clin Vaccine Immunol*. 2008; 15(7):1042–53. [PubMed: 18448618]
- Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol*. 2005; 79(5):2814–22. [PubMed: 15709000]
- Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci U S A*. 2004; 101(5):1356–61. [PubMed: 14745020]
- Frace AM, Klimov AI, Rowe T, Black RA, Katz JM. Modified M2 proteins produce heterotypic immunity against influenza A virus. *Vaccine*. 1999; 17(18):2237–44. [PubMed: 10403591]
- Francis T Jr, Salk JE, Brace WM. The protective effect of vaccination against epidemic influenza B. *J Am Med Assoc*. 1946; 131:275–8. [PubMed: 21027918]
- Fu TM, Freed DC, Horton MS, Fan J, Citron MP, Joyce JG, Garsky VM, Casimiro DR, Zhao Q, Shiver JW, Liang X. Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. *Virology*. 2009a; 385(1):218–26. [PubMed: 19070878]
- Fu TM, Grimm KM, Citron MP, Freed DC, Fan J, Keller PM, Shiver JW, Liang X, Joyce JG. Comparative immunogenicity evaluations of influenza A virus M2 peptide as recombinant virus like particle or conjugate vaccines in mice and monkeys. *Vaccine*. 2009b; 27(9):1440–7. [PubMed: 19146898]
- Gessner JE, Heiken H, Tamm A, Schmidt RE. The IgG Fc receptor family. *Ann Hematol*. 1998; 76(6):231–48. [PubMed: 9692811]
- Glaser CA, Gilliam S, Thompson WW, Dassey DE, Waterman SH, Saruwatari M, Shapiro S, Fukuda K. Medical care capacity for influenza outbreaks, Los Angeles. *Emerg Infect Dis*. 2002; 8(6):569–74. [PubMed: 12023911]
- Graves PN, Schulman JL, Young JF, Palese P. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. *Virology*. 1983; 126(1):106–16. [PubMed: 6189287]
- Harper SA, Fukuda K, Uyeki TM, Cox NJ, Bridges CB. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep*. 2004; 53(RR-6):1–40. [PubMed: 15163927]
- Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH. The molecular basis of the specific anti-influenza action of amantadine. *EMBO J*. 1985; 4(11):3021–4. [PubMed: 4065098]
- Heinen PP, Rijsewijk FA, de Boer-Luijtz EA, Bianchi AT. Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. *J Gen Virol*. 2002; 83(Pt 8):1851–9. [PubMed: 12124449]
- Heusser CH, Anderson CL, Grey HM. Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. *J Exp Med*. 1977; 145(5):1316–27. [PubMed: 300783]
- Horvath A, Toth GK, Gogolak P, Nagy Z, Kurucz I, Pecht I, Rajnavolgyi E. A hemagglutinin-based multipeptide construct elicits enhanced protective immune response in mice against influenza A virus infection. *Immunol Lett*. 1998; 60(2–3):127–36. [PubMed: 9557954]

- Huber VC, Lynch JM, Bucher DJ, Le J, Metzger DW. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J Immunol.* 2001; 166(12): 7381–8. [PubMed: 11390489]
- Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, Makarova N, Perez DR, Macdonald GH, McCullers JA. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. *Clin Vaccine Immunol.* 2006; 13(9):981–90. [PubMed: 16960108]
- Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, Tang J, McDonald W, Song L, Evans RK, Umlauf S, Tussey L, Powell TJ. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine.* 2008; 26(2):201–14. [PubMed: 18063235]
- Ionescu RM, Przysiecki CT, Liang X, Garsky VM, Fan J, Wang B, Troutman R, Rippeon Y, Flanagan E, Shiver J, Shi L. Pharmaceutical and immunological evaluation of human papillomavirus viruslike particle as an antigen carrier. *J Pharm Sci.* 2006; 95(1):70–9. [PubMed: 16315228]
- Iwatsuki-Horimoto K, Horimoto T, Noda T, Kiso M, Maeda J, Watanabe S, Muramoto Y, Fujii K, Kawaoka Y. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. *J Virol.* 2006; 80(11):5233–40. [PubMed: 16699003]
- Jayasekera JP, Moseman EA, Carroll MC. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *J Virol.* 2007; 81(7):3487–94. [PubMed: 17202212]
- Jegerlehner A, Schmitz N, Storni T, Bachmann MF. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *J Immunol.* 2004; 172(9):5598–605. [PubMed: 15100303]
- Jennings R, Smith TL, Spencer RC, Mellersh AM, Edey D, Fenton P, Potter CW. Inactivated influenza virus vaccines in man: a comparative study of subunit and split vaccines using two methods for assessment of antibody responses. *Vaccine.* 1984; 2(1):75–80. [PubMed: 6531953]
- Jimenez GS, Planchon R, Wei Q, Rusalov D, Geall A, Enas J, Lalor P, Leamy V, Vahle R, Luke CJ, Rolland A, Kaslow DC, Smith LR. Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. *Hum Vaccin.* 2007; 3(5):157–64. [PubMed: 17637571]
- Kashyap AK, Steel J, Oner AF, Dillon MA, Swale RE, Wall KM, Perry KJ, Faynboym A, Ilhan M, Horowitz M, Horowitz L, Palese P, Bhatt RR, Lerner RA. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proc Natl Acad Sci U S A.* 2008; 105(16):5986–91. [PubMed: 18413603]
- Kilbourne ED, Murphy JS. Genetic studies of influenza viruses. I. Viral morphology and growth capacity as exchangeable genetic traits. Rapid in ovo adaptation of early passage Asian strain isolates by combination with PR8. *J Exp Med.* 1960; 111:387–406. [PubMed: 13755924]
- Klenk HD, Stickl H. Which influenza vaccination is the best - with whole virus, split vaccine or subunit vaccines? *Med Welt.* 1981; 32(25):1008–9. [PubMed: 7278577]
- Krystal M, Elliott RM, Benz EW Jr, Young JF, Palese P. Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proc Natl Acad Sci U S A.* 1982; 79(15):4800–4. [PubMed: 6956892]
- Lalor PA, Webby RJ, Morrow J, Rusalov D, Kaslow DC, Rolland A, Smith LR. Plasmid DNA-based vaccines protect mice and ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus. *J Infect Dis.* 2008; 197(12):1643–52. [PubMed: 18513153]
- Laver WG, Air GM, Dopheide TA, Ward CW. Amino acid sequence changes in the haemagglutinin of A/Hong Kong (H3N2) influenza virus during the period 1968–77. *Nature.* 1980; 283(5746):454–7. [PubMed: 6153236]
- Laver WG, Gerhard W, Webster RG, Frankel ME, Air GM. Antigenic drift in type A influenza virus: peptide mapping and antigenic analysis of A/PR/8/34 (HON1) variants selected with monoclonal antibodies. *Proc Natl Acad Sci U S A.* 1979; 76(3):1425–9. [PubMed: 86990]
- Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Nguyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. Avian flu: isolation of drug-resistant H5N1 virus. *Nature.* 2005; 437(7062):1108. [PubMed: 16228009]

- Li C, Jaentschke B, Song Y, Wang J, Cyr TD, Van Domselaar G, He R, Li X. A simple slot blot for the detection of virtually all subtypes of the influenza A viral hemagglutinins using universal antibodies targeting the fusion peptide. *Nat Protoc.* 2010; 5(1):14–9. [PubMed: 20010723]
- Liu W, Peng Z, Liu Z, Lu Y, Ding J, Chen YH. High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity. *Vaccine.* 2004; 23(3):366–71. [PubMed: 15530682]
- Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J Virol.* 2004; 78(22): 12665–7. [PubMed: 15507653]
- McCown MF, Pekosz A. The influenza A virus M2 cytoplasmic tail is required for infectious virus production and efficient genome packaging. *J Virol.* 2005; 79(6):3595–605. [PubMed: 15731254]
- McCown MF, Pekosz A. Distinct domains of the influenza a virus M2 protein cytoplasmic tail mediate binding to the M1 protein and facilitate infectious virus production. *J Virol.* 2006; 80(16):8178–89. [PubMed: 16873274]
- Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS, Hierholzer JC. Studies on inactivated influenza vaccines. II. Effect of increasing dosage on antibody response and adverse reactions in man. *Am J Epidemiol.* 1970; 92(4):248–56. [PubMed: 5458217]
- Mozdzanowska K, Feng J, Eid M, Zharikova D, Gerhard W. Enhancement of neutralizing activity of influenza virus-specific antibodies by serum components. *Virology.* 2006; 352(2):418–26. [PubMed: 16777168]
- Mozdzanowska K, Zharikova D, Cudic M, Otvos L, Gerhard W. Roles of adjuvant and route of vaccination in antibody response and protection engendered by a synthetic matrix protein 2-based influenza A virus vaccine in the mouse. *Virol J.* 2007; 4:118. [PubMed: 17974006]
- Murphy BR, Coelingh K. Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. *Viral Immunol.* 2002; 15(2):295–323. [PubMed: 12081014]
- Nava GM, Attene-Ramos MS, Ang JK, Escorcía M. Origins of the new influenza A(H1N1) virus: time to take action. *Euro Surveill.* 2009; 14(22)
- Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med.* 1999; 5(10):1157–63. [PubMed: 10502819]
- Neuberger MS, Rajewsky K. Activation of mouse complement by monoclonal mouse antibodies. *Eur J Immunol.* 1981; 11(12):1012–6. [PubMed: 7327198]
- Ohmit SE, Victor JC, Teich ER, Truscon RK, Rothhoff JR, Newton DW, Campbell SA, Boulton ML, Monto AS. Prevention of symptomatic seasonal influenza in 2005–2006 by inactivated and live attenuated vaccines. *J Infect Dis.* 2008; 198(3):312–7. [PubMed: 18522501]
- Okuno Y, Isegawa Y, Sasao F, Ueda S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J Virol.* 1993; 67(5):2552–8. [PubMed: 7682624]
- Palache AM, Beyer WE, Luchters G, Volker R, Sprenger MJ, Masurel N. Influenza vaccines: the effect of vaccine dose on antibody response in primed populations during the ongoing interpandemic period. A review of the literature. *Vaccine.* 1993; 11(9):892–908. [PubMed: 8212834]
- Palese P, Compans RW. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. *J Gen Virol.* 1976; 33(1):159–63. [PubMed: 978183]
- Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF. Human infection with influenza H9N2. *Lancet.* 1999; 354(9182):916–7. [PubMed: 10489954]
- Plotkin JB, Dushoff J. Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. *Proc Natl Acad Sci U S A.* 2003; 100(12):7152–7. [PubMed: 12748378]
- Poehling KA, Edwards KM, Weinberg GA, Szilagyi P, Staat MA, Iwane MK, Bridges CB, Grijalva CG, Zhu Y, Bernstein DI, Herrera G, Erdman D, Hall CB, Seither R, Griffin MR. The underrecognized burden of influenza in young children. *N Engl J Med.* 2006; 355(1):31–40. [PubMed: 16822994]

- Prabhu N, Prabakaran M, Ho HT, Velumani S, Qiang J, Goutama M, Kwang J. Monoclonal antibodies against the fusion peptide of hemagglutinin protect mice from lethal influenza A virus H5N1 infection. *J Virol*. 2009; 83(6):2553–62. [PubMed: 19109379]
- Quinnan GV, Schooley R, Dolin R, Ennis FA, Gross P, Gwaltney JM. Serologic responses and systemic reactions in adults after vaccination with monovalent A/USSR/77 and trivalent A/USSR/77, A/Texas/77, B/Hong Kong/72 influenza vaccines. *Rev Infect Dis*. 1983; 5(4):748–57. [PubMed: 6622889]
- Robertson JS, Nicolson C, Newman R, Major D, Dunleavy U, Wood JM. High growth reassortant influenza vaccine viruses: new approaches to their control. *Biologicals*. 1992; 20(3):213–20. [PubMed: 1457106]
- Sagawa H, Ohshima A, Kato I, Okuno Y, Isegawa Y. The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular region. *J Gen Virol*. 1996; 77 (Pt 7):1483–7. [PubMed: 8757990]
- Salk JE, Pearson HE, Brown PN, Francis T. Protective effect of vaccination against induced influenza B. *J Clin Invest*. 1945; 24(4):547–53. [PubMed: 16695244]
- Schnell JR, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature*. 2008; 451(7178):591–5. [PubMed: 18235503]
- Slepishkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine*. 1995; 13(15):1399–402. [PubMed: 8578816]
- Solovoyov A, Palacios G, Briese T, Lipkin WI, Rabadan R. Cluster analysis of the origins of the new influenza A(H1N1) virus. *Euro Surveill*. 2009; 14(21)
- Song JM, Van Rooijen N, Bozja J, Compans RW, Kang SM. Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. *Proc Natl Acad Sci U S A*. 2011a; 108(2):757–61. [PubMed: 21187388]
- Song JM, Wang BZ, Park KM, Van Rooijen N, Quan FS, Kim MC, Jin HT, Pekosz A, Compans RW, Kang SM. Influenza virus-like particles containing M2 induce broadly cross protective immunity. *PLoS One*. 2011b; 6(1):e14538. [PubMed: 21267073]
- Steel J, Lowen AC, Wang T, Yondola M, Gao Q, Haye K, Garcia-Sastre A, Palese P. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio*. 2010; 1(1)
- Stokes J, Chenoweth AD, Waltz AD, Gladen RG, Shaw D. Results of immunization by means of active virus of human influenza. *J Clin Invest*. 1937; 16(2):237–43. [PubMed: 16694474]
- Stouffer AL, Acharya R, Salom D, Levine AS, Di Costanzo L, Soto CS, Tereshko V, Nanda V, Stayrook S, DeGrado WF. Structural basis for the function and inhibition of an influenza virus proton channel. *Nature*. 2008; 451(7178):596–9. [PubMed: 18235504]
- Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science*. 1998; 279(5349):393–6. [PubMed: 9430591]
- Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol*. 2009; 16(3):265–73. [PubMed: 19234466]
- Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, Fukuda K. Influenza-associated hospitalizations in the United States. *JAMA*. 2004; 292(11):1333–40. [PubMed: 15367555]
- Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K. Mortality associated with influenza and respiratory syncytial virus in the United States. *Jama*. 2003; 289(2):179–86. [PubMed: 12517228]
- Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, Hogan RJ, Wu Z, Benton KA, Tumpey TM, Epstein SL. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. *Emerg Infect Dis*. 2007; 13(3):426–35. [PubMed: 17552096]

- Treanor JJ, Tierney EL, Zebedee SL, Lamb RA, Murphy BR. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. *J Virol.* 1990; 64(3): 1375–7. [PubMed: 2304147]
- Wang R, Song A, Levin J, Dennis D, Zhang NJ, Yoshida H, Koriazova L, Madura L, Shapiro L, Matsumoto A, Mikayama T, Kubo RT, Sarawar S, Cheroutre H, Kato S. Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. *Antiviral Res.* 2008; 80(2):168–77. [PubMed: 18598723]
- Wang TT, Tan GS, Hai R, Pica N, Ngai L, Ekiert DC, Wilson IA, Garcia-Sastre A, Moran TM, Palese P. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proc Natl Acad Sci U S A.* 2010a; 107(44):18979–84. [PubMed: 20956293]
- Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, Palese P. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog.* 2010b; 6(2):e1000796. [PubMed: 20195520]
- Wei CJ, Boyington JC, McTamney PM, Kong WP, Pearce MB, Xu L, Andersen H, Rao S, Tumpey TM, Yang ZY, Nabel GJ. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science.* 2010; 329(5995):1060–4. [PubMed: 20647428]
- Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature.* 1981; 289(5796):366–73. [PubMed: 7464906]
- Wong SS, Yuen KY. Avian influenza virus infections in humans. *Chest.* 2006; 129(1):156–68. [PubMed: 16424427]
- Wrammert J, Koutsouanos D, Li GM, Edupuganti S, Sui J, Morrissey M, McCausland M, Skountzou I, Hornig M, Lipkin WI, Mehta A, Razavi B, Del Rio C, Zheng NY, Lee JH, Huang M, Ali Z, Kaur K, Andrews S, Amara RR, Wang Y, Das SR, O'Donnell CD, Yewdell JW, Subbarao K, Marasco WA, Mulligan MJ, Compans R, Ahmed R, Wilson PC. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Exp Med.* 2011; 208(1):181–93. [PubMed: 21220454]
- Wright, P.; Webster, R. Orthomyxoviruses. In: Knipe, DM.; Howley, PM., editors. *Fields Virology.* 4. Vol. 2. Philadelphia: Lippincott Williams & Wilkins press; 2001. p. 1533-1579.
- Wright PF, Cherry JD, Foy HM, Glezen WP, Hall CB, McIntosh K, Monto AS, Parrott RH, Portnoy B, Taber LH. Antigenicity and reactogenicity of influenza A/USSR/77 virus vaccine in children--a multicentered evaluation of dosage and safety. *Rev Infect Dis.* 1983; 5(4):758–64. [PubMed: 6353530]
- Wu F, Huang JH, Yuan XY, Huang WS, Chen YH. Characterization of immunity induced by M2e of influenza virus. *Vaccine.* 2007; 25(52):8868–73. [PubMed: 18061317]
- Wu F, Yuan XY, Huang WS, Chen YH. Heterosubtypic protection conferred by combined vaccination with M2e peptide and split influenza vaccine. *Vaccine.* 2009a; 27(43):6095–101. [PubMed: 19056447]
- Wu F, Yuan XY, Li J, Chen YH. The co-administration of CpG-ODN influenced protective activity of influenza M2e vaccine. *Vaccine.* 2009b; 27(32):4320–4. [PubMed: 19410621]
- Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J Virol.* 1988; 62(8):2762–72. [PubMed: 2455818]
- Zhao G, Du L, Xiao W, Sun S, Lin Y, Chen M, Kou Z, He Y, Lustigman S, Jiang S, Zheng BJ, Zhou Y. Induction of protection against divergent H5N1 influenza viruses using a recombinant fusion protein linking influenza M2e to *Onchocerca volvulus* activation associated protein-1 (ASP-1) adjuvant. *Vaccine.* 2010; 28(44):7233–40. [PubMed: 20732469]

Table 1

M2-based influenza vaccines¹

Types	Carrier	Vaccination	Challenge Subtypes	References
M2	M2 protein (rBV), GST fusion (<i>E. coli</i>)	M2 (9 µg) /IFA/IP / 3 times (mice) M2-GST fusion	H1N1 (A/Taiwan/1/86), H2N2 (A/Ann Arbor/6/60), H3N2 (A/Hong Kong/1/68)	(Frace et al., 1999; Slepishkin et al., 1995)
M2	M2 DNA/rAd NP DNA/rAd vaccines	50 µg DNA/ 10 ¹⁰ particles rAd /IM (mice, ferrets)	H1N1 (A/PR/8/34, A/FM/1/47), H5N1 (A/Thailand/SP-83/04, A/Vietnam/1203/04)	(Jimenez et al., 2007; Lalor et al., 2008; Tompkins et al., 2007)
M2e	Peptide (synthetic)	M2e peptides /IFA, Aluminium/ CpG ODN (mice)	H1N1 (A/PR/8/34)	(Wu et al., 2007; Wu et al., 2009b)
M2e	Protein (conjugates)	M2e-KLH (40 µg) /CFA (mice)	H1N1 (A/PR/8/34, A/FM/1/47)	(Tompkins et al., 2007)
M2e	Protein (conjugates)	M2e-KLH, M2e-OMPC (20–100 µg) /CFA, QS1, Aluminum adjuvants (mice, ferrets, rhesus monkeys)	H1N1 (A/PR/8/34) H3N2 (X-31) H5N1 (A/Hong Kong/97)	(Fan et al., 2004)
M2e	Fusion proteins (<i>E. coli</i>)	M2e-GST (mice, rabbit) M2e-CTA1-DD (5 µg)/ IN/ (mice). M2e-tGCN4 (10 µg)/ MPL, Alhydrogel/CTA1-DD adjuvants/ IP, IN. M2e-ASP-1 (20 µg)/ IM, 3 times	H1N1 (A/PR/8/34) H3N2 (A/Victoria/3/75, X47) H5N1 (A/Vietnam/1203/04, A/Shenzhen/406H/06)	(Liu et al., 2004) (Eliasson et al., 2008) (De Filette et al., 2008) (Zhao et al., 2010)
M2e	VLPs (<i>E. coli</i>)	M2e-HBVc /CTA1-DD, LT(R192G), QS1, ISA720 adjuvants IP, IN/ twice, 3 times	H1N1 (A/PR/8/34) H3N2 (A/Victoria/3/75, X47)	(De Filette et al., 2006a; De Filette et al., 2005; Fiers et al., 2004; Heinen et al., 2002; Neirynck et al., 1999)
M2e	VLPs (<i>E. coli</i>)	M2e-PaMV (100 µg)/ Alum, M2 peptide/VLP adjuvants	H1N1 (A/WSN/33)	(Denis et al., 2008)
M2e	HPV VLPs (Yeast) conjugates	M2e-HPV (45 µg)/ Alum adjuvant/ IM, twice	H1N1 (A/PR/8/34), H3N2 (X-31, reassortant A/Aichi/68)	(Ionescu et al., 2006)
M2e	Liposome	M2e-liposomes (15–60 µg)/ MPL adjuvant/ 2 – 3 times	H1N1 (A/PR/8/34), H5N1 (A/HK/483/97), H6N2 (X-88), H9N2 (A/HK/1073/99),	(Ernst et al., 2006)
M2	M2 VLPs (rBV)	M2 VLPs (10 µg)/ IN, 2 times	H1N1 (A/PR/8/34), H3N2 (A/Philippines/82) H5N1 (reassortant A/Vietnam/1203/04)	(Song et al., 2011b)

¹ M2: whole influenza M2 protein. M2e: extracellular domain peptide of M2. IFA, incomplete Freund's adjuvant. CFA, complete Freund's adjuvant. rBV, recombinant baculovirus. rAd, IP, intraperitoneal. IN, intranasal. IM, intramuscular. KLH, keyhole limpet hemocyanin. CpG ODN, CpG oligodeoxy nucleotide adjuvants. GST, glutathione S-transferase. CTA-DD, genetic fusion protein adjuvant consisting of the cholera toxin (CT) subunit A1 (CTA1). tGCN4, four-chain coiled-coil leucine zipper domain of the yeast transcription factor *GCN4* (transgenic general control nondepressible-4). ASP-1, Onchocerca volvulus activation associated protein-1 adjuvant. MPL, monophosphoryl lipid A adjuvant. VLPs, Virus-like particles. HBVc, Hepatitis B virus core, HPV, Human papillomavirus. LT(R192G), mutant of heat-labile endotoxin adjuvant. PaMV, Petunia asteroid mosaic tombusvirus. X-88 (HA-A/Turkey/Massachusetts/3740/76, NAA/Aichi/6/68, and other genes from A/PR8/34) (H6N2). H5N1 reassortant A/Vietnam/1203/04 (HA and NA from A/Vietnam/1203/04, and other genes from A/PR8/34). Subtypes: Subtypes of challenge influenza viruses tested.

Table 2Universal influenza vaccines based on the HA protein[/]

Types	Carrier	Vaccination	Challenge Subtypes	References
Fusion Peptide	Peptide (synthetic)	Peptides (100 µg) /CFA, IFA/SC (mice)	H1N1 (A/PR/8/34)	(Horvath et al., 1998)
Fusion Peptide	Protein (conjugates)	Influenza B HA fusion Peptide-OMPC (1 µg peptides) /QS21, Aluminum adjuvants/ IM (mice)	B/Ann Arbor/4/55 B/Hong Kong/330/2001 B/Yamanashi/ 166/1998	(Bianchi et al., 2005)
Fusion Peptide	Peptide – protein conjugates	Synthetic Peptides-KLH (mice) / Broadly cross-reactive antibodies	H1-H13 subtype HA proteins	(Li et al., 2010)
Head-less HA	Transfected cells	Transfected cells (1×10 ⁶ cells) expression headless HA (A/Okuda/57 (H2N2))/IP/	H1N1 (A/FM/1/47)	(Sagawa et al., 1996)
Head-less HA	DNA/VLPs	Genetically engineered DNA/VLP vaccines/ IM, Freund's adjuvants (mice)	H1N1 (A/PR/8/34)	(Steel et al., 2010)
HA2 peptide	Peptide – protein conjugates	Synthetic Peptides-KLH (25 µg)/ SC/(mice)	H3N2 (X31), H1N1 (A/PR/8/34)	(Wang et al., 2010b)

[/] CFA, complete Freund's adjuvant. IFA, incomplete Freund's adjuvant. SC, subcutaneous. IP, intraperitoneal. IM, intramuscular. OMPC, outer membrane protein complex. KLH, keyhole limpet hemocyanin. DTT, dithiothreitol. VLPs, Virus-like particles. Subtypes: Subtypes of challenge influenza viruses tested.