Biodrugs 2006; 20 (6): 335-340
1173-8804/06/0006-0335/$39.95/0

Evolving Strategies for the Prevention of Influenza Infection

Potential for Multistrain Targeting

Brian D. Livingston, Debbie Higgins and *Gary Van Nest*

Dynavax Technologies Corp., Berkeley, California, USA

Abstract

Approved influenza vaccines based on the induction of antibodies to hemagglutinin are strain specific and cumbersome to manufacture. Several alternative vaccine strategies based on the induction of humoral responses against the external domain of the M2 protein, as well as cellular responses against nucleoprotein, have the potential to target multiple strains of influenza. A universal vaccine would be a major advancement in the prevention of influenza infection as it would alleviate the need for tailored vaccines to control seasonal influenza epidemics while simultaneously providing a level of protection against potential pandemic strains.

The mechanisms of protective immunity induced by approved influenza vaccines are well understood. Current vaccines, which contain three components representing circulating influenza A strains H1N1 and H3N2 and an influenza B virus, effectively stimulate strong antibody responses against the viral surface glycoproteins. The antibody response is primarily directed against the hemagglutinin (HA), which confers sterilizing immunity by preventing viral infection. To a lesser degree the vaccines stimulate responses against the viral neuraminidase (NA), which inhibit release of progeny virus from the infected host cells. Annual immunization effectively prevents homologous influenza infection in children and adults, although the vaccines are less effective in elderly patients.

One shortcoming of the current influenza vaccines relates to the inability to provide protection against influenza strains that are antigenically distinct. Influenza A viruses are subtyped on the basis of antigenicity of the viral surface proteins; 16 HA and 9 NA subtypes are known to exist. While relatively few subtypes infect humans, HA subtypes H1, H2, and H3 and NA subtypes N1 and N2 have widely circulated in the population, and there is significant amino acid sequence variation in HA and NA of individual subtypes. The high mutation rate associated with transcription of the viral RNA genome results in the continuous introduction of amino acid changes in the viral antigens, particularly in the regions of the HA and NA that are exposed to selective immune pressure. Analysis of mutation frequencies has found that typically three to four amino acid substitutions are introduced per year.[1] The antigenic drift resulting from the accumulation of amino acid substitutions reduces vaccine efficacy; consequently the components of the vaccine must be periodically updated to match the anticipated predominant circulating strains of influenza. This seasonal production of influenza vaccine is costly, requiring a reassortment with a high-yield strain or, in the case of the live attenuated vaccine, a cold-adapted strain to generate a master seed strain for manufacturing. These complexities in manufacturing have resulted in limited vaccine availability. Moreover, this production process is lengthy, requiring up to 9 months to complete, which precludes easily adapting the vaccine to include influenza strains corresponding to the prevailing strains in the general population. As recently as the 2003–4 influenza season the vaccine composition ineffectively reflected the principal infective strain, which resulted in suboptimal vaccine protection.[2]

A graver problem with the current vaccine strategy concerns treating potential pandemic strains of influenza. Influenza pandemics occur when viral strains expressing an HA for which individuals have no pre-existing immunity gain the ability to productively infect the human population. The entry of the H1N1 subtype into the human population in 1918 was responsible for >40 million deaths worldwide.[3] Subsequent pandemic outbreaks in 1957 and 1968, corresponding with the emergence of H2N2 and H3N2 viruses, respectively, were also associated with high mortality rates.[4]

Aquatic birds can be infected with all known influenza A strains. Most infections in waterfowl do not result in death, allowing these animals to serve as reservoirs of viral selection and reassortment as a result of co-infection with multiple influenza subtypes. This reservoir has the capability to produce an avian influenza strain that can enter into the human population and trigger a pandemic. In the last decade there have been over ten documented cases of transmission of avian influenza strains to humans.[5] These infections have encompassed a diversity of influenza subtypes including H5N1,[6,7] H7N7,[8,9] as well as H9N2.[10,11] While in most cases infections were restricted to <100 individuals, the recurring instances of avian influenza infection in humans serve as a reminder of the ever-present risk that an influenza strain expressing a novel HA, and possibly NA, that can be effectively spread between humans may enter the global population. While forecasts on the spread and mortality resulting from the emergence of a pandemic influenza strain differ, most estimates agree the impact on public health would be severe. Although antiviral therapies can control disease if administered prior to or early in infection, the practicalities of drug stockpiling and distribution would likely limit their effective application in a scenario of a quickly spreading influenza pandemic.[12] Consequently, vaccination represents the most effective means of combating infection; however, applying the current vaccine strategy to the development of a pandemic influenza vaccine reiterates the problem of strain selection. There is no way to predict which influenza strain will cause the next pandemic. A virus originating from avian reservoirs could express a variety of HA and NA antigens. In addition, there is the potential for the re-emergence of an H2N2 strain in humans. Since this viral subtype exited human circulation in 1968, a growing proportion of the population lacks the immunologic memory to provide protection from infection with this influenza subtype.

The development of a pandemic vaccine is further complicated by the relatively poor immunogenicity of vaccines based on avian influenza isolates. Clinical trials with subunit vaccines designed to target H5N1 subtypes found that at least two doses were required to induce a neutralizing antibody response.[13] Combination of the vaccine with MF-59 adjuvant significantly increased the seroconversion rates and geometric mean antibody titers, and reduced the required antigen dose; nonetheless, two doses of vaccine were required to provide a level of response expected to provide protection.[14] Similar results have been found with experimental vaccines targeting H9N2 influenza subtypes.[15,16]

The challenges in the production of vaccines against possible pandemic strains, as well as the limitations of the approved vaccines against circulating strains, could be addressed by the development of a universal influenza vaccine with multistrain targeting capacity. There are a number of points in the viral life cycle that could be targeted by vaccines with multistrain application (figure 1). These include humoral-based vaccine strategies that could prevent influenza attachment to the respiratory epithelial cell or release of the progeny virus, as well as vaccines designed to elicit cellular immunity that could reduce the levels of influenza replication by lysis of the infected cell.

1. Humoral Immunity-Based Strategies for Targeting Multiple Influenza Strains

As vaccine-induced antibody responses clearly prevent influenza infection, the ideal universal vaccine would elicit a humoral response directed against a conserved influenza antigen. Although the distal immunodominant receptor binding site of HA is variable and subject to continual antigenic drift, the portion of antigen proximal to the viral membrane exhibits a higher degree of conservation, ranging from 50% to 80% homology between subtypes. For antibody-mediated protection to be effective the epitope must be accessible for binding. These membrane proximal conserved regions are exposed in the HA precursor present on the plasma

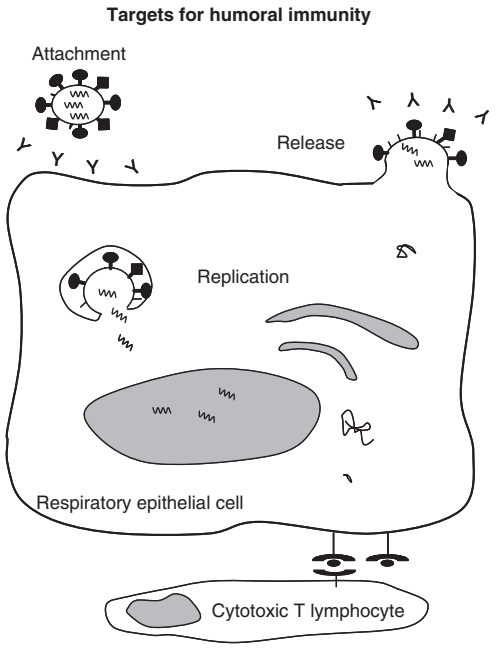

Fig. 1. Points in the influenza life cycle that could be targeted by potential multistrain vaccines. Targets for humoral immunity include conserved antigens accessible on the virion or on the membrane of the infected cell. Vaccine-induced antibodies recognizing these targets could confer sterilizing immunity by preventing attachment or ameliorate disease by limiting the amount of progeny virus released. Vaccines designed to induce cellular immunity might also provide a means of controlling infection by reducing viral replication through the elimination of infected cells by cytotoxic T lymphocytes.

membrane prior to cleavage by extracellular proteases, which induces a conformational change that conceals this portion of the antigen. Murine studies using a multiple-antigenic peptide corresponding to the conserved cleavage site found that this immunogen did confer a degree of protection to influenza A/PR/8/34 infection.[17,18] Other conserved antibody determinants may exist in HA; however, use of these regions in vaccine design may be complicated by additional conformational considerations.

An alternative influenza surface antigen that may serve as a target for humoral-based vaccine strategies capable of inducing immunity against multiple strains of influenza is the matrix 2 protein (M2). This 97 amino acid transmembrane protein forms homotetramers that function as ion channels involved in the release of the influenza ribonucleoprotein complex from the viral membrane after fusion. In addition, they are involved in regulating the pH in the vesicles where the viral antigens undergo maturation as they are transported from the endoplasmic reticulum to the plasma membrane of the host cells.[19,20] M2 is expressed in the plasma membrane of infected cells at high density, similar to that of HA molecules; however, the density of M2 is reduced during the release of progeny virus.[21] On average, influenza contains 10 M2 tetramers per virion compared with approximately 400 HA trimers and 100 NA tetramers.[22] The antigenic contribution of M2 is further reduced by the relatively small size of the ectodomain, referred to as M2e, which consists of 23 amino acids. In comparison, the ectodomains of HA and NA consist of over 500 and 400 amino acids, respectively. As a consequence of these factors, M2e-specific antibodies are rarely observed following influenza infection and antibodies directed against M2e fail to bind viral particles or prevent infection.[23] Nevertheless, M2e antibodies recognize influenza-infected cells and reduce plaque size, an effect attributed to an alteration in the localization of the antigen during the release of virus from the infected cell. Despite the inability to provide sterilizing immunity, passive transfer of M2e-specific antibodies protects against lethal influenza challenge in animal models.[24]

The potential for developing humoral-based vaccine strategies capable of targeting multiple influenza strains on induction of M2e antibodies is particularly compelling given the high degree of amino acid conservation for this antigen. Detailed analysis of hundreds of M2e sequences has found that the nine N-terminal and four C-terminal amino acids are nearly invariant in all influenza subtypes, including H1–H7 and H9 isolates.[25] Amino acids 10–20 exhibit a greater degree of variability, on average approximately 75% conservation; however, the variation is largely a reflection of host restriction. Consensus M2e sequences from human influenza strains are highly conserved. For example, M2e in A/New Caledonia/20/99 (H1N1) and A/California/7/2004 (H3N2), the influenza strains used in the 2005–6 vaccine formulation, have complete sequence identity. Analysis of the 1918 pandemic H1N1 influenza reveals that there is only one amino acid substitution compared with the currently utilized vaccine strains. Antibodies directed against M2e from human influenza strains, such as PR8, are able to react with M2e peptides representing a variety of human isolates.[25] Moreover, experimental data suggest that the extent to which M2e can mutate to escape immune pressure may be limited. Zharikova and colleagues[26] have found that M2e mutants isolated from severe combined immune deficiency (SCID) mice protected from influenza infection by passive transfer of M2e-specific antibodies were limited to one of two amino acid substitutions at distinct positions.

In order to develop a truly universal influenza vaccine, one capable of providing protection against both circulating human influenza strains and potential pandemic strains, the vaccine will probably need to incorporate peptides to account for the variation found in M2e from avian influenza subtypes. An avian M2e consensus sequence, based primarily on H5, H7, and H9 subtypes, bears three notable amino acid substitutions, compared with the human consensus, within the 15 N-terminal residues containing the primary epitope recognized by protective antibodies.[25] Antibodies against the human influenza M2e sequence do not effectively recognize avian-derived peptides containing an isoleucine for threonine substitution at position 11.[25,27] These results suggest that a single M2e peptide may not be able to provide universal protection against all influenza subtypes or potential escape mutants; however, given the small size of the peptide and the limited number of peptides that would need to be incorporated into the vaccine, a number of feasible designs are conceivable.

The benefits of M2 vaccination were initially demonstrated in murine studies that found that immunization with recombinant full-length M2 was able to confer protection from homologous and heterologous influenza challenge.[28] Subsequent vaccine studies have largely focused on the induction of M2e-specific antibody response using peptide conjugate vaccines consisting of M2e recombinantly or chemically linked to carrier proteins such as hepatitis B virus core antigen,[29] glutathione-S transferase (GST),[30] keyhole limpet hemocyanin, or Neisseria meningitidis outer membrane protein complex.[27] These vaccines are immunogenic in a variety of animal models, inducing M2e-specific antibody responses when delivered intramuscularly, intraperitoneally, or intranasally.[27,31,32] The majority of these M2e conjugate vaccines are inherently active; however, the immunogenicity is dependent on vaccine structure and formulation. Epitope density appears to play a significant role as constructs bearing multiple copies of M2e exhibit enhanced immunogenicity. GST fusion proteins containing 16 copies of M2e are immunogenic after a

single immunization in either mice or rabbits, whereas related constructs bearing four copies are comparatively poorly immunogenic.[33] Similar results have been observed with synthetic multiple-antigenic peptide vaccines.[34] As has been the case historically in the field of vaccine development, use of an adjuvant-containing formulation increases immunogenicity, and a variety of animal studies have found that delivery of M2e vaccine constructs with adjuvants such as alum, Montanide ISA-720, QS-21, or CpG-containing immunostimulatory oligonucleotides increases antibody titers.[27,31,34]

The multistrain targeting potential of M2e-based vaccine strategies and the passive transfer of nonhuman primate serum containing high M2e antibody titers has been demonstrated in mice and ferrets.[27,30] In these studies, animals exhibit reduced viral replication in the lower respiratory tract, accelerated influenza clearance, and are protected against both H1 and H3 influenza subtypes. Protection appears to correlate with a high IgG2a titer, suggesting that T-helper type 1 (T_h1) responses play an important role in protection.[32,35] Not surprisingly, given the expression and distribution of the target antigen, M2e vaccines do not prevent morbidity; animals exhibit significant weight loss following challenge. The relative severity of these effects are difficult to extrapolate to a clinical situation as the influenza challenge doses in animal models typically far exceed the levels of naturally occurring exposure in humans. The clinical efficacy of M2e vaccination remains untested in humans; however, the data from multiple animal model systems support the rationale and the potential for the development of an easily manufactured vaccine that would not require annual updating and merits investigation in human clinical trials.

2. Cellular Immunity-Based Strategies for Targeting Multiple Influenza Strains

Although cellular immune responses are known to contribute to the control of numerous viral infections, their role in protection against influenza infection remains controversial. Influenza-specific CD8+ cytotoxic T lymphocytes (CTL) limit viral replication and protect against challenge in murine models.[36-38] Mice lacking MHC class I molecules have delayed viral clearance and increased mortality after influenza challenge.[39] Moreover, the magnitude of the CTL response has been shown to directly correlate with the extent of protective immunity.[40] In humans, influenza-specific T-cell responses are correlated with improved viral clearance.[41] Despite the data indicating the contribution of cellular immune responses in the control of influenza infection, under some circumstances these responses may in fact have detrimental effects. In a T-cell receptor transgenic mouse model, where approximately 90% of the peripheral T cells are specific for a nucleoprotein (NP) epitope, high titer viral challenges resulted in exacerbated viral pathology, an effect attributed to the production of inflammatory cytokines by lymphocytes infiltrating the airway epithelium; however, at lower challenge doses, the presence of NP-specific T cells enhanced viral clearance.[42]

While antibody responses to influenza are primarily directed against the variable surface antigens, cellular immune responses are frequently directed against internal viral antigens, which are highly conserved between divergent strains of both human and avian influenza subtypes. For example, the NP sequence of A/PR/8/34 (H1N1) bears approximately 90% amino acid identity to a consensus NP sequence generated based on nearly 1000 influenza strains isolated since 1990. Furthermore, the amino acid sequence variation in NP sequences between subtypes is typically conserved. Amino acid substitutions commonly found in the immunodominant H-2^b-restricted CD8+ T-cell epitope present in A/PR/8/34 (H1N1), $NP_{366-374}$, have little impact on MHC class I binding.[43] Cross-protection studies in mice have shown that recognition of epitopes in the NP protein can confer heterosubtypic immunity. Immunization with an H9N2 virus expressing an NP antigen 98% homologous to the NP of a challenge H5N1 virus results in protection that is T-cell mediated.[43] Related murine studies have found that the recall CD8+ T-cell memory established by immunization with an H1N1 virus provides increased protection from lethal H7N7 virus challenge.[44]

DNA immunization studies have provided perhaps the most direct evidence on the potential role of cellular immune responses to confer protective efficacy to influenza infection. In animal models, *in situ* antigen expression resulting from DNA immunization effectively induces broad cellular immune responses.[45] Mice immunized with DNA encoding the A/PR/8/34 (H1N1) NP have decreased viral lung titers, less weight loss, and increased survival following heterologous challenge with A/HK/68 (H3N2).[46] While DNA immunization resulted in the induction of NP-specific antibodies, *in vivo* depletion and passive transfer experiments clearly demonstrated that the CD8+ T cells were the effectors of protection.[47] Collectively these data suggest that cellular immune responses play a significant role in controlling influenza infection and support the rationale of developing vaccines designed to induce cellular immune responses directed against conserved antigens that will confer a degree of protection against multiple strains of influenza.

To date, clinical studies have not been conducted with a vaccine designed to solely induce a cellular immune response in the absence of a neutralizing antibody response; however, human data also suggest that cellular immune responses play an important role in controlling influenza infection. Recent studies have shown that the expansion of CD8+ T cells is reduced in the elderly and that

these responses are better correlates of vaccine protection in this more difficult-to-treat population.[48,49] As for the extent to which cellular immune responses may provide a degree of multistrain protection, human studies have observed that previously infected individuals are partially protected against subsequent infection with a heterosubtypic virus.[50,51] As compared with traditional vaccines, live attenuated vaccines might be more effective at inducing CTL responses, although definitive data have not been reported. Alternatively, the co-delivery of conserved influenza antigens, such as NP, or even individual epitopes, formulated with adjuvants that would promote a cellular immune response, could represent an effective strategy for augmenting this facet of the immune response.

3. Conclusions

In contrast to traditional influenza vaccines, neither the M2e nor the cellular immunity vaccine approaches discussed would provide sterilizing immunity. Consequently, combination approaches may ultimately be required to provide effective protection from infection by the spectrum of influenza subtypes. The induction of coordinated humoral and cellular immune responses against conserved viral antigens would be a powerful approach to address an unmet need in the prevention of both seasonal and potential pandemic influenza infections.

Acknowledgments

No sources of funding were used to assist in the preparation of this review. The authors have no conflicts of interest that are directly relevant to the content of this review.

References

1. Smith DJ, Lapedes AS, de Jong JC, et al. Mapping the antigenic and genetic evolution of influenza virus. Science 2004; 305 (5682): 371-6
2. Prevention CfDCa. Update: influenza activity United States, 2004-2005 season [online]. Available from URL: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5408a1.htm [Accessed 2006 Nov 6]
3. Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med 2002; 76 (1): 105-15
4. Cox NJ, Subbarao K. Global epidemiology of influenza: past and present. Ann Rev Med 2000; 51: 407-21
5. Horimoto T, Kawaoka Y. Influenza: lessons from past pandemics, warnings from current incidents. Nat Rev Microbiol 2005; 3 (8): 591-600
6. Subbarao K, Klimov A, Katz J, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 1998; 279 (5349): 393-6
7. Claas EC, Osterhaus AD, van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998; 351 (9101): 472-7
8. Fouchier RA, Schneeberger PM, Rozendaal FW, et al. 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
9. Koopmans M, Wilbrink B, Conyn M, et al. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 2004; 363 (9409): 587-93
10. Guo YJ, Krauss S, Senne DA, et al. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 2000; 267 (2): 279-88
11. Peiris M, Yuen KY, Leung CW, et al. Human infection with influenza H9N2. Lancet 1999; 354 (9182): 916-7
12. Kandel R, Hartshorn KL. Prophylaxis and treatment of influenza virus infection. BioDrugs 2001; 15 (5): 303-23
13. Treanor JJ, Wilkinson BE, Masseoud F, et al. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine 2001; 19 (13-14): 1732-7
14. Nicholson KG, Colegate AE, Podda A, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001; 357 (9272): 1937-43
15. Stephenson I, Nicholson KG, Gluck R, et al. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet 2003; 362 (9400): 1959-66
16. Hehme N, Engelmann H, Kunzel W, et al. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. Med Microbiol Immunol (Berl) 2002; 191 (3-4): 203-8
17. Bianchi E, Liang X, Ingallinella P, et al. Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. J Virol 2005; 79 (12): 7380-8
18. Horvath A, Toth GK, Gogolak P, et al. 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
19. Sugrue RJ, Hay AJ. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology 1991; 180 (2): 617-24
20. Steinhauer DA, Wharton SA, Skehel JJ, et al. Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. Proc Natl Acad Sci U S A 1991; 88 (24): 11525-9
21. Lamb RA, Zebedee SL, Richardson CD. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell 1985; 40 (3): 627-33
22. 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
23. Hughey PG, Roberts PC, Holsinger LJ, et al. Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology 1995; 212 (2): 411-21
24. Treanor JJ, Tierney EL, Zebedee SL, et al. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol 1990; 64 (3): 1375-7
25. Liu W, Zou P, Ding J, et al. Sequence comparison between the extracellular domain of M2 protein human and avian influenza A virus provides new information for bivalent influenza vaccine design. Microbes Infect 2005; 7 (2): 171-7
26. Zharikova D, Mozdzanowska K, Feng J, et al. Influenza type A virus escape mutants emerge in vivo in the presence of antibodies to the ectodomain of matrix protein 2. J Virol 2005; 79 (11): 6644-54
27. Fan J, Liang X, Horton MS, et al. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine 2004; 22 (23-24): 2993-3003
28. Slepushkin VA, Katz JM, Black RA, et al. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine 1995; 13 (15): 1399-402
29. Neirynck S, Deroo T, Saelens X, et al. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999; 5 (10): 1157-63
30. Frace AM, Klimov AI, Rowe T, et al. Modified M2 proteins produce heterotypic immunity against influenza A virus. Vaccine 1999; 17 (18): 2237-44
31. De Filette M, Min Jou W, Birkett A, et al. Universal influenza A vaccine: optimization of M2-based constructs. Virology 2005; 337 (1): 149-61
32. De Filette M, Fiers W, Martens W, et al. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. Vaccine. Epub 2006 Jun 12

33. Liu W, Peng Z, Liu Z, et al. 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
34. Mozdzanowska K, Feng J, Eid M, et al. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. Vaccine 2003; 21 (19-20): 2616-26
35. Fiers W, De Filette M, Birkett A, et al. A "universal" human influenza A vaccine. Virus Res 2004; 103 (1-2): 173-6
36. Mackenzie CD, Taylor PM, Askonas BA. Rapid recovery of lung histology correlates with clearance of influenza virus by specific CD8+ cytotoxic T cells. Immunology 1989; 67 (3): 375-81
37. Taylor PM, Askonas BA. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. Immunology 1986; 58 (3): 417-20
38. Kuwano K, Tamura M, Ennis FA. Cross-reactive protection against influenza A virus infections by an NS1-specific CTL clone. Virology 1990; 178 (1): 174-9
39. Bender BS, Croghan T, Zhang L, et al. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J Exp Med 1992; 175 (4): 1143-5
40. Fu TM, Guan L, Friedman A, et al. Dose dependence of CTL precursor frequency induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge. J Immunol 1999; 162 (7): 4163-70
41. McMichael AJ, Gotch FM, Noble GR, et al. Cytotoxic T-cell immunity to influenza. N Engl J Med 1983; 309 (1): 13-7
42. Moskophidis D, Kioussis D. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. J Exp Med 1998; 188 (2): 223-32
43. O'Neill E, Krauss SL, Riberdy JM, et al. Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice. J Gen Virol 2000; 81 (Pt 11): 2689-96
44. Christensen JP, Doherty PC, Branum KC, et al. Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8 (+) T-cell memory. J Virol 2000; 74 (24): 11690-6
45. Whitton JL, Rodriguez F, Zhang J, et al. DNA immunization: mechanistic studies. Vaccine 1999; 17 (13-14): 1612-9
46. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259 (5102): 1745-9
47. Ulmer JB, Fu TM, Deck RR, et al. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J Virol 1998; 72 (7): 5648-53
48. Deng Y, Jing Y, Campbell AE, et al. Age-related impaired type 1 T cell responses to influenza: reduced activation ex vivo, decreased expansion in CTL culture in vitro, and blunted response to influenza vaccination in vivo in the elderly. J Immunol 2004; 172 (6): 3437-46
49. McElhaney JE, Xie D, Hager WD, et al. T cell responses are better correlates of vaccine protection in the elderly. J Immunol 2006; 176 (10): 6333-9
50. Sonoguchi T, Naito H, Hara M, et al. Cross-subtype protection in humans during sequential, overlapping, and/or concurrent epidemics caused by H3N2 and H1N1 influenza viruses. J Infect Dis 1985; 151 (1): 81-8
51. Epstein SL. Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. J Infect Dis 2006; 193 (1): 49-53

Correspondence and offprints: Dr *Brian D. Livingston,* Dynavax Technologies Corp., 2929 Seventh St., Ste. 100, Berkeley, CA 94710, USA.
E-mail: blivingston@dvax.com