Published in final edited form as:
Hum Vaccin. 2010 February 24; 6(2): .

Egg-independent vaccine strategies for highly pathogenic H5N1 influenza viruses

Aseem Pandey, **Neetu Singh**, **Suryaprakash Sambhara**[1,§], and **Suresh K. Mittal**[§]
Department of Comparative Pathobiology, School of Veterinary Medicine, and Bindley Bioscience Center, Purdue University, West Lafayette, IN, USA

[1] Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

The emergence of a highly pathogenic H5N1 influenza virus in Hong Kong in 1997 and the subsequent appearance of other H5N1 strains and their spread to several countries in south-east Asia, Africa, the Middle East, and Europe has evoked fear of a global influenza pandemic. Vaccines offer the best hope to combat the threat of an influenza pandemic. However, the global demand for a pandemic vaccine cannot be fulfilled by the current egg-based vaccine manufacturing strategies, thus creating a need to explore alternative technologies for vaccine production and delivery. Several egg-independent vaccine approaches such as cell culture-derived whole virus or subvirion vaccines, recombinant protein-based vaccines, virus-like particle (VLP) vaccines, DNA vaccines and viral vector-based vaccines are currently being investigated and appear promising both in preclinical and clinical studies. The present review will highlight the various egg-independent alternative vaccine approaches for pandemic influenza.

Keywords

H5N1 influeza; cell-derived vaccine; egg-independent; pandemic influenza; viral vector

INTRODUCTION

Currently circulating H5N1 avian influenza viruses (“avian flu” or “bird flu”) represent a potential pandemic threat, as it is only a matter of time before these viruses acquire the necessary genetic changes enabling efficient human-to-human transmission.1 H5N1 viruses have diverged into antigenically distinct clades and subclades, adding another “unknown” to the nature of the H5N1 pandemic influenza strain.2 The global fatality rate of 75% due to H5N1 infections in humans was alarmingly high in 2008.3 It is estimated that a 1918-like influenza pandemic could result in over 50 million deaths worldwide even though the availability of health care facilities are far better than they were nine decades ago.4

We are presently in the midst of an ongoing 2009 H1N1 influenza A pandemic5 which has spread to more than 100 countries with approximately 134,000 laboratory-confirmed cases and 816 deaths reported to date.6 This pandemic is providing real-time information regarding the dynamics of a modern day pandemic, fortunately without a high fatality rate. The sudden emergence (April 2009) and unprecedented global spread (WHO declared a

[§]Correspondence: Suresh K. Mittal, Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA, Tel: 765-496-2894, Fax: 765-494-9830, mittal@purdue.edu, Suryaprakash Sambhara, Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA, Tel: 404-639-3800, Fax: 404-639-5180, ssambhara@cdc.gov.

pandemic in June 2009) of this new H1N1 virus in less than 3 months as compared to approximately a year as seen in the timelines of previous pandemics7 demonstrate the role of human mobility leading to the rapid emergence of an influenza pandemic. Certainly the current pandemic is testing the effectiveness of pandemic preparedness plans (devised for a potential H5N1 pandemic) with regard to early detection and containment of disease outbreaks, school closures, travel restrictions, effective coordination among various agencies, and the availability of effective vaccines for mass immunization. It has also exposed the difficulties in implementing some of the measures for containing the spread of the virus.

New H1N1 influenza vaccine production in eggs (the conventional method) is ongoing, and it is anticipated that over 120 million doses will be available in the U.S. before the regular flu season. However, vaccine manufacturers in their early attempts to grow the virus in eggs have reported issues with virus yields.8 A single dose of the new H1N1 vaccine may not elicit a strong enough protective immune response suggesting the requirement for a two-dose formulation. In a pandemic situation where timely manufacture of vaccines is extremely important, it is very clear that conventional egg-based vaccine production is not the solution for a highly virulent human pandemic strain such as H5N1. The timeline from strain identification to vaccine availability is about 4–6 months, a duration which could prove fatal in a pandemic.

The development of a timely, cost-effective vaccine represents a major challenge in preparing for a possible H5N1 influenza pandemic. With existing manufacturing capacity, 413 million doses of a trivalent seasonal influenza vaccine are being produced every year for the entire world population9. The number of doses of a monovalent pandemic influenza vaccine given at a similar dosage level as the trivalent seasonal influenza vaccine would be 1,239 million doses. In a pandemic situation, the vaccine demand will be at least 5–10-fold that of the current global seasonal influenza vaccine production capability and achieving this target is further challenged by the low yield per egg of H5N1 vaccine strains and the need to have multiple inoculations to elicit protective levels of immunity. Moreover, the supply of embryonated eggs for vaccine production may be adversely affected in an H5N1 pandemic since the virus is lethal to egg-laying poultry. Apart from production issues, delivery of a vaccine to the global population also poses a challenge further compounded by the fact that the majority of vaccine manufacturers are located in developed countries.

Varied strategies for egg-independent vaccine production will greatly expand existing manufacturing capacity and offer flexibility for rapid scale-up in the case of an impending pandemic. Vaccine manufacturers have obtained comparable or higher virus yields in well-characterized certified cell lines compared to those obtained in embryonated eggs.10,11 Furthermore, the development and use of a serum/protein free synthetic media to grow cells has also minimized a potential safety concern regarding the spread of transmissible spongiform encephalopathies or other adventitious agents.12 To increase the global influenza vaccine production capacity, different vaccine approaches should be considered.13,14 The following sections will highlight various egg-independent vaccine approaches for pandemic preparedness.(Fig 1).

CELL-DERIVED VACCINES

The vaccine industry currently possesses the necessary technical expertise for the large scale production of vaccines in certified cell lines, and major vaccine manufacturers are at various stages of developing cell-derived vaccines for seasonal and pandemic influenza.15–21 In 2006, in response to a potential pandemic threat, the U.S. Department of Health and Human Services (DHHS) awarded contracts to GlaxoSmithKline, MedImmune, Novartis Vaccines

& Diagnostics, Sanofi Pasteur and DynPort Vaccine to develop cell-based influenza vaccines in the US.22 More recently, DHHS awarded a contract to Novartis to establish the first U.S. facility to manufacture cell-based vaccines for seasonal and pandemic influenza.23 Novartis has already licensed their first cell based seasonal flu vaccine “Optaflu” for the European market.24

Conventional egg-based influenza vaccines involve the annual selection of influenza virus strains that are closely matched antigenically to the circulating epidemic viruses. Reassortant viruses are generated containing the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) gene segments from the closely matched circulating strain and the other six gene segments from a high growth master donor strain, A/PR/8/34 (A/Puerto Rico/8/34). High-growth influenza B donor strains do exist (e.g., B/Lee/40), however, the reassortants do not generally grow better than the wild-type viruses. A wild-type strain is therefore used for the influenza B component of the vaccine that necessitates adaptation to eggs for high growth. Adaptation of influenza viruses has been shown to induce amino acid changes in the hemagglutinin protein.25,26,27

Although antigenic variants due to egg adaptation are detected early in vaccine seed virus selection, generating reassortants sometimes poses problems due to poor growth of the original virus in eggs. However, the viruses grown in cell culture usually maintain their antigenicity.28–30 A comparative study between an inactivated influenza vaccine grown in Madin-Darby canine kidney (MDCK) cells and an influenza vaccine grown in eggs suggested that the cell culture-derived vaccine induced a more robust humoral immune response leading to superior protection against challenge in a ferret model compared with egg-grown vaccine.31 However, a clinical trial demonstrated comparable immune responses with an influenza subunit vaccine produced either in MDCK cells or eggs.32 Other significant advantages of cell-derived influenza vaccines are the reduced risk of exogenous contamination in closed scalable bioreactors, and, more importantly, their independence from a continuous egg supply from certified poultry flocks.33

However, potential introduction of adventitious agents or oncogenic elements at any stage of the vaccine manufacturing process would compromise the vaccine safety, and therefore, this concern has to be addressed for all cell-based vaccine production systems. Adventitious agents in influenza vaccines may originate from the original clinical sample or be introduced during growth of the reference strains in eggs. Removal of intact cells and degradation of residual DNA during vaccine processing are some of the required quality control measures to minimize the risk of oncogenicity.34 A risk-assessment model demonstrated that MDCK cell culture-based subunit influenza vaccine manufacturing process is highly efficient in minimizing the risk of adventitious agent to a million-fold below infectious levels.34 Similar to embryonated eggs, MDCK cells also act as an effective filter for a wide range of adventitious agents that might be introduced during vaccine production.34

At present, African green monkey kidney (Vero), MDCK and human PER.C6 cell lines are approved to produce influenza vaccines, and several cell culture-derived influenza vaccines have been tested in pre-clinical and clinical studies.35–37 A cell culture-derived inactivated whole virus vaccine containing clade 1 (A/Vietnam/1203/04) and clade 2 (A/Indonesia/05/05) viruses induced cross-clade protective responses against divergent H5N1 viruses in a mouse model.36 A Vero cell-derived inactivated whole virus (A/Vietnam/1203/2004) vaccine produced in a BSL3 facility was shown to be safe and well tolerated in humans.38 In this study, a two-dose regimen of 7.5 microgram of vaccine formulation without an adjuvant resulted in virus neutralization titers of ≥ 20 in 76% of the subjects by day 42. Furthermore, cross-neutralizing antibody titers (≥ 20) were observed in 76% of subjects against A/Hong Kong/156/97 (clade 0) and in 45% against A/Indonesia/05/05 (clade 2).38

Two phase III trials by Baxter are currently underway to assess the immune responses as well as the safety and tolerability of an H5N1 influenza vaccine (whole virion, Vero cell-derived inactivated influenza vaccine containing H5N1 HA antigen) in adult and elderly populations.39,40

Immune responses for cell culture-derived vaccine appear to be encouraging in both animal and human studies. With the advantages of a reduced risk of contamination in closed scalable bioreactors, they are presently being researched by major pharmaceutical manufacturers. However, great care must be taken to address the issue of possible introduction of adventitious agents or oncogenic elements in the manufacturing process.

RECOMBINANT PROTEIN-BASED VACCINES

The technology of baculovirus expressed recombinant proteins produced in cultured insect cells is a viable means to generate large amounts of recombinant immunogenic proteins.41 In the case of influenza, this approach can result in large amounts of purified recombinant HA protein devoid of egg-derived allergens in a short duration (6–8 weeks) following identification of the target influenza strain.42 Baculoviruses have a narrow host range, and therefore, do not replicate in mammalian cells, but are capable of efficiently transducing them.43 In addition, the protein expression is very efficient with one of the strongest promoters (polyhedrin) found in the nature.44 Baculovirus recombinant proteins expressed in insect cells are correctly folded since these cells also support many of the posttranslational modifications that occur in mammalian cells.45 However, insect cell-based production of recombinant glycoproteins suffer a major limitation due to the lack of complex-type *N*-glycans containing terminal sialic acid residues.45 This may affect the immunogenicity of insect cell-derived influenza antigens. Nevertheless, several approaches are being pursued to address this issue.46,47

Baculovirus expressed recombinant HA has also been shown to be well tolerated and immunogenic in a number of clinical trials.42,48–50 A seasonal trivalent recombinant HA vaccine from Protein Science was safe and immunogenic in a healthy adult population, although at a three-fold higher dose rate than that of an egg-derived seasonal flu vaccine. Nonetheless, it demonstrated the potential feasibility of this approach to be used for the development of a pandemic flu vaccine.51 In another study, a two dose regimen (90 microgram) of a purified recombinant HA derived from a clade 0 H5N1 virus (A/Hong Kong/156/97) was well tolerated, and 52% of the naive subjects generated potentially protective titers of 1:80 by day 42.52 In a follow up study after 8 years involving the same subjects (now primed), a single dose of an egg-derived inactivated subvirion clade 1 vaccine (RG A/Vietnam/1203/04) generated seroconversion (≥1:40) in 68% of the primed subjects compared to 43% of unprimed subjects receiving two doses of 90 micrograms of the vaccine, suggesting the importance of priming the population during a pre-pandemic period. 53

Clinical trials have shown the potential of recombinant protein-based vaccines as a viable potential pandemic influenza vaccine. To enhance the immunogenicity and dose sparing of recombinant HA protein-based vaccine formulations, the use and development of novel and improved adjuvants with an enhanced safety profile will be important. The addition of conserved internal protein/s in the vaccine formulation containing recombinant HA may further broaden the vaccine efficacy.

VIRUS-LIKE PARTICLES (VLP)-BASED VACCINES

Virus-like particles (VLP) are composed of highly organized particles that self-assemble from virus-derived structural proteins and are devoid of the viral nucleic acid. Currently, this

approach is getting much attention for a pandemic influenza vaccine owing to its safety and immunogenic attributes. A VLP vaccine mimics live virus with regard to its interactions at the cellular level but is completely non-infectious. VLP have been shown to possess excellent adjuvant properties capable of enhancing humoral and cellular immune responses.54 They can be produced in established protein expression host systems such as yeast, insect or mammalian cells. The baculovirus expression system and insect cells form the most promising VLP technology for viral vaccines.55,56

The availability of a VLP-based vaccine for human papillomavirus (Gardasil) has demonstrated the safety of this approach for human use. A H5N1 influenza VLP vaccine expressing HA, NA & M1 of clade 1 (A/VN/1203/2004) or clade 2 (A/Indonesia/05/2005) was used to immunize mice with two doses (0.6 or 3 microgram), and it protected mice against lethal challenge with homologous and heterologous reassortant H5N1 viruses.57 Interestingly, a single immunization with either of the two vaccine amounts was also protective even in the absence of detectable neutralizing antibodies. Further, the immune responses generated by the clade 1 VLP vaccine (A/Vietnam/1203/04) was shown to be long lasting (30 weeks) and protected mice against homologous H5N1 challenge.58

A candidate clade 2 influenza VLP vaccine induced hemagglutination inhibition (HI) antibodies against the homologous H5N1 clade 2.1 strain, as well as heterologous strains from H5N1 clades 1, 2.2, and 2.3 in a dose-dependent manner in ferrets.59 The vaccine was protective against homologous or heterologous virus challenge even at a low vaccine dose (0.6 microgram of HA) given twice. The vaccine also reduced the virus shedding in the vaccinated animals.59 More recently, mucosal administration of a VLP vaccine containing HA, NA and M1 of the 1918 H1N1 virus not only protected against lethal challenge with the reconstructed 1918 H1N1 virus but also offered heterosubtypic cross-protection against a lethal H5N1 virus challenge in mice and ferrets.60

A Phase I/IIa clinical trial for evaluation of safety and immunogenicity of a clade 2 H5N1 (A/Indonesia/05/2005) VLP vaccine showed that it was well tolerated and immunogenic.10 The study evaluated individuals from 18 to 40 years of age who received two injections of 15, 45, or 90 micrograms of the vaccine or a placebo. There were dose-dependent increases in HI titers. In the 90 microgram group, 63% of subjects achieved a four-fold or greater (≥1:40) rise in HI titers compared to the baseline.10 A multivalent VLP incorporating HA from different subtypes broadened the protection coverage.61

Despite some encouraging pre-clinical and clinical results, VLP strategy warrants further stringent clinical evaluation to assess neutralizing and cross-neutralizing immune responses.. Importantly, clinical studies should also be done to evaluate the role of adjuvants in further enhancing the immunogenicity and dose-sparing to make this strategy feasible.

DNA VACCINES

A DNA vaccine involves administration of plasmid DNA carrying one or more genes representing antigenic proteins. The immunogenicity of a DNA vaccine is dependent, to a great extent, on the delivery method used. The most efficacious approach for DNA immunization is the bombardment with particles coated with DNA (gene-gun) or electroporation into the epidermal layer of skin.62,63 Unlike conventional vaccines, a DNA plasmid can be easily manipulated to incorporate gene inserts representing single/multiple antigens and immunostimulatory molecules to enhance the magnitude and type of immune responses. DNA vaccines have demonstrated both an excellent safety profile64 and the ability to induce humoral and cellular immune responses. DNA vaccination usually elicits a strong Th 1 response.65

The first demonstration of protective efficacy of a DNA vaccine in an animal model was reported for influenza in 1993.66 Despite all its advantages, clinical development of DNA vaccines has been hampered by the inability to induce consistent, high-level immune responses in humans.67 The development of new delivery systems that enhance the immunogenicity of DNA vaccines would be critical for their clinical use.68 For pandemic influenza vaccine development, this strategy can be rapidly adapted to emerging influenza variants. The ease of introducing multiple antigens and immunostimulants offer flexibility in designing improved vaccine formulations.64

A DNA vaccine based on the consensus sequence of HA generated neutralizing antibodies against clade 1 and clade 2 H5N1 viruses and offered complete protection in mice against both clades.69 A similar study using an HA consensus sequence-based DNA vaccine induced protective neutralizing antibody titers against clade 1 and clade 2 viruses in ferrets and showed significant reductions in virus shedding after challenge.70 This vaccine also induced cross-clade neutralizing antibody titers in a primate model. DNA vaccines based on the conserved nucleoprotein and matrix protein have been shown to induce a cross-protective cell-mediated immune response thereby reducing the morbidity associated with the disease.71,72 Electroporation of synthetic DNA resulted in robust induction of cross-reactive cellular and humoral immune responses capable of providing protection from influenza infection in rhesus macaques.73 Vaxfectin (a cationic lipid delivery system74,75)-formulated DNA vaccine encoding the HA-derived from A/Vietnam/1203/04, was protective in mice and ferrets in a two dose regimen.76,77 However, only a single dose of a Vaxfectin-formulated multivalent (HA + NP + M2) DNA vaccine was equally protective in ferrets.

Preliminary results of an ongoing clinical trial using a trivalent Vaxfectin-formulated DNA vaccine encoding the HA of A/Vietnam/1203/04 and the consensus sequences of two highly conserved influenza proteins, NP and M2, delivered intramuscularly or with a Biojector 2000 needle-free injection system showed promising safety and immunogenicity results.78 At least 67% of the subjects developed protective levels of antibody response (HI titers ranged from 40 to 640) by Day 56 in higher dose cohorts receiving 0.5 mg or 1 mg vaccine doses given twice.78 Similarly, another clinical trial involving a three dose regimen of a DNA plasmid encoding HA from a more recent strain of H5N1 (A/Indonesia/05/05) given intramuscularly via Biojector is currently underway.79

DNA vaccines have a good safety profile in clinical trials, but while testing has shown significant development of immune responses, this was accomplished only with several doses. In the event of a pandemic, however, a three dose regimen may not be feasible.

VIRAL VECTOR-BASED VACCINES

The viral vector-based vaccine approach involves the insertion of the genetic material encoding the important antigen/s into the genome of a harmless virus resulting in the generation of a recombinant virus. These modified viruses serve as gene delivery systems to efficiently carry the gene/s of interest into the host cells for expression of immunogenic antigen/s. Thus, viral vector-based vaccines function as live vaccines, but do not involve the complete pathogen. The antigens are expressed in infected cells similar to a natural infection, thereby inducing both humoral and cell-mediated immune responses. In addition, the inherent ability of certain viral vectors to enhance innate immune responses may boost the efficacy of such vaccines. Viral vectors serve as a natural nanoparticle-based gene delivery system. These vectors can be grown to high titers in certified cell lines in a short period of time and without the safety challenges associated with the production of highly pathogenic viruses. Furthermore, this approach can also be used to deliver multiple antigens

and/or genetic adjuvants at the same time to broaden the protection coverage. As a tool for producing large amounts of vaccine quickly in an influenza pandemic, viral vector-based vaccines hold considerable potential.

Adenovirus vectors

Adenovirus (Ad)-based vectors have several features that are desirable in a vaccine vector. The virus is nonpathogenic, infects both dividing and non-dividing cells, grows to high titers in cell culture, enables high levels of transgene expression and lacks the ability to integrate into the host genome.80 Ad vectors have been shown to exert an adjuvant effect by stimulating the innate immune system by both Toll-like receptor-dependent and independent pathways.81[Sharma and Mittal unpublished data]. Interaction of Ad capsids with cellular receptors induces expression of pro-inflammatory cytokines/chemokines, which results in recruitment of effector cells of the innate and adaptive immune system to the site of vector delivery.82 These cytokines also activate functions of antigen-presenting cells (APCs). Several preclinical and clinical studies using Ad vector-based vaccines for measles, severe acute respiratory syndrome (SARS), human immunodeficiency virus, hepatitis B and Ebola have highlighted the versatility of this approach.83–87

With regard to influenza virus, our studies have demonstrated that a human adenovirus (HAd)-based H5N1 vaccine expressing HA gene of HK/156/97 elicited significantly high levels of virus-neutralizing antibody titers in a mouse model.88 The vaccine also elicited a significantly high frequency of HA-518 (a conserved CD8 T cell epitope in all human H5N1 viruses isolated since 1997) epitope-specific interferon-gamma secreting $CD8^+$ T cells and offered complete protection against lethal challenge with homologous as well as antigenically distinct H5N1 influenza viruses.88 The generation of robust cell-mediated immune responses by the vaccine is particularly important as it has been shown to play a role in viral clearance and shortening the duration of illness.89

A similar study conducted in mice and chickens also demonstrated the efficacy of HAd-based H5N1 vacccine.90 The HAd-H5HA vaccine induced durable serological responses, long-term persistence of epitope-specific $CD8^+$ T-cell responses and conferred long-term protective immunity for at least 12 months post-immunization in mice.91 Interestingly, two doses of vaccine as little as 1×10^6 plaque forming unit (p.f.u.) was completely protective. 91 The protective efficacy of HAd-based H5N1 vaccine can be further broadened by including HA from clade 1 and clade 2 viruses, as well as conserved NP, in the vaccine formulation, thereby offering complete protection in mice against challenge with either clade 1 or clade 2 viruses.92

In a similar study, a replication incompetent complex Ad vector (CAdVax) devoid of E1, E3, and most of E4 regions of the Ad genome and carrying multiple genes (HA, NA, and M1) from clade 1 virus (CAdVax-FluAv) was shown to be 100% protective in mice against challenge with both clade 1 and clade 2 viruses.93 Ad5 vector-based delivery system for influenza vaccine has been shown to be safe and immunogenic in humans.94,95 In a clinical trial involving 24 healthy human subjects, intranasal administration of a two dose regimen of 5×10^8 virus particles expressing HA from A/PR/8/34 resulted in a fourfold increase in HI titers in 83% of the subjects. A topical application of 10–1000-fold higher viral particles resulted only in 33–67% seroconversion.95,94

One of the potential problems with Ad vectors is the role of vector immunity in blunting the immune response generated against the antigen delivered by HAd5 vectors. Several pre-clinical studies have addressed vector immunity,80,96 but to date this has not been effectively established in clinical studies. Moreover, the impact of Ad vector immunity on an HAd-based HIV vaccine in a phase I/II clinical trial was shown to be overcome by

increasing the vaccine dose.97 However, the much publicized failure of Merck's replication–defective HAd5 HIV-1 vaccine trial (STEP trial) has raised some concerns regarding the use of HAd5 vector as vaccine delivery system.98،99 The trial showed the lack of vaccine efficacy. Surprisingly there was a higher incidence rate (2.3%) of HIV-1 acquisition in vaccinees with baseline HAd5-specific neutralizing antibodies compared with controls.98،99 However, the scientific community is still debating the potential explanations100،101،102 of the observed results of the STEP trial. Whether the findings were unique to HIV pathogenesis is yet to be determined conclusively. Moreover, the implications of the STEP trial's findings on the use of other rare human or nonhuman Ad serotypes should be favorable since the basic principle behind their use is a low or no seroprevalence in the human population.

Nonetheless, several alternative approaches are being explored to circumvent the limitations of human Ad vectors, such as the use of nonhuman Ad vector based vaccines80،103،104 or the use of DNA priming and an Ad vector boost.105،106 Our studies have shown that a bovine Ad subtype 3 (BAd) vector-based H5N1 vaccine offered complete protection in a mouse model in the presence of high levels of preexisting HAd vector immunity.104 In addition, the prime-boost strategy using HAd5/BAd vectors resulted in significant higher levels of immune responses compared to responses generated with either vector alone, suggesting the importance of two vector systems in inducing better immune responses.104

Advantages of Ad-based vaccines are numerous, and results of both animal studies and clinical trials have shown an Ad-vector-based delivery system for influenza vaccine to be safe and immunogenic in humans.94،95 However, the safety and efficacy of the HAd5-based H5N1 vaccine needs to be thoroughly tested in clinical trials involving individuals of varied age groups and health status.

Alphavirus vectors

Alphaviruses are positive strand RNA viruses, and the vectors derived from them are also known as "replicons" due to the self-amplification of the vector RNA in the cytoplasm of infected cells. Alphavirus vectors have been primarily developed using the Venezuelan equine encephalitis, Sindbis, and Semliki Forest viruses.107 The attractive features of these vaccine vectors are the high levels of antigen expression leading to development of robust cellular, humoral and mucosal immune responses, the induction of innate immune responses through the double-stranded RNA intermediates, and the absence of preexisting immunity in humans against these vectors.108،109 Alphavirus vector-based vaccines have shown protection in numerous models for infectious diseases including influenza, human papilloma, and Ebola. 110،111،112

In one study, alphavirus replicon particles containing the HA gene from an H5N1 isolate (A/HK/156/97) was protective in chickens at a dose of 10^7 infectious units.111 Alphavirus-like replicon particles expressing the HA of A/Wyoming/03/2003 (H3N2) was immunogenic in preclinical studies in mice, rabbits and macaques.113 A phase 1 clinical trial involving 216 healthy adults immunized with one or two doses of a low or high concentration of the same H3N2 vaccine induced protective HI titers in 77 and 80% of subjects receiving a single low or high antigen dose of vaccine, respectively.114 A second immunization in these individuals enhanced seroprotective responses to 86% for both dosage levels and extended the duration of T cell response as compared to the single immunization. No significant differences in immune responses were observed between subcutaneous and intramuscular routes of immunization.114

The advantages inherent in alphavirus replicon particles such as a lack of preexisting immunity in humans indicates that this vaccine strategy should be further investigated. Since

an initial clinical study showed promising results, H5N1 vaccine candidates developed using this technology should be tested extensively for safety and efficacy in clinical trials.

Newcastle disease virus (NDV) vectors

With the advent of the reverse genetics system, the manipulation of NDV (a negative sense RNA virus) has become straightforward (similar to other DNA and positive-sense RNA viruses) and has helped to develop NDV as a safe and efficacious vaccine vector.115 NDV naturally infects via the mucosal surfaces of respiratory and alimentary tracts thus making it well suited for the control of respiratory virus infections such as influenza. The needle-free intranasal route of inoculation also makes it suitable for mass immunization. NDV is highly attenuated for replication in primates and is restricted to the respiratory tract, precluding concerns associated with potential spread to distal sites. The high level of host range restriction in primates offers a potential advantage to being developed as a vaccine vector for humans. In addition, NDV-based vectors grow to high titers in certified Vero cells.116

Several preclinical studies in mouse and chicken models have demonstrated the immunogenicity and protective efficacy of a NDV-vectored influenza vaccine against highly pathogenic avian influenza.117,118 Recently, DiNapoli et al have demonstrated the utility of an NDV vectored vaccine for human use in a nonhuman primate model.119 They engineered a live attenuated NDV-based vaccine (NDV-HA) expressing HA of highly pathogenic H5N1 virus (A/Vietnam/1203/2004) and showed that the vaccine was highly attenuated in nonhuman primates. A single inoculation by both the intranasal and intratracheal routes with 10^7 p.f.u. of NDV-HA per site induced substantial serum IgG and mucosal IgA responses.119

Although the safety of the vector has been demonstrated in the mouse, chicken and, importantly, in nonhuman primate models, there are concerns regarding the safety associated with the recombination between a vaccine vector and its circulating wild-type counterpart. Additionally, there are issues regarding the foreign gene insertion site in the NDV genome which affects the immunogenicity and vaccine efficacy.120

Vesicular stomatitis virus (VSV) vectors

Several naturally occurring or recombinant strains of VSV have been developed as potential therapeutic and vaccine vectors for various diseases.121–124 The low seroprevalance of VSV in humans makes it suitable as a vector for human use. Its immunogenicity and safety profile has been evaluated in several preclinical trials.125–127 VSV has a broad host range due to its utilization of phosphatidylserine, an universal component of cell-surface membranes, for virus entry.128 Using mice, Schwartz et al.129 showed that a recombinant VSV vector expressing HA of an H5N1 virus isolated in 1997 induced robust neutralizing antibody titers against the homologous and more recent antigenically different H5N1 viruses. This vaccine provided protection up to 7 months against lethal challenge with the homologous H5N1 virus even in animals receiving a single dose of the vaccine.129 Efficacy of a VSV-based vaccine expressing HA of A/FPV/Rostock/34 (H7N1) in place of the VSV G gene was also evaluated in chickens.130 The vaccinated birds were protected against challenge with a homologous H7N1 virus but not against an H5N2 virus.130

A VSV-vector vaccine has the potential to protect against different subtypes of avian influenza viruses in poultry. However, while low seroprevalance in humans is a definite advantage to using VSV as a potential pandemic influenza vaccine, its value in designing a pandemic influenza vaccine for humans requires more preclinical studies before clinical trials on humans would be feasible.

Poxvirus vectors

Vaccinia virus, a member of the family *Poxviridae* was successfully used to eradicate small pox. Poxviruses are the largest viruses known, and vectors derived from them can accommodate large/multiple gene inserts (>25kb).131 Modified vaccinia Ankara (MVA) vectors are highly attenuated with comparable immunogenicity and recombinant gene expression in mammalian cells indicating their safety and usefulness as vaccine vectors.132⁻134 Immunogenicity and safety of MVA vectors has been demonstrated in several preclinical and clinical trials for HIV, malaria and small pox.135⁻138

MVA recombinants expressing influenza virus antigens have been evaluated in several preclinical trials.139⁻141 Immunization with two doses (10^8 p.f.u.) of a MVA expressing A/Vietnam/1194/04 HA, given at 4 weeks part, induced strong antibody responses in mice.142 Furthermore, the mice were protected from infection when challenged with the homologous clade 1 (A/VN/1194/04) or clade 2 (A/Indonesia/05/05) virus.142 The same vaccine (MVA-HA-VN/04) also induced protective immune responses in nonhuman primates (Cynomolgus macaques) against clade 1 and clade 2 viruses.143 Another vaccinia virus-based multivalent H5N1 influenza vaccine expressing HA, NA & NP from A/Vietnam/1203/04 and M1 & M2 from A/CK/Indonesia/PA/2003 and adjuvanted with IL-15 elicited protective neutralizing antibody titers (1:80) against both clade 1 and clade 2.2 viruses in mice.144 The vaccine induced influenza-specific antibody response was detectable in appreciable amounts for 14 months post-immunization.144 Similarly, a single dose of a replication defective vaccinia H5N1 vaccine based on A/Vietnam/1203/2004 induced substantial cell-mediated immune (CMI) responses and provided cross-clade protection in mice.145

The pre-clinical data on studies using poxviruses as vectors is encouraging. Given the large size of poxviruses able to accommodate multiple gene inserts and the proven record as a vaccine against small pox, the strategy of using poxviruses as vectors warrants further clinical evaluation as an H5N1 pandemic vaccine.

CONCLUSION

Developing effective vaccines in a timely manner remains our best option for influenza pandemic preparedness. Egg-dependent influenza vaccine approaches may not be able to meet the huge global demand in a pandemic scenario, due to lack of manufacturing capacity as well as potential problems with the availability of embryonated chicken eggs. Therefore, an egg-independent vaccine manufacturing technology/infrastructure will not only augment the existing vaccine production capability but also offer shorter production timelines than egg-based technologies (Fig. 2). The ongoing 2009 H1N1 influenza pandemic can be seen struggling with the challenge to meet the global vaccine demand with primarily egg-based strategies.

In a pandemic scenario, there will be a need for improvising regulatory requirements for vaccine approval and facilitating a fast-track approval of vaccines made using current or evolving technologies. Over the years, regulatory agencies in the US and Europe have come up with strategies and guidelines for accelerated approval of pandemic vaccines.146⁻148 Strategies with a “mock-up” or “pandemic-like” vaccine have been outlined wherein vaccine manufacturers go through a special advance licensing process, producing a “core dossier” of safety and immunogenicity data on their vaccines and manufacturing processes prior to a pandemic. Then, if warranted, they may apply for a “pandemic variation” to switch to the subtype covered by the vaccine to the pandemic virus. Nonetheless, harmonization of the diverse regulatory pathways in different countries for the licensure of a pandemic influenza vaccine would further simplify the regulatory requirements for a vaccine manufactured using new technologies or with new adjuvant formulations. One of the major concerns in the

endorsement of new vaccine technologies is the lack of official calibrated reagents and assays, thus contributing to significant delays in the approval process.

Cell-derived whole virus and subunit influenza vaccines are in advanced phases of clinical trials149 (Table 1) and have demonstrated safety and efficacy. They have also been approved for use in several countries. Other approaches such as VLP, DNA vaccines and viral vector-based vaccines appear very attractive as these carrier systems favorably impact the innate immune system and generate strong humoral and cell-mediated immune responses. VLP-based vaccines for influenza are fast gaining acceptance and are likely to be approved in Europe and the US within 2–3 years.150

The development of a Pandemic Influenza Preparedness Plan includes more factors than the development and stockpiling of vaccines. In addition to vaccine programs, plans will need to ensure the availability of antiviral and antibiotics drugs and to utilize passive immune therapy with human polyclonal antibodies against common epitopes to treat infected individuals in order to curtail the spread of a pandemic. Other non-pharmaceutical interventions such as regulating travel, social distancing and personal hygiene will also help to mitigate the severity of a pandemic. Furthermore, additional strategies need to be developed to ensure that infants less than six months old, immunocompromised individuals and the elderly are especially protected against a pandemic virus, since they comprise the most vulnerable populations and do not respond adequately to vaccines. Existing vaccine capacity can be further increased by dose-sparing and by developing novel adjuvants that will not only reduce the amount of antigen required for protection but also will help achieve the desirable objective of a single shot pandemic vaccine, most likely in a primed population. New vaccine technologies, however, are one of the most important tools in pandemic preparedness with their potential to meet the global demand for vaccine manufacturing and stockpiling.

Acknowledgments

This work was supported by Public Health Service grant AI059374 from the National Institute of Allergy and Infectious Diseases to SKM and an NVPO grant to SS. We are thankful to Jane Kovach for her secretarial assistance.

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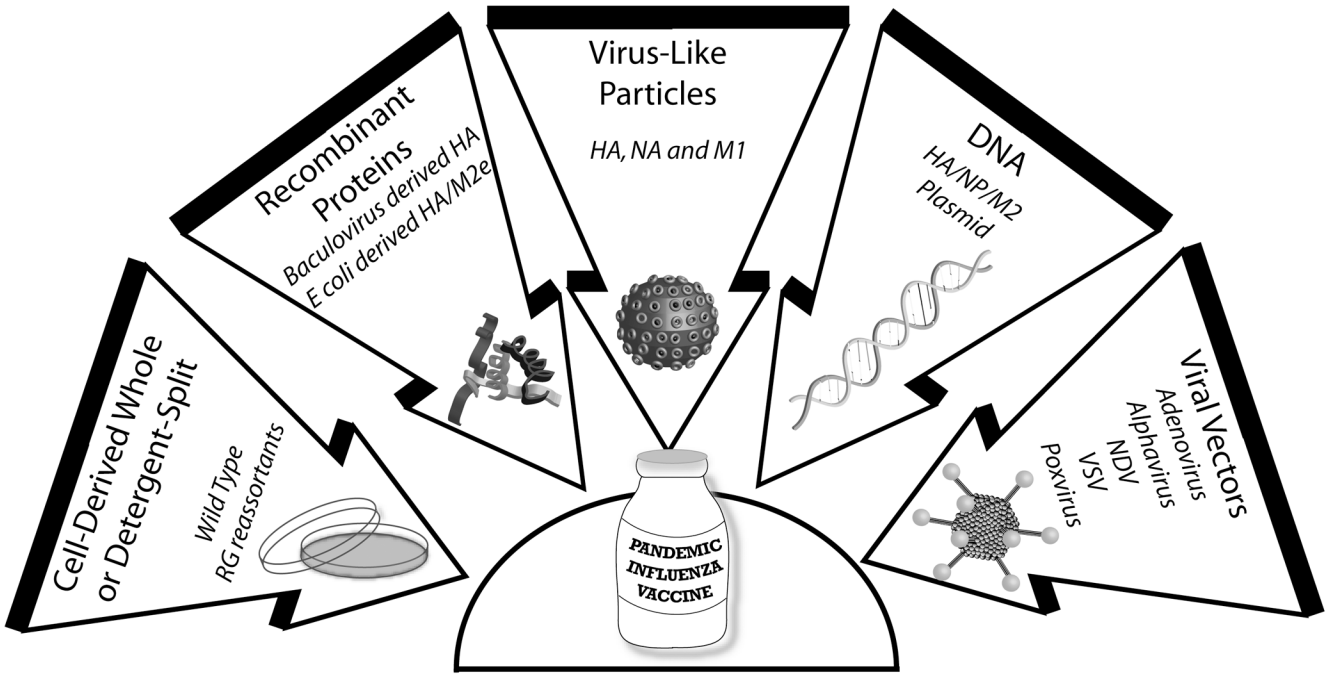

Fig. 1.
Egg-independent pandemic influenza vaccine strategies.

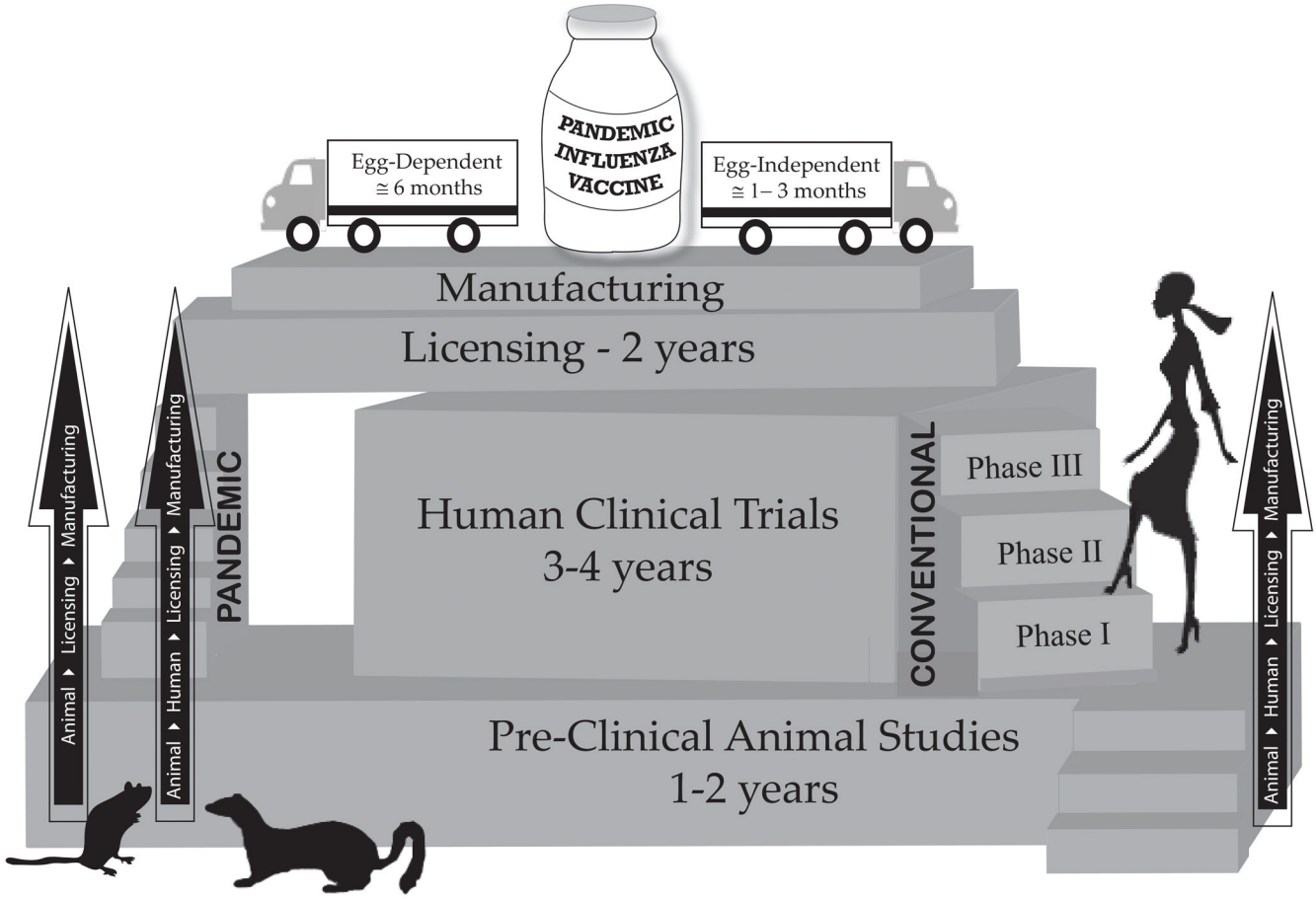

Fig 2.
Pandemic influenza vaccine development: From pre-clinical testing to vaccine distribution.

Table 1

Egg-independent influenza vaccine strategies that are at various stages of clinical trial.

Approach	Subtype	Virus strain and/or gene/s	Substrate	Company	Clinical phases	References
Inactivated whole virus	H5N1	A/Vietnam/1203/2004	Vero cells	Baxter, Austria	I/II, III	38, 39, 40
Inactivated whole virus	H5N1	A/Vietnam/1194/2004	Primary Monkey Kidney cells	Vabiotech, Vietnam	I	149
Inactivated split virus	H7N1	A/chicken/Italy H7N1xPR8(RD-3)	PER. C6 cells	Sanofi Pasteur, France	I	20
Inactivated subunit	H5N1	A/Vietnam/1194/2004	MDCK cells	Solvay Pharmaceuticals, Netherlands	I	19
Live-attenuated (NS1 deleted virus)	H5N1	A/Vietnam/1203/2004	Vero cells	Green Hill Biotechnology, Austria	I	149
Recombinant M2	Consensus	Consensus M2/M2e sequence/motif	E.coli	Cytos Biotechnology, Switzerland	I	19
Recombinant HA	H5N1	(i)A/Hong Kong/156/97 and A/Hong Kong/ 483/97 (ii)A/Vietnam/1203/2004	SF9 Insect cells	Protein Sciences, USA; UMN Pharma, Japan	I/II; I	52, 149
VLP	H5N1	HA, NA & M1 from A/Indonesia/05/2005	SF9 Insect cells	Novavax, USA	I/II	10
DNA vaccine	H5N1	(i) HA, NP, M2 from A/Vietnam/1203/2004 (ii) HA of A/Indonesia/05/2005	E.coli	Vical, USA; NIH, USA	I; I/II	78;79
Adenovirus vector	H1N1	HA from A/PR/8/34	293 cells	Vaxin Inc, USA	I	95
Alphavirus vector	H3N2	HA from A/Wyoming/03/2003	Vero Cells	Alphavax, USA	I	114