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New Strategies for the Development of H5N1 Subtype Influenza Vaccines

Progress and Challenges

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Abstract

The emergence and spread of highly pathogenic avian influenza (H5N1) viruses among poultry in Asia, the Middle East, and Africa have fueled concerns of a possible human pandemic, and spurred efforts towards developing vaccines against H5N1 influenza viruses, as well as improving vaccine production methods. In recent years, promising experimental reverse genetics-derived H5N1 live attenuated vaccines have been generated and characterized, including vaccines that are attenuated through temperature-sensitive mutation, modulation of the interferon antagonist protein, or disruption of the M2 protein. Live attenuated influenza virus vaccines based on each of these modalities have conferred protection against homologous and heterologous challenge in animal models of influenza virus infection. Alternative vaccine strategies that do not require the use of live virus, such as virus-like particle (VLP) and DNA-based vaccines, have also been vigorously pursued in recent years. Studies have demonstrated that influenza VLP vaccination can confer homologous and heterologous protection from lethal challenge in a mouse model of infection. There have also been improvements in the formulation and production of vaccines following concerns over the threat of H5N1 influenza viruses. The use of novel substrates for the growth of vaccine virus stocks has been intensively researched in recent years, and several candidate cell culture-based systems for vaccine amplification have emerged, including production systems based on Madin-Darby canine kidney, Vero, and PerC6 cell lines. Such systems promise increased scalability of product, and reduced reliance on embryonated chicken eggs as a growth substrate. Studies into the use of adjuvants have shown that oil-in-water-based adjuvants can improve the immunogenicity of inactivated influenza vaccines and conserve antigen in such formulations. Finally, efforts to develop more broadly cross-protective immunization strategies through the inclusion of conserved influenza virus antigens in vaccines have led to experimental vaccines based on the influenza hemagglutinin (HA) stem domain. Such vaccines have been shown to confer protection from lethal challenge in mouse models of influenza virus infection. Through further development, vaccines based on the HA stem have the potential to protect vaccinated individuals against unanticipated pandemic and epidemic influenza virus strains. Overall, recent advances in experimental vaccines and in vaccine production processes provide the potential to lower mortality and morbidity resulting from influenza infection.

1. Introduction

1.1 Influenza A

Influenza viruses are classified in the family of *Orthomyxoviridae*, and comprise viruses possessing negative-sense, single-stranded, segmented RNA genomes. The most serious infections of humans occur with viral strains of the genus *Influenzavirus A*.[1] In particular, influenza A subtypes H1N1 and H3N2 currently cause significant human morbidity and mortality,[2,3] with an estimated 10–15% of the US population infected, and an average of approximately 30 000 associated deaths occurring annually.[4,5] Globally, as many as 500 000 fatalities may occur each year.[6] In addition, influenza A viruses have the propensity to cause periodic pandemics, as novel strains of influenza virus emerge from avian or other animal reservoirs into immunologically naive human populations. Three such influenza pandemics occurred in the 20th century:[7] in 1918,[8] 1957,[9] and 1968.[10] The 21st century has already witnessed one pandemic event, in 2009.[11,12] The disease burden associated with such pandemics can be enormous: in the order of 50 million deaths occurred during the 1918 influenza pandemic, and up to 4 million deaths were associated with the H2N2 strain introduced into the human population in 1957.[9] Such historical precedents provide compelling impetus to construct and maintain public health systems that can adequately combat future pandemics. However, while influenza pandemics are considered to be inevitable, the identity of any specific pandemic strain is notoriously difficult to predict before the event. Furthermore, while vaccination is considered the pre-eminent public health measure to protect populations against pandemic influenza, it can take 4–6 months or more from the time of strain selection to deliver vaccine using current manufacturing technologies,[13,14] and the antigenic diversity present in influenza viruses restricts the utility of currently licensed vaccines to a small number of specific strains. Thus, as the global spread of a pandemic influenza virus strain may occur in a considerably shorter time span than 6 months, the need to make improvements to the vaccine production chain is clear. With this impetus, several technologies are being developed or have recently become available that may shorten the lead-time to vaccine delivery and that aim to make the production chain more secure.

These advances include generating vaccine seed viruses using reverse genetics techniques, amplifying vaccine seed viruses in non-traditional substrates such as Vero and Madin-Darby canine kidney (MDCK) cell cultures, lowering the need for antigen by using dose-sparing measures such as the inclusion of adjuvants, or improving the route of delivery. Other approaches include the use of recombinant vaccine vectors, which do not rely on growth of influenza virus to create the vaccine. These include the use of DNA vaccination, virus-like particle (VLP)-based vaccination, use of virus vectors such as vaccinia, adenovirus and others, and using recombinant bacterial protein as antigen.

Finally, progress has been made recently in efforts to generate more broadly protective vaccine by targeting conserved epitopes maintained within the influenza virus genome.

1.2 H5N1 Influenza Virus as a Possible Pandemic Threat

Highly pathogenic avian influenza virus (HPAIV) of the H5N1 subtype caused a series of outbreaks of influenza disease in poultry in Asia between 1997 and 2003.[15-17] These outbreaks were accompanied by infrequent human infections,[18] which resulted in severe disease or death in the individuals concerned.[19-21] By 2003, H5N1 viruses had become endemic in poultry in Asia,[22] resulting in the deaths of millions of unprotected poultry through infection or culling,[23] as well as causing sporadic fatal cases in humans[24,25] through contact with poultry. The geographic range of the virus endemicity in poultry increased by 2006 to include areas of Africa, and the periodic human infections associated with HPAIV H5N1 outbreaks continue. The WHO reports that up until 20 January 2011, 518 human cases of H5N1 infection had been confirmed, with a mortality rate of around 60%.[26]

Due to the susceptibility of certain individuals to infection, there exists the potential for the virus to adapt within a human host through mutation or reassortment with a seasonal influenza strain, resulting in the genesis of a pathogenic virus possessing a transmissible phenotype. While the likelihood of such an event arising is considered to be of very low probability, its consequences could potentially be grave. Concerns regarding an influenza pandemic originating from an HPAIV strain have prioritized the H5N1 subtype of influenza virus for vaccine development.

In the event of a worst-case influenza pandemic scenario, it has been estimated that over a billion doses of vaccine may be required globally, and delivered within, at the most, 4–6 months, in order to fully prevent a public health disaster.[27] Current manufacturing methods and capacities are not adequate to allow such a pandemic response. Vaccine strains have to be identified, and seed stocks generated and then tested for immunogenicity and safety, before large-scale production can commence.[13,14] The final stocks then have to be tested, packaged, and distributed before use. As mentioned above, this process can take 4–6 months or more using current technologies.[27] The timeline of production can furthermore expand due to a number of factors that are difficult to control; the vaccine virus strain may (i) be slow growing; (ii) grow to low titers; (iii) incorporate low levels of hemagglutinin (HA) antigen; (iv) cause the death of the growth substrate; or (v) change immunologically by adapting to the growth substrate. In addition, if the substrate itself is limited, this will cause further logistical difficulties. In a situation where a pandemic influenza virus is circulating, time to vaccine delivery is of the essence. Several of these vaccine production concerns would be applicable to an H5N1 virus strain, and has given rise to a recent upsurge in interest in the development of novel H5N1 influenza vaccines and vaccine production technologies.

This article focuses on the current state of research on H5N1 vaccines and vaccine production technologies, summarizes advances, and assesses how these developments may ultimately improve public health.

2. Advances in Influenza Vaccine Development

2.1 Reverse Genetics-Derived Vaccines

Several distinct strategies for the generation of live attenuated influenza virus (LAIV) vaccines have emerged through the use of reverse genetics techniques to introduce attenuating mutations into the influenza virus genome. These strategies include (i) the directed introduction of mutations in the polymerase genes resulting in a temperature-sensitive phenotype; (ii) the modulation of the interferon antagonist protein, nonstructural protein 1 (NS1), to create viruses unable to prevent the induction of a cellular anti-viral state; and (iii) the disruption of the cytoplasmic tail of the M2 protein of influenza virus, resulting in viruses that cannot assemble efficiently. An advantage of reverse genetics-based approaches is the ability to specifically tailor the growth characteristics of live attenuated vaccine viruses, in order to optimize the balance between immunogenicity and safety. An additional application of reverse genetics techniques is the generation of seed viruses destined to be used as inactivated split or subunit vaccines.

The production of HPAIV H5N1 vaccines involves issues specific to the subtype. These viruses are highly pathogenic, not only to the conventional embryonated egg substrate, but also potentially to manufacturing workers. In addition, HPAIV

have select agent status in the US, which restricts their use to highly stringent containment facilities and work practices. The use of reverse genetics techniques allows swift, targeted modification of these viruses at the necessary loci, in order to attenuate virulence, improve growth properties, maintain immunogenicity, and allow production of vaccine to occur at a lower containment level. Thus, reverse genetics techniques are well suited to meet the need for a rapid response to vaccine demand in a pandemic situation, and the advantages that reverse genetics techniques offer may be particularly useful in the case of an H5N1-origin pandemic.

2.2 Cold-Adapted, Temperature-Sensitive (ca/ts), Live Attenuated Influenza Virus (LAIV) Vaccines

Cold-adapted temperature-sensitive (ca/ts) LAIV vaccines are presently licensed for use in the US. Originally, these vaccines were generated by classical reassortment methods, such that the constituent viruses possessed the antigenic proteins HA and neuraminidase from the viral strains of interest and the remaining proteins from ca/ts master donor strains.[28] In the US, the two master donor strains used were A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66. Three ca/ts viruses comprised each final vaccine such that it included the antigenic proteins of the H1N1, H3N2, and influenza B strains predicted to circulate for a given influenza season. These master donor strains continue to be used to generate vaccines by classical reassortment techniques.

2.2.1 Reverse Genetics-Derived ca/ts LAIV Vaccines

More recently, LAIV vaccines for seasonal influenza virus strains generated by reverse genetics have also been licensed in the US. These vaccines still comprise the same master donor strains,[29,30] reassorted with the antigenic proteins of circulating influenza strains, but are obtained using recombinant DNA technology, thereby increasing the efficiency and precision of the virus generation process. First-generation, plasmid-based, reverse genetics-derived viruses were obtained through transfection of 12–16 plasmids into virus-permissive cell co-cultures, in which eight plasmids encode the negative-sense viral RNAs, driven from a polymerase (pol) I promoter, and the remaining plasmids encode viral mRNAs, driven from a pol II promoter.[31,32] When transfected into cells with compatible pol I and pol II proteins, these plasmids generate the necessary protein and viral RNA products to allow the recovery of infectious virus. The technique has been subsequently refined through the use of ambisense plasmids, which contain both pol I and pol II promoters flanking the DNA encoding viral gene segments of interest.[33] This approach reduces to eight the number of plasmids required in the rescue transfection, thereby improving efficiency of virus rescue. A further reduction in the number of plasmids required was achieved in 2005, with the advent of four- and five-plasmid-based rescue systems.[34] The licensure of ca/ts vaccines for seasonal influenza that utilize reverse genetics techniques facilitates efficient and predictable generation of the virus of interest, and represents an improvement in the vaccine production chain.

2.2.2 H5N1 Subtype ca/ts LAIV Vaccines

Although currently unlicensed, several experimental reverse genetics ca/ts LAIV vaccines specifically targeting H5N1 subtype viruses that show promise in animal models have been developed in approximately the last decade. Two candidate reverse genetics ca/ts LAIV vaccines designed to protect against A/Hong Kong/97 (H5N1) were generated in 1999.[35] The constituent viruses, possessing the A/Hong Kong/156/97 or A/Hong Kong/483/97 HA and neuraminidase proteins, with attenuated cold-adapted A/Ann Arbor/6/60 internal gene segments, were modified to remove the polybasic cleavage site from the HA proteins (a typical attenuating modification performed on reverse genetics-derived live attenuated H5N1 vaccines), resulting in trypsin-dependent *in vitro* growth. The viruses maintained a ca/ts phenotype, grew to in excess of 10^8 EID_{50} (50% egg infectious dose)/mL in embryonated chicken eggs (ECEs), and were shown to be safe and immunogenic in a ferret model. Safety was demonstrated by an absence of viral growth in ferret lung following inoculation with 10^7 EID_{50} of virus. In 4-week-old chickens, vaccination with either vaccine was safe and provided 75–100% protection from a lethal homologous challenge, and 50% protection from a lethal heterologous H5N1 virus challenge.

Further ca/ts LAIV H5N1 vaccines – generated using essentially the same strategy, but possessing surface proteins derived from strains arising in Hong Kong in 1997 and 2003, or in Vietnam in 2004 – gave similarly encouraging results.[36] These vaccines also demonstrated trypsin-dependent *in vitro* growth, and were safe and immunogenic in mice and ferrets. Mice were protected from a stringent (5000 MLD_{50} [50% mouse lethal dose]) challenge with homologous or heterologous H5N1 virus following one dose of vaccine. This protection was observed despite an absence of serologic correlates of protection. Two doses of vaccine, administered 4 weeks apart, furthermore resulted in a complete absence of viral replication in the lungs of mice and ferrets following homologous or heterologous H5N1 challenge 4 weeks after vaccine boost.

In 2009, an open-label human vaccine trial in healthy adults demonstrated the safety of the Hong Kong-2003 and Vietnam-

2004 strain-based vaccines.[37] However, replication of the vaccine strains was minimal, and serum-based correlates of protection were absent, even after two doses of vaccine were administered. In follow-up studies, the authors demonstrated that over-attenuation of vaccine may be related to removal of the polybasic cleavage site of the HA, in the context of the ca/ts internal gene constellation.[38] Although traditional correlates of protection based on hemagglutination inhibition activity were not detected in sera, both vaccines were immunogenic, generating virus-specific IgA in approximately half of the study participants. The lack of a significant hemagglutination inhibition response observed in both animal studies and human vaccine trials highlights the difficulty of determining reliable correlates of protection when using LAIV as opposed to traditional inactivated vaccines. To allow meaningful evaluation of these vaccines in humans, more research into novel correlates of protection for LAIV is clearly needed.

In parallel studies involving reverse genetics-derived ca/ts LAIV H5N1, Fan et al.[39] demonstrated immunogenicity and protective efficacy in both mice and non-human primates, further suggesting that ca/ts LAIV vaccines are potentially efficacious against HPAIV H5N1. In these studies, the elicitation of neutralizing antibody, as well as T-cell responses, was observed. Taken together with the data of Subbarao and co-workers,[35,36] these results suggest that a diverse range of immunologic responses contribute to protection induced by ca/ts LAIV vaccines.

Overall, ca/ts vaccines represent a promising approach to protect against H5N1 influenza. Data from studies on ca/ts vaccines targeting other influenza viral subtypes, as well as data from the experimental vaccines discussed here, suggest that these vaccines provide protection that may be broader, more balanced, and longer lasting than traditional inactivated vaccines.[40-42] Further research is, however, needed into the optimization of these vaccines, as well as the identification of correlates of protection applicable to LAIV vaccines.

2.3 Non-Structural Protein 1 (NS1)-Truncated Influenza Vaccines

An alternative strategy to ca/ts vaccines for the generation of experimental LAIV vaccine is through the modulation of the interferon antagonist protein NS1 of influenza virus. Viruses lacking the NS1 protein (delNS1) are unable to antagonize the host interferon response, rendering them highly attenuated in interferon-competent hosts, while remaining capable of growth in substrates with a compromised interferon response, such as Vero cells, as well as in immunocompromised hosts, such as *STAT1* knockout mice.[43] Initial studies demonstrated that, while being non-pathogenic, such delNS1 viruses were in fact too attenuated to be optimal as an LAIV vaccine, providing protection to mice from lethal challenge only when used at a high inoculum dose of 10^6 plaque-forming units (PFU).[43] Further work showed that partial truncation of the NS1 open reading frame, such that it encoded the first 99 N-terminal amino acids (NS1-99), yielded a virus with diminished NS1 function. This NS1-99 virus was attenuated relative to wild-type, but induced a more robust antibody response in the host than the delNS1 variant.[44] Lower doses of NS1-99 virus were protective in mice following lethal challenge with wild-type PR8 virus relative to delNS1-vaccinated animals.[44]

LAIV vaccines containing NS1 truncations have been constructed in the context of influenza virus strains associated with multiple host species, and these, in turn, have been evaluated in several model systems, including equine,[45,46] porcine,[47-49] avian,[50] murine,[51] and macaque.[52] The results of these studies were summarized in a recent review article.[53] In these diverse model systems and natural hosts, truncated NS1-based LAIV vaccines have consistently shown attenuated growth, induced host immunity, and provided protection from morbidity or mortality following challenge with influenza virus.

2.3.1 H5N1 Subtype, NS1-Truncated, LAIV Vaccines

Experimental NS1-truncated LAIV vaccines against H5N1 influenza have been characterized. Steel et al.[54] generated a panel of vaccine candidate viruses derived from A/VN/1203/04 virus in which the NS1 protein was truncated such that it expressed N-terminal 73, 99, or 126 amino acids, in combination with removal of the polybasic cleavage site from the HA protein, and alteration of the polymerase subunit PB2 to affect replicative ability. The resulting viruses all demonstrated trypsin-dependent growth *in vitro*, and were highly attenuated in mice relative to wild-type A/VN/1203/04 virus. One dose of vaccine was sufficient to fully protect mice against a lethal homologous challenge, and generate virus-neutralizing titers in serum. In poultry, vaccination with 10^6 EID_{50} of an NS1-99-containing virus was sufficient to elicit full protection against homologous lethal challenge, and partial protection against a heterologous H5N1 virus challenge. Moreover, shedding of challenge virus from trachea of protected birds was below the limit of detection, suggesting challenge virus was neutralized at the site of inoculation.

2.3.2 NS1-Truncated, LAIV Vaccines in Humans

LAIV attenuated through disruption of interferon antagonist functions provide a different modality from ca/ts vaccines, and may be better suited for use in specific population groups, such as the elderly, who may not respond as well to ca/ts vaccines

as younger individuals.[55] It has been speculated that the ca/ts viruses may be too attenuated to achieve optimum protection in the elderly, due to the presence of pre-existing immunity.

In addition to further pre-clinical testing, clinical studies are now required to demonstrate the safety and efficacy of NS1-modulated vaccines in humans. To this end, a recent phase I clinical trial using a reverse genetics-derived, NS1-truncated variant of A/New Caledonia/99 (H1N1) virus demonstrated that the vaccine was well tolerated, safe, and immunogenic in male volunteers.[56] Interestingly, sera obtained from trial participants receiving vaccine was found to contain neutralizing antibodies reactive against the homologous strain as well as the heterologous isolates A/Solomon Islands/2006 (H1N1) and A/Brisbane/59/2007 (H1N1).

2.3.3 Preventing Reassortment between NS1-Truncated, LAIV Vaccines and Circulating Strains

A perceived concern relating to NS1-modulated LAIV is their potential for genetic reassortment with wild-type virus, which could lead to phenotypic reversion to pathogenicity. As it is thought that influenza virus genome segments are packaged by means of a segment-specific mechanism,[1] packaging signals encoded within the viral RNA of truncated and full-length non-structural segments would be expected to be degenerate. However, by modulating the packaging sequences, it may be possible to generate a vaccine virus that is unable to reassort with wild-type viruses, thereby improving the stability of NS1-truncated strains. A recent publication by Gao and Palese[57] has demonstrated the feasibility of this approach. Therein, a recombinant virus possessing HA and non-structural segments in which the respective packaging signals were exchanged was generated and characterized. The authors of the study showed that, compared with a wild-type control, the packaging mutant virus did not exchange gene segments efficiently with a virus possessing wild-type packaging sequences.

In a number of contexts, NS1-modulated vaccines have been demonstrated to stimulate a robust and relatively broad immune response and to provide protection against influenza infection. The experimental evidence to date furthermore suggests that one dose of vaccine may be sufficient to safely generate a protective response, and that this response confers increased cross-protection against strains compared with conventional inactivated vaccines.

2.4 M2-Modulated Influenza Vaccines

The M2 protein of influenza virus functions as an ion channel allowing acidification of virions in the endosome, thereby facilitating uncoating and release of viral RNA into the cytoplasm.[1] The cytoplasmic tail of M2 has also been shown to be important in virus assembly as well as pathogenesis.[58-62] Based on the essential functions of this protein in the viral life-cycle, Kawaoka and colleagues[63-66] have developed a strategy for viral attenuation through truncation of the M2 cytoplasmic tail.

2.4.1 H5N1 Subtype, M2-Truncated, LAIV Vaccines

Watanabe et al.[65] have demonstrated that A/VN/1203/04 (H5N1) virus, genetically altered through an 11 amino acid truncation of the M2 cytoplasmic tail as well as deletion of the HA polybasic cleavage site (VN1203M2del11), has attenuated growth in lungs and nasal turbinates of mice. Moreover, the MLD_{50} value of this mutant virus was increased approximately 10^5-fold relative to the wild-type virus in a mouse model. Nonetheless, when cultured in eggs, the mutant virus retained the efficient *in vitro* growth phenotype of wild-type A/VN/1203/04 virus. Vaccine-challenge studies with mice vaccinated intranasally using live VN1203M2del11 virus demonstrated sterilizing protection from 100 MLD_{50} challenge with homologous or heterologous A/Indonesia/7/05 (H5N1) virus.

2.4.2 A Vaccine Based on Lengthening the M2 Cytoplasmic Tail

Similar phenotypic traits to those seen upon truncation of the M2 protein were observed following extension of the M2 cytoplasmic tail. These studies were performed using a reverse genetics-derived strain of A/WSN/33 (H1N1) in which the cytoplasmic tail of the M2 protein was altered by the introduction of a carboxy-terminal epitope tag (WSN M2myc).[62] This virus grew to titers of 10^8 $TCID_{50}$ (50% tissue culture infective dose)/mL in MDCK cells, but induced no lethality in mice inoculated intranasally with 10^5 PFU of virus, and had lower viral load in the lungs than did wild-type virus. In mice infected intranasally with WSN M2myc virus, and subsequently challenged with 20 MLD_{50} of homologous wild-type virus, complete protection from death was observed.

Thus, M2-modulated LAIV presents a useful phenotype of high growth in cell culture combined with attenuated growth *in vivo*, while retaining immunogenicity in animal models. Attenuation by protein truncation means the chance of reversion to virulence is low; however, the strategy shares the criticism with NS1 truncation vaccines that reassortment may lead to a reversion to wild-type phenotype.

2.5 Bivalent Influenza Vaccines

A further strategy utilized for the generation of LAIV vaccines involves the replacement of the endogenous influenza virus

neuraminidase with a heterologous protein. The introduced protein must provide neuraminidase activity to allow the completion of the viral life-cycle, and, if selected appropriately, can confer immunity in the vaccinated host to a second infectious agent. Thus, using an influenza virus-based, reverse genetics system it is possible to generate bivalent live attenuated virus vaccines.

Kawaoka and colleagues[67] demonstrated in a mouse model that a bivalent vaccine virus, based on the PR8 strain and incorporating the HA-neuraminidase protein from a paramyxovirus, was attenuated, and effectively protected mice against lethal challenge with mouse-adapted influenza and Sendai viruses. Laboratory studies in both mouse and chicken models have furthermore shown that a bivalent influenza virus utilizing the same strategy provides protection against both HPAIV H5N1 and the avian paramyxovirus, Newcastle disease virus (NDV).[68,69] Since it allows two important pathogens to be targeted with a single vaccine, this bivalent approach has the potential to greatly decrease the costs associated with vaccination campaigns. When considering vaccination of poultry, in particular, such economic concerns are of high importance and can determine whether an immunization program is undertaken or not.

2.6 Inactivated Influenza Vaccines

Reverse genetics can also be used for the rational and efficient generation of seed stocks for inactivated vaccine production. By using this approach, Subbarao et al.[70] generated an experimental H5N1 vaccine virus with the internal genes derived from A/PR/8/34, as used in currently licensed inactivated seasonal vaccines, and the surface proteins from A/HK/491/97 (H5N1). Inactivated vaccine derived from this virus was shown to be safe, immunogenic, and protective in both mouse and chicken models.

2.7 Cell Culture-Based Vaccine Production

In order to improve the efficiency, security, and reliability of the vaccine production chain, the development of alternative substrates for the growth of influenza virus has seen intensive activity in recent years.

2.7.1 Embryonated Chicken Eggs as a Substrate

Traditionally, in the US, Europe, and elsewhere, ECEs have been the substrate for the selection, amplification, and production of influenza virus vaccine strains. ECEs are well characterized for the growth of influenza virus, and have proven useful over many years. Nonetheless, recent focus on influenza vaccine production in the light of pandemic threats has highlighted certain shortcomings relating to the ECEs. Notably, there is a constrained and relatively inflexible supply of specific pathogen-free ECEs, which in turn limits vaccine production capacity.[13,71] This fixed capacity prevents immediate scale-up in response to a rapidly spreading global outbreak and may be exacerbated in the case of a pandemic triggered by HPAIV, since laying hens may be affected by the pandemic strain. In addition, the downstream processing of influenza virus obtained from the ECEs is cumbersome and labor intensive, and the logistics of growing influenza virus in the ECEs requires careful planning to ensure sufficient egg supplies.

In addition to these better-defined limitations, recently there have been significant difficulties with the isolation of some H3 subtype human influenza viruses in eggs.[13,72] These problems have led to vaccine production delays and could result in vaccine strain mismatch if the epidemic strain of choice cannot be cultured in eggs.[73] The passage of isolated seasonal influenza viruses in eggs can furthermore lead to adaptive changes in the influenza virus genome,[74] which may alter the immunogenicity of the vaccine strain and thereby reduce match to the circulating strain.[13,75] Finally, the use of eggs is not suited for the growth of reverse genetics-derived vaccines, which require federally approved and licensed cell culture systems.

2.7.2 Advantages of Cell Culture-Based Systems

While working towards a reduction in reliance on growth of influenza virus in the ECEs, it has been noted that influenza viruses are also amenable to growth in certain transformed continuous cell lines such as MDCK and Vero cells. Using such cell lines as a growth substrate for influenza vaccine virus has several potential advantages, including improved scalability of the production process over eggs, resulting in the ability to meet high global demand during pandemic outbreaks, as well as being capable of meeting an anticipated incremental increase in vaccine demand due to changes in the recommendations for vaccine coverage. Furthermore, closed bioreactors used for large-scale cell culture can potentially reduce the risk of contamination of vaccine with adventitious agents. Cell culture growth may eliminate the need for the generation of high growth reassortants, as human influenza viruses typically grow to higher titers in a cell culture than in the ECEs without adaptation, but this approach may lead to regulatory hurdles relating to approved vaccine strain usage, and possibly to the need for higher biocontainment levels during virus production.[13]

2.7.3 Cell Culture-Based Approaches in Use and in Development

The advantages of cell culture-based systems have resulted in the investigation of several cell lines for amplification of virus

stocks for vaccine production. In particular, MDCK, Vero, and PerC6 cells[76-79] have gained the focus of attention. In addition, Sf9-derived cells have been used to generate protein for an HA-based influenza vaccine.[80] Sf9 cells are a cell line originally derived from the fall armyworm, *Spodoptera frugiperda*.

Novartis obtained regulatory approval in the EU in 2007 to manufacture an inactivated subunit vaccine for seasonal influenza (Optaflu®), which is produced in a patented MDCK cell line (MDCK 33016). A similar, MDCK grown, inactivated subunit vaccine (Influvac®, Solvay Pharmaceuticals)[81] gained licensure in the Netherlands in 2001, although this vaccine was not ultimately taken to market. Data from several clinical trials conducted using these vaccines demonstrated broadly comparable safety and immunogenicity profiles to egg-grown vaccine subjected to the same processing.[79,82-85]

MDCK cell lines have been shown to be amenable to modification to permit suspension growth[86-88] and to be capable of growth in defined serum-free media. Several vaccine manufacturers have generated master strains, and cell banks, of MDCK cells, which grow in suspension and yield high titers of influenza virus.[79] Hussain et al.[89] have described a high yielding MDCK cell line, which allows the growth of ca/ts influenza virus to similar titers as those obtained from the ECEs.

The use of Vero cells for influenza vaccine production is also being pursued. Baxter has several Vero cell culture-derived vaccine trials completed or underway. A recently published phase III clinical trial demonstrated comparable safety and immunogenicity of a Vero-cell-culture-derived influenza vaccine to the egg-grown vaccine for pH1N1 influenza.[90] In addition, similarly positive safety and immunogenicity data were gleaned from a phase II vaccine trial for a Vero cell-derived H5N1 vaccine.[91]

S. frugiperda-derived cells have been used to grow recombinant baculovirus, which expresses HA protein. Using this system, three times more protein can be included in the final seasonal vaccine formulation without adverse reactions relative to egg-derived vaccine.[80] Studies have indicated that FluBlok®, a vaccine manufactured using this approach, may be more immunogenic and provide broader protection than conventional vaccine.[92] Several clinical trials involving FluBlok® have been performed,[92,93] and the vaccine is awaiting a decision on licensure in the US.

2.7.4 Drawbacks of Cell Culture-Based Vaccine Production

While improving the security of the vaccine production chain, the use of transformed cells to grow influenza virus carries risks associated with the potential for growth of contaminating micro-organisms, as well as theoretical risks pertaining to the known tumorigenicity in mice of cell lines such as MDCK.[82] For these reasons, strict regulatory requirements need to be satisfied[13,82] before cell lines can be granted licensure for use in the production of influenza vaccines.

Overall, cell culture-based approaches offer a promising alternative to traditional egg-based influenza virus vaccine manufacture and have the potential to alleviate a number of logistical concerns with current methods. Some cell lines have already been adopted and are in use by the vaccine industry. Cell-based approaches are likely to be increasingly used, but they have their own challenges, which will need to be monitored closely.

2.8 Virus-Like Particles (VLPs) as Influenza Vaccines

Replication-defective influenza VLPs can be generated through the transfection of plasmid DNA into mammalian or insect cells. Such VLPs are characterized by spontaneous self-assembly and budding from transfected cells, following the intracellular expression of the structural proteins HA, neuraminidase, and matrix, or combinations thereof.[94-96] The morphologic and antigenic similarity of influenza VLPs to influenza virions has encouraged research into their potential use as a vaccine platform.[95] The potential advantages of VLPs over conventional inactivated vaccines include cutting out the need to work with isolated virus strains or egg-based amplification systems. Furthermore, as discussed below, some data suggest that VLPs may induce broader and longer-lasting immune responses than currently licensed inactivated vaccines.

2.8.1 Hemagglutinin-Containing VLPs

In pre-clinical laboratory studies, VLPs incorporating the major antigen of H5N1 influenza virus, the HA protein, in combination with neuraminidase and matrix proteins, have been shown to be protective, immunogenic, and safe in mice.[97,98] An interesting variation to this approach involves the inclusion of the minor surface protein of influenza virus, M2, in the VLPs. Most likely because this protein is more highly conserved among strains, incorporation of M2 into VLPs was shown to broaden the protective response supplied by an H1N1 VLP.[99]

2.8.2 M2-Based VLPs

VLPs containing matrix and M2 protein, but not HA, were found to provide an adjuvanting effect in mice when combined with a conventional inactivated influenza vaccine. The response to vaccination was broadened in the presence of M2 VLPs, allowing protection against both H5N1 and H3N2 viruses.

Furthermore, the immune response induced using this combinatorial approach lasted for at least 7 months.[100]

2.8.3 Routes of VLP Administration

Studies on intranasal delivery of VLPs have shown that immunization by this route can stimulate a protective immune response against influenza in mice. The breadth of heterologous protection conferred by this immunization strategy was improved by the addition of flagellin or other adjuvants.[101,102]

It has furthermore been shown that intradermal delivery of influenza VLPs against H5N1, using microneedles, stimulates a protective immune response in mice.[97] Microneedles are micron-scale needles, which penetrate the stratum corneum to deliver antigen into the dermis.[103] Protective immune responses were also observed following microneedle-mediated H1N1 vaccination.[104] Vaccination provided immunity that was demonstrated to be long-lasting and superior to conventional inactivated vaccine in inducing protective immunity.[105,106] Additional experimental microneedle-delivered VLP vaccines targeting H5N1 influenza virus have elicited immunity, providing complete protection from lethal challenge in mice.[107,108]

Overall, VLP-based approaches offer a promising alternative to traditional inactivated influenza virus vaccine and have the potential to broaden as well as lengthen immune responses relative to those provided by conventional influenza vaccination.

2.9 DNA-Based Influenza Vaccines

A further method of vaccination that has received increased attention in recent years is that of DNA vaccination.[109] The method typically consists of injecting plasmid DNA encoding antigen directly into the muscle or dermal tissue of the vaccine recipient. Cells in the tissue take up the DNA, and protein antigen is expressed and presented intracellularly by the host. This approach has the potential to provide significant advantages over traditional vaccine delivery methods, including lower costs. Plasmid DNA can be generated very inexpensively and on a large scale from *Escherichia coli*-based culture. Following purification, plasmid DNA can be stored without a cold chain, reducing the cost of vaccine distribution. This may be of particular importance in the developing world.

2.9.1 Performance in Animal Models

Relative to traditional inactivated influenza vaccine, DNA vaccination using HA has been shown to generate balanced humoral and cell-mediated immune responses in small animal models,[110] and research has provided evidence of long-lasting protection in mice.[111] Recent studies by Laddy et al.[112] have demonstrated that DNA vaccination delivered by *in vivo* electroporation generated strong humoral and cellular immune responses in primates, and markedly reduced viral shedding following challenge with H5N1 influenza virus. In related studies, broad cross-protection was observed in mice, ferrets, and primates by vaccinating animals with DNA encoding a consensus, as opposed to any strain-specific, HA sequence.[113] Epstein et al.[114] took an alternative approach by using plasmids encoding the conserved influenza proteins; vaccination of this type conferred partial immunity against H5N1 influenza challenge in mice, but protection was limited to low challenge doses with moderately virulent virus strains. Similar data were generated by Rao et al.,[115] who demonstrated that DNA vaccination with internal protein plasmids conferred limited immunity to influenza, and that inclusion of the major antigenic protein, HA, was required for optimal protection against lethal challenge with H5N1 in mice and ferrets.

2.9.2 Performance in Humans

The safety and immunogenicity of DNA vaccines against seasonal influenza has been demonstrated in phase I clinical trials.[116,117] Furthermore, a recent phase I clinical trial involving an H5N1 influenza-specific DNA vaccine also demonstrated safety and immunogenicity.[118] Immune responses as measured by hemagglutination inhibition titers following H5 HA DNA vaccination were similar to those observed following immunization with conventional inactivated H5N1 vaccine, suggesting further improvement in immunogenicity through adjuvant use or other means may be desirable.

DNA vaccines have been shown to be safe and provide protection from influenza morbidity and mortality in a number of animal studies. The data from clinical trials demonstrate that these vaccines are also immunogenic in humans, but it remains to be seen whether DNA vaccination in its current form will reach licensure. The use of electroporation as a delivery method may warrant further study as a means of improving the immunogenicity of DNA vaccines targeting influenza viruses.

2.10 Virus Vectors as Influenza Vaccines

In recent years, researchers have explored the application of replication-incompetent or nonpathogenic viruses as antigen delivery platforms. Several potential viral vector systems are at various stages of development and were recently the subject of a review.[119] H5N1-specific experimental vaccines inducing protective immunity in small animal models have been evaluated, including modified vaccinia virus Ankara,[120,121]

NDV,[68] vesicular stomatitis virus,[122-124] baculovirus,[125-127] and adenovirus-based systems.[111,128,129] Such delivery platforms have the potential to improve the efficiency of vaccine production, as they are adaptable to many antigens, require only the sequence of the antigen of interest, and are amenable to standardization with bioreactor-based processes. A phase I clinical trial has shown an A/PR/8/34-based adenovirus vaccine to be safe and immunogenic.[130]

2.11 Adjuvants

Adjuvants act to stimulate innate immune responses or enhance the presentation of antigen and, through these mechanisms, increase the immune response to vaccination. Various adjuvants, including oil-in-water emulsion adjuvants such as MF59 and AS03, have been shown to be effective in enhancing the immune response to influenza vaccine.[131-133] In the EU, adjuvanted influenza vaccine has been shown to be safe over years of use and after administration of millions of doses. A recent review by O'Hagan[134] has discussed MF59 adjuvanted influenza vaccination in detail.

Oil-in-water adjuvants have been shown to improve immunogenicity and reduce the need for antigen, in clinical trials of inactivated H5N1 vaccine.[135,136] Recently, studies of the immune response to adjuvanted vaccines have shed light on the mechanisms behind the augmented responses, providing rational strategies for the design of improved vaccines.[137,138]

2.12 Efforts Towards a Universal Influenza Vaccine

Monoclonal antibodies that bind to the HA stalk domain and are both broadly cross-reactive and neutralizing have recently been identified.[139-144] A key feature of these antibodies is that they map to epitopes that comprise membrane proximal portions of both the HA1 and HA2 subunits of the HA protein and, importantly, these epitopes are relatively conserved among strains. Vaccination with a modified HA that lacks the globular head domain and maintains the integrity of the stalk region has been shown to elicit anti-sera that is cross-reactive against multiple subtypes of HA, and provides protection against lethal influenza virus challenge.[145] Furthermore, cross-neutralizing antibodies to influenza virus HA were elicited in mice by sequential vaccination with drifted HA antigens, suggesting the immune response can be directed towards more conserved epitopes.[146] These data suggest that, through optimization of antigen delivery and immunogenicity, modified HA molecules could form the basis for a broadly protective influenza virus vaccine. This approach has been supported by recent studies demonstrating that broadly neutralizing antibodies directed to the stalk domain of the HA can be generated in individuals, following natural influenza virus infection or immunization.[147-149] Future efforts should focus on strategies to elicit a stronger response to these broadly conserved epitopes following vaccination.

3. Conclusions

Following a long period of relative stasis in influenza virus vaccine production methods, the last decade or so has seen significant advances in both basic research and production methods. The application of reverse genetics techniques, together with cell culture-based production practices, has streamlined vaccine production. Research into alternative forms of vaccine and a better understanding of immune responses to vaccination promise to deliver new vaccines that provide longer-lasting and broader protection from disease. Steps have already been taken in this direction. Ultimately, gains in vaccine efficacy and improvements in the efficiency and security of production processes will translate into lower mortality and morbidity resulting from influenza infection.

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