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Clinical vaccine development for H5N1 influenza

Christopher H Clegg[*,1], **Joseph A Rininger**[2], and **Susan L Baldwin**[3]

[1]TRIA Bioscience Corp., Suite 250, 1616 Eastlake Avenue East, Seattle, WA 98102, USA

[2]CaroGen Corporation, 295 Washington Ave, Suite 4N, Hamden, CT 06518, USA

[3]Infectious Disease Research Institute, Suite 400, 1616 Eastlake Avenue East, Seattle, WA 98102, USA

Abstract

H5N1 is a highly pathogenic avian influenza virus that can cause severe disease and death in humans. H5N1 is spreading rapidly in bird populations and there is great concern that this virus will begin to transmit between people and cause a global crisis. Vaccines are the cornerstone strategy for combating avian influenza but there are complex challenges for pandemic preparedness including the unpredictability of the vaccine target and the manufacturing requirement for rapid deployment. The less-than-optimal response against the 2009 H1N1 pandemic unmasked the limitations associated with influenza vaccine production and in 2010, the President's Council of Advisors on Science and Technology re-emphasized the need for new recombinant-based vaccines and adjuvants that can shorten production cycles, maximize immunogenicity and satisfy global demand. In this article, the authors review the efforts spent in developing an effective vaccine for H5N1 influenza and summarize clinical studies that highlight the progress made to date.

The continuing risks of influenza infection

Influenza viruses are highly contagious airborne pathogens that belong to the *Orthomyxoviridae* family of RNA viruses. Infection is initiated when viral hemagglutinin (HA) proteins bind sialic acidlinked glycoproteins on respiratory epithelium of birds and mammals. Unlike some viral infections that stimulate lifelong immunity, influenza viruses have evolved mechanisms that promote point mutations in genes encoding the two surface proteins, HA and neuraminidase (NA), which help evade host immune responses. This antigenic drift selects for newly emerging strains that cause recurrence of seasonal epidemics. The worldwide prevalence during such seasonal epidemics can vary between 5 and 15% causing 3–5 million cases of severe illness and up to 500,000 deaths per year with

[*]Author for correspondence: Tel.: +1 206 819 9413, cclegg@triabio.com.

90% of deaths and hospitalizations occurring in the elderly [101,1]. Influenza viruses also mutate through genetic reassortment providing opportunities for the virus to cross species barriers into immunologically naive hosts including humans. Pandemic influenza infection has occurred intermittently over the centuries and the last three in 1918, 1957 and 1968 killed nearly 50 million people combined. Fortunately, the recent outbreak of a novel H1N1 virus in 2009 was relatively mild with limited morbidity and mortality, although a disproportionate number of deaths occurred among children and young adults.

H5N1 viruses

Avian influenza causes two forms of disease in poultry, one common and mild, the other rare but highly lethal. While 16 HA and nine NA virus subtypes infect birds, the highly pathogenic viruses are typically H5 and H7 subtypes, which are distinguishable by a set of basic amino acids in the HA cleavage site [2]. The highly pathogenic H5N1 virus continues to spread in bird populations worldwide and has appeared in more than 60 countries across Asia, Europe and Africa. Avian influenza viruses are normally speciesspecific, but five strains (H5N1, H7N3, H7N7, H7N9 and H9N2) have infected humans. While disease with most of these strains is generally mild, H5N1 and H7N9 infections are the notable exceptions. H5N1 infection has caused over 600 deaths with a 60% fatality rate [3–5], and at the time of writing, 22 people have died with a 20% death rate from the recent H7N9 outbreak. H5N1 viruses are rapidly evolving and have diverged into ten distinct clades, each with multiple subclades, and there is great concern that H5N1 will eventually acquire the ability to transmit between humans [6]. This worry is compounded by the fact that a relatively small number of mutations permit aerosol H5N1 transmission between ferrets without causing a significant change in virulence [7,8]. Vaccination is the most effective tool for preventing influenza and in response to the emerging H5N1 threat in 2005, the US National Strategy for Pandemic Influenza delineated preparedness goals that included building a domestic production capacity sufficient to vaccinate the entire US population within 6 months. A second goal involved stockpiling millions of 'prepandemic' vaccines targeted against circulating H5N1 strains with the hope that the supply could provide sufficient protection against newly emerging viruses while antigenically matched vaccines could be produced.

Inactivated H5N1 vaccines

Influenza HA is the primary vaccine antigen for inducing protective antibody responses and vaccines are standardized to HA content. The initial H5N1 vaccines were produced using the same manufacturing methods and regulatory approval criteria as seasonal influenza vaccines, which involves production of a virus seed stock optimized for growth in chicken eggs and inactivation. Chemical treatment with formalin or β-propiolactone followed by a detergent-based disruption, or splitting, preserves the virus and HA sufficiently to elicit a protective immune responses against seasonal influenza viruses [9]. Whole inactivated viruses that are not split as well as live-attenuated viruses are reportedly better immunogens for seasonal influenza vaccines, although recent evidence with live-attenuated H5N1 viruses suggests that the removal of HA multibasic cleavage site diminishes its effectiveness [10–12]. Importantly, inactivated whole and split viruses are not highly purified and have

significant quantities of residual egg/host proteins, DNA and RNA that are greater than those allowable for recombinant protein-based vaccine candidates.

The immunogenicity criteria for licensure of pandemic influenza vaccine candidates are based primarily on the induction of hemagglutination inhibition (HI) antibody titers (TABLE 1). The USA has two primary criteria, whereas the European authorities have outlined three benchmarks [102,103]. Considerations surrounding licensure criteria may also be made for manufacturers with a licensed seasonal vaccine that use the same process to manufacture a pandemic vaccine coupled with the severity of the threat to human health [102]. The first US FDA-approved H5N1 vaccine, an inactivated split virus using the clade 1 A/Vietnam/1203/2004 strain, induced a 'seroprotective' HI titer in 58% of study participants after two 90-µg immunizations (TABLE 2). This constitutes a sixfold higher dose than that routinely used in seasonal vaccines and is, therefore, considered suboptimal for any large-scale vaccination program. Similar results were obtained with additional egg-based H5N1 inactivated subunit and split virion vaccine candidates from licensed seasonal vaccine manufacturers (GSK and Novartis), where doses lower than 90 µg failed to achieve approvable endpoints.

As an alternative to egg-based virus production, the US Department of Health and Human Services has supported the development of cell-based H5N1 vaccines, which entails high-titer virus production using suspension growth of MDCK and Vero cells in serum-free media [13]. This technology eliminates many of the limitations associated with egg-based methods including supply of certified eggs, adaptation of virus seed stocks and potentially slow growth with unreliable yields. Cell culture technologies facilitate uniform and predictable vaccine production, although this methodology has its own challenges related to facility startup costs, including appropriate biocontainment measures and potential delays in production scale-up. It remains to be determined whether cell-based methods will significantly enhance the turn-around time for vaccine production since both methodologies entail purification and inactivation of whole virus. However, cell-based vaccines may provide a more immunogenic vaccine based on a clinical seroconversion rate of 62% in adult subjects immunized with 2×7.5 µg injections of an inactivated whole virion vaccine produced in Vero cells (Vepacel, Baxter) [14]. These Phase III data are superior to unadjuvanted egg-derived split virus vaccines and, with accompanying safety findings, served as the basis for vaccine licensure in the EU.

A need for vaccine adjuvants

The ideal H5N1 vaccine will induce a robust protective immune response with minimal antigen and provide cross-protection against viruses from different clades. To achieve this goal, the vaccine must engage mechanisms that effectively prime and induce adaptive immunity in the naive host. Immunological priming is complicated by the fact that vaccine effectiveness will differ with age, race and health status. For instance, children are more susceptible to infection, have increased rates of morbidity and accelerate virus transmission within the general population. For these reasons, children are among the top priority group for broad immunization programs to combat pandemic influenza. Another important population is the elderly, which accounts for more than 90% of seasonal influenzarelated

deaths and hospitalizations. While vaccines are highly effective in younger adults, they are much less effective in the elderly due to age-related losses in immune function, including unproductive priming and recall, weakened T-cell help and the eventual skewing in B-cell repertoire [15]. Their increased susceptibility to infection presents a major public health challenge as the proportion of elderly people reaches 20% worldwide by 2050 [104].

Vaccine adjuvants augment the breadth of protective immunity and significantly improve vaccine manufacturing capacity by decreasing vaccine dose and dosage requirements. Adjuvants accelerate priming and the induction of CD4 T-helper cells that enhance cross-neutralizing antibody responses as well as longlived bone marrow-derived plasma cells and memory B cells. These T-helper cells also regulate the expansion, maintenance and recall of antiviral CD8 cytotoxic T cells that are critical for clearance and recovery from infection. The primary strategy for improving vaccine performance involves the stimulation of innate immune pathways that facilitate antigen uptake by antigen presenting cells. Some of the adjuvants that have been tested with H5N1 influenza vaccines are summarized below.

Aluminum salts

The first and only licensed adjuvant in the USA between 1920 and 2009 was aluminum-based mineral salts, which have an acceptable safety profile, a low cost and are best used for inducing antibody responses. It was originally thought that the particle nature of antigen adsorbed to alum created aggregates at the site of injection that favor uptake by APCs [16]. However, deciphering alum's mechanism of action may not yet be complete. For instance, lysosomal disruption caused by the engulfed salts reportedly activates an intracellular stress response mediated, in part, by HSP70 and the NOD-like receptor family pyrin domain 3 inflammasome [17,18]. Additional evidence argues that the strong binding between alum and the dendritic cell (DC) plasma membrane stimulates clustering of ITAM immunoreceptors and activation of Syk, PI3K and ERK phosphorylation cascades [19]. More recent work indicates that alum mediates host DNA entry into the cytoplasm of DCs, where it engages receptors that promote DC maturation and MHC class II presentation [20]. It is well recognized that alum preferentially induces IL-4 and the development of Th2 CD4 T cells and the differentiation of B cells that secrete primarily IgG1 and IgE antibody isotypes. One drawback of alum is that it has little effect on Th1-type responses and cytolytic T cells that are critical for antiviral protection and clearance. Several studies have tested the dose-sparing capacity of aluminum salts formulated with subvirion and whole virus A/H5N1 vaccines in healthy adults, but meaningful adjuvant effects were not consistently observed (TABLE 2). Similarly, alum was less effective than emulsions in augmenting the immunogenicity of recombinant H5N1 vaccines (see below).

Oil-in-water emulsions

To date, the most successful class of adjuvant for H5N1 vaccines are oil-in-water (o/w) emulsions formulated with shark-derived squalene, that is microfluidized in buffer and surfactants to generate oil particles suspended in water averaging 100–160 nm in diameter. The best characterized emulsion is MF59 (Novartis, Basel, Switzerland), which is approved in Europe for use in a seasonal influenza vaccine for the elderly (Fluad®), a split H1N1 A/California/107/2009 vaccine (Focetria®) and a prepandemic H5N1 influenza vaccine

originally derived from an inactivated A/Vietnam/1194/2004 (Aflunov®). AS03 (GlaxoSmithKline Biologicals, London, UK) contains squalene combined with α-D-tocopherol (vitamin E) and polysorbate 80 and is approved in the EU for use with the H5N1 influenza vaccine (Prepandrix®) and a H1N1 vaccine (Pandemrix®) [21]. AF03 (Sanofi Pasteur, Lyon, France), a third squalene-based o/w emulsion prepared with two emulsifiers (polyoxyethylene cetyl-stearyl ether and sorbitan oleate) and mannitol, had received EU approval with the inactivated influenza vaccine, Humenza®, although it has since been withdrawn from use in the EU [22]. Emulsions like MF59 appear to mediate their adjuvant effect by inducing a chemokine-driven gradient at the site of injection that recruits leukocyte infiltration, enhanced antigen uptake and transport to local lymph nodes for subsequent presentation to antigen-activated lymphocytes [23].

In humans, MF59, AS03 and AF03 enhance seroprotective antibody responses to inactivated H5N1 viral vaccine and mediate approximately 30-fold dose sparing following two administrations (TABLE 2) [24–28]. Moreover, these studies also show that heterologous prime–boost vaccination with emulsions stimulate cross-priming and the production of cross-reactive antibody titers. As an example, addition of AS03 to 3.8 μg of an inactivated split virus vaccine induced significant neutralization titers against clade 1 virus in 86% of the subjects and induced a high level of cross-protection (75–85%) against three drifted clade 2 viruses [29]. Similarly, addition of AF03 to 1.9 μg of the Sanofi split virion vaccine stimulated effective antibody responses in 72% of the subjects, whereas the seroconversion rate of 7.5 μg of vaccine alone was only 34% [28]. Emulsions are thought to prime Th2/Th0 T-cell immunity leading to strong B-cell antibody responses and recent work suggests that they induce broadly reactive CD4 T cells that accurately predict the rise of neutralizing antibody titers after booster immunizations [30]. Importantly, emulsions induce antibody epitope spreading from the HA2 portion of HA to large conformational domains in the globular HA1 region of HA, and these newly induced antibodies correlate with broad cross-clade neutralization [31]. Vaccine responses in infants and young children are also markedly improved with emulsions, which reinforce the need to develop effective adjuvants for pediatric use [32]. Despite the fact the MF59 and AS03 are both approved in the EU, their deployment in the USA has been slow due to a conservative position adopted by the FDA regarding potential toxicity and the lack of long-term safety data.

Glucopyranosyl lipid adjuvant

Th1-mediated immune responses play a critical role in preventing viral infection and progression within the host [33–35]. Th1 CD4 T cells express IFN-γ and TNF-α which inhibit virus replication directly; these 'helper' T cells also broaden antibody responses and induce B-cell memory, as well as regulate antiviral CD8 cytotoxic T cells that clear infected cells. The most direct method for inducing Th1 adaptive immune responses involves activation of Tolllike receptors (TLRs) that recognize and bind pathogen-derived sugars, proteins, lipids and nucleic acids [36,37]. These receptors stimulate DC maturation and are required for normal innate and adaptive immunity. To date, the best validated adjuvant target is TLR4, which is unique amongst the TLR family in that downstream signaling occurs via both the MyD88-dependent pathway and the type I interferon pathway [38]. TLR4 agonists are highly effective adjuvants in experimental and clinical settings with the most advanced

product being MPL (monophosphoryl lipid A), which is a component of the hepatitis B vaccine licensed within the EU (Fendrix®) and is also contained in the HPV vaccine, Cervarix®, which received US FDA approval in October 2009 [39].

Glucopyranosyl lipid adjuvant (GLA) is a synthetic TLR4 agonist that may offer improvements over MPL, which is processed from *Salmonella minnesota* as a detoxified bacterial lipopolysaccharide. The natural product, MPL, contains a heterogeneous mix of hexa-, penta- and tetra-acylated lipid A moieties, only one of which (the hexa-acylated form) was shown in a similar product to bind human TLR4 efficiently [40,41]. As a pure synthetic hexaacylated molecule, GLA has a higher specific activity for human TLR4 than MPL and potently stimulates human DC activation and cytokine production *in vitro* [42]. In a very practical sense, GLA production could also be more easily scalable than natural products such as MPL, and could potentially meet worldwide demand if/when a pandemic flu outbreak were to occur. GLA can be formulated with various components to improve vaccine compatibility including o/w emulsions (GLA-stable emulsion [GLA-SE]), an aqueous formulation containing DPPC-based micelles (GLA-AF) and GLA adsorbed to alum. Experimental vaccines containing GLA demonstrate enhanced immunogenicity in a variety of preclinical animal models [37]. In the context of influenza, GLA-SE significantly enhanced the immunogenicity of the seasonal vaccine, Fluzone®, as measured by increases in HI titers, dose-sparing and broadened cross-reactivity to drifted strains of virus [43]. Moreover, Fluzone + GLA-SE stimulated a dose-dependent increase in HI titers in nonhuman primates, and safe doses of GLA-SE have been established in a recent Phase I clinical study [44]. In addition, split virus vaccine and GLASE-stimulated peripheral blood mononuclear cells from elderly adults were shown to enhance T-cell responses in this population [45]. All of these factors helped establish the rationale for testing GLA-SE/GLA-AF as an adjuvant for H5N1 pandemic influenza vaccines (see below).

ISCOMATRIX®

This adjuvant contains purified fractions of *Quillaia saponaria* extract (ISCOPREP saponin), cholesterol and phospholipid and forms cage-like structures, typically 40–50 nm in diameter, that can be mixed directly with most vaccines [46]. ISCOMATRIX (CSL Research, Parkville, Australia) is another example of an integrated adjuvant system that facilitates antigen delivery within lymphoid tissues and stimulates DC maturation and adaptive immunity. It induces a mixed Th1/Th2 antibody response and robust CD4+ and CD8+ T-cell activation in mice and humans. ISCOMATRIX adjuvant is antigen dose-sparing in influenza models and has been shown to provide single dose, prolonged protection against lethal H5N1 virus challenge in ferrets [47]. To date, more than 1600 subjects have been dosed with this adjuvant across approximately 16 clinical studies, although results with an H5N1 vaccine have not yet been reported.

Recombinant H5N1 vaccines

Manufacturing pandemic vaccines by traditional methods is time consuming, expensive and requires an impractical number of eggs to immunize people worldwide. As evidenced by the 2009 emergence of swine H1N1, there was tremendous pressure to produce large quantities

of a 'first wave' pandemic vaccine and significant delays and inefficiencies were unmasked. This lessthan-optimal response raised new awareness of the limitations associated with influenza vaccine production, which in turn, resulted in specific recommendations by the President's Council of Advisors on Science and Technology for re-engineering the influenza vaccine production enterprise in the USA [105]. Central to this plan is an aggressive effort in developing new vaccines based on recombinant DNA technology and strong support for testing these vaccines in the presence of adjuvants that will maximize antigen immunogenicity and increase vaccine supply (dose sparing). Current manufacturing strategies for H5N1 vaccines exploit a variety of host producer cell systems that are designed for inexpensive production and rapid response [48]. Some of the recombinant strategies and corresponding vaccine candidates that are currently being developed are outlined in TABLE 3 and are further discussed below.

Recombinant HA

The production of a purified recombinant HA (rHA) protein has been the focus of the majority of recombinant vaccine technologies, given the importance and standardization of HA content for calibrating vaccine formulation. HA's native conformation is a trimeric envelope glycoprotein and purifying HA as a stable and immunogenic protein has been a fundamental challenge. The leading rHA vaccine candidate is produced by Protein Sciences Corp (Meriden, CT, USA). This process utilizes recombinant baculovirus and infection of SF+ insect cells, which are notable for high yielding expression of appropriately folded protein and post-translational modifications similar to those in mammalian cells [49]. This system has been used to produce both seasonal and avian H5 influenza vaccines which, following purification under nondenaturing conditions (90%), form large oligomeric rosette-like structures predominantly in the 30–40 nm range [50]. The seasonal influenza vaccine (Flublok®, approved in January 2013) is produced as a full length HA_0 form rather than processed HA1 and HA2 subunits, whereas the rHA from H5N1 influenza strains (PanBlok®; Protein Sciences Corp., CT, USA) is predominantly cleaved at the multibasic site that is characteristic of H5N1 HA protein sequence.

PanBlok is the most advanced recombinant HA in clinical development with the first H5N1 clinical trial conducted after the initial outbreak of human infections in 1997 (TABLE 3). In this study, subjects received two doses of 25-, 45- or 90-μg rHA (A/Hong Kong/156/2007) or 90 μg followed by a 10-μg boost, at intervals of either 21, 28 or 42 days. The best responses achieved with this unadjuvanted vaccine was the 2×90-μg dose group, although immunogenicity was relatively poor with only 52% of subjects seroconverted at a 1:80 titer [51]. Subsequent studies conducted in Japan tested two doses of 5, 15 and 45 μg of rHA (A/Vietnam/1203/2004) adjuvanted with alum. Again the vaccine was poorly immunogenic with serum conversion rates below 25% and alum failed to increase HI titers [52]. The most recent study conducted in the USA tested an adjuvanted clade 2 A/Indonesia/05/2005 rHA using GLA-SE. Preclinical studies demonstrated that GLA-SE, relative to emulsion, accelerated induction of a Th1-mediated antibody response that broadened protection against heterosubtypic H5N1 virus challenge in mice and ferrets [53]. In the Phase I clinical study, all adjuvant doses, 3.8, 7.5, 15 and 45 μg, met FDA licensure criteria, whereas the 135- and 45-μg nonadjuvanted dose levels failed to meet immunogenicity criteria. This

demonstrated a 36-fold dose-sparing capacity with the GLA-SE-adjuvanted rHA vaccine (TABLE 3). Unfortunately, the study design did not allow for a delineation of responses by GLA versus that of the emulsion. A Phase II clinical trial with this H5N1 pandemic candidate using an o/w emulsion-based adjuvant is approaching completion.

rHA is also being produced (Fraunhofer, NJ, USA) via transient expression in tobacco plants using a recombinant RNA virus. Plant-based production is fast, economical and eliminates standard cell culture-based technologies [54]. The first study with this antigen platform evaluated doses of 15, 45 and 90 μg with alum or 90 μg alone of A/Indonesia/05/2005 rHA antigen [55]. The plant-derived rHA showed a good safety profile; however its immunogenicity was lower than a comparable amount of insect cell-produced rHA. The highest response elicited was in the nonadjuvanted 90-μg dose group, suggesting that the antigen may not be appropriately folded.

Escherichia coli expression is a preferred protein production system because of its low cost, speed in fermentation and homogeneity of product. *E. coli* represents a convenient and powerful tool for vaccine production, assuming that the primary bottlenecks for this process, such as the potential for poor product solubility and the lack of post-translation modifications, (i.e., glycosylation), are properly addressed. There are two promising *E. coli*-based production systems also in development that express the globular head of HA (HA1). The most advanced candidate is a chimeric protein consisting of HA1 fused to flagellin of *Salmonella typhimurium* (fljB; STF2). Flagellin has been described as a TLR5 ligand consisting of domains 0, 1, 2 and 3, where domain 1 contains the TLR5-binding site. A single molecule incorporating both antigen and adjuvant results in enhanced antigen presentation and induction of humoral and cell-mediated immune responses [56]. Clinical results with seasonal influenza have demonstrated that doses of 2.5 and 4 μg in healthy adults and the elderly, respectively, produced significant immunogenicity [57]. Dose-limiting adverse events were also observed starting with the 8-μg formulation and others at doses >15 μg [57]. Initial clinical data with an A/Indonesia flagellin construct yielded seroprotection in 49% of subjects at 1.5–2.5 μg doses in adults (18–49 years) but only 15% in adults aged 50–64 [58]. Results of a new Phase I clinical trial encompassing a broad dose range with H5N1 are pending (TABLE 3). The second *E. coli* recombinant candidate is the selective expression and purification of the HA1 subunit or rHA1 which has been extensively characterized for functional activity and forms structures similar to the insect cell-produced antigen [59,60]. This construct has shown utility in preclinical models but is not yet in clinical development.

Virus-like particles

Virus-like particles (VLPs) mimic the structure of authentic virus particles and are characterized by spontaneous self-assembly and budding following DNA transfection of mammalian, insect, yeast and plant cells. VLPs form large particles approximately 100 nm in diameter and offer a clear advantage over conventional inactivated vaccines by eliminating the need to work with isolated virus strains or egg-based amplification systems, and may induce broader and longer-lasting immune responses. Novavax Inc. (Rockville, MD, USA) is developing VLP-based influenza vaccines produced in insect cells using a

recombinant baculovirus coexpressing HA, M1 (matrix) and NA proteins from respective seasonal and pandemic viruses [61]. In preclinical models, these VLPs induce high-titer serum HI antibodies and elicit complete protection in mice against cross-clade H5N1 influenza virus challenge [62]. Initial H5N1 studies with the baculoviral VLP vaccine candidate incorporated doses of 15, 45 and 90 μg (based on HA content) from A/Indonesia/05/2005 without adjuvant. The vaccine induced dose-dependent immune responses that, unlike the other rHA vaccines with the A/Indonesia/05/2005 antigen, met licensure criteria at the 90-μg dose with a 61% seroconversion rate at the 90-μg dose group [60]. Analogous to the egg-produced vaccine, this dose is suboptimal for mass production and vaccination upon pandemic outbreak. As a result, two Phase I trials with undisclosed adjuvants have been conducted [106]. Preliminary results showed that 86 to 100% of subjects receiving adjuvanted vaccine at all dose levels, including a 3.8-μg dose, demonstrated seroconversion rates with either a fourfold rise in HI titer or a titer of ≥1:40 from a negative baseline. In addition, a 45-μg unadjuvanted dose resulted in HI titers ≥40 in >82% of subjects, which would fulfill Center for Biologics Evaluation and Research criteria for accelerated approval.

Medicago Inc. (Montreal, Quebec, Canada) is developing influenza VLPs that express HA in tobacco plants using a plant *Agrobacterium* viral vector and a 5-day production cycle where uncleaved HA_0 molecules spontaneously assemble into VLPs between the plasma membrane and the cell wall, after which they are processed and purified. A commercial facility has recently been built in Research Triangle Park in the USA, where 120 million doses of pandemic influenza vaccine a year are anticipated. The plant-derived VLP, adjuvanted with alum, was tested clinically at doses of 5-, 10- and 20-μg HA and demonstrated dosedependent increases in immunogenicity with seroconversion rates ranging from 42 to 58% [63]. In addition, a follow-up study of the plant VLPs produced from the A/Indonesia/H5N1 HA gene were tested in a Phase II clinical trial in 18–60 year-old subjects using two doses of 20-μg HA adjuvanted with aluminum hydroxide and administered 21 days apart. The vaccine was well tolerated except for local adverse events (pain) in 60% of the volunteers. Seroprotection and seroconversion rates were 37–64% with the greatest immunogenic response demonstrated at the 20-μg dose [64]. These plant VLPs are currently being evaluated in a Phase 1 clinical trial with a GLA adjuvant formulation [107]. This trial is also designed to compare administration of the vaccine by both the intramuscular and intradermal routes, and is, to our knowledge, the first to test an adjuvanted vaccine intradermally in humans.

Vaccine safety

The risk-to-benefit ratio of any pandemic influenza vaccine must be very low given the large number of healthy individuals intended for immunization. Vaccine safety is influenced by the inherent toxicity of the product itself, the presence of impurities and contaminants, as well as potential toxicity associated with the corresponding immune response. Additional regulatory concerns that may be highly variable between vaccines are the interactions between specific adjuvant–antigen combinations and the potential for triggering autoimmune reactions and acute phase responses. Accordingly, the FDA, EMA and WHO have established well-defined guidelines governing vaccine safety that include preclinical toxicology and mechanism of action studies and large-scale epidemiological studies in

human populations [65,66]. To date, the most scrutinized component of adjuvanted H5N1 vaccines has been shark-derived squalene used in the emulsion-based adjuvants MF59, AS03 and AF03 [67]. Although MF59 does not increase the risk of autoimmune disease based on the safety data collected from the tens of millions of recipients receiving MF59 adjuvanted influenza vaccines [68]. However, the potential for adverse reactions is real and best exemplified by the AS03-adjuvanted influenza A(H1N1)pdm09 vaccine, Pandemrix, which appears to be a precipitating factor for narcolepsy in some children, especially in individuals with a *HLA-DQB1*0602* haplotype [69,70]. Importantly, this condition was not observed with other nonadjuvanted-inactivated H1N1 vaccines or with the MF59-adjuvanted inactivated H1N1 vaccine, Focetria. The causal agent(s) in Pandemrix responsible for this effect are not yet known, but it demonstrates the complex interplay between vaccine formulation and host genetic background, the need to simplify antigen and adjuvant compositions, and the importance of worldwide vaccine safety surveillance.

Conclusion

There are numerous scientific, technological and economic challenges in providing universal coverage of a pandemic influenza vaccine on a global scale. Ideally, these vaccines will be rapidly and inexpensively manufactured and have a long shelf life. They will be safe, and generate robust cross-clade protective immune responses using minimal antigen in children, adults and the elderly. Much has been learned over the past 15 years that help define the scope and limitations in pandemic preparedness as well as how to create these high-performance vaccines. Importantly, recombinant H5N1 vaccines that focus primarily on HA expression appear as effective as whole inactivated virus in eliciting seroprotective antibody responses when used with an adjuvant. These innovative vaccines improve pandemic readiness significantly. By eliminating virus seed stocks, production timelines of recombinant vaccines are shortened by as much as 50%, their yield in doses is increased considerably and bulk cost per dose is reduced substantially [71,105]. While each recombinant platform offers distinct advantages, the choices made in HA gene modification, expression and purification create new challenges in largescale manufacturing that have yet to be resolved conclusively. It remains to be determined how these platforms might impact HA activity and whether incorporating additional H5N1 antigens provide superior protection. Regardless of the relative merits of virus-based and recombinant vaccine manufacturing, all available technologies will be needed in order to produce a world supply of vaccine within 6 months of a pandemic outbreak, and it is clear from the clinical data that vaccine adjuvants will play a central role in achieving this goal. Our knowledge of how adjuvants regulate adaptive immunity has grown considerably since the introduction of alum. Oil-in-water emulsions represent the 'best in class' adjuvant for pandemic influenza that improves priming and the induction of heterosubtypic HI antibody responses, which in turn, leads to a significant increase in dose-sparing capacity. In addition, TLR agonists may represent an important strategy for adjuvanting H5N1 vaccines. Preparation for a pandemic crisis has emphasized the need for better, faster and more efficient ways to develop vaccines. Significant scientific and technological strides have been taken in preparing for what may be an inevitable event.

Expert commentary

There are numerous scientific, technological and economic challenges in providing universal coverage of a pandemic influenza vaccine on a global scale. Much has been learned over the past 15 years that has helped define our limitations in pandemic preparedness and how to create effective vaccines that can be manufactured more rapidly and elicit protective antibody responses safely in people. Importantly, adjuvanted recombinant H5N1 vaccines that focus primarily on HA expression appear as effective as whole inactivated virus in early to mid-stage clinical studies. Several recombinant vaccine platforms are being developed, although it is too early to know which technology might provide superior immune responses. Our understanding of how adjuvants regulate adaptive immunity is much improved and it is clear that adjuvants will play a central role in maximizing vaccine performance. Oil-in-water emulsions are currently the 'best in class' adjuvant for H5N1 vaccines. The threat of pandemic influenza has driven our need to find better, faster and more efficient ways to develop vaccines. Significant scientific and technological strides have been taken in preparing for what may be an inevitable event.

Five-year view

The safety and efficacy of adjuvanted recombinant H5N1 vaccines will be confirmed in Phase III clinical studies, technology platforms will be prioritized and manufacturing processes will be more efficient. New additional clinical endpoints will be developed pertaining to other viral antigens that will support vaccine licensure. Stockpiles of prepandemic vaccines and adjuvants will be significantly larger and the world will be much better prepared for a rapid response against pandemic influenza.

Acknowledgments

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Key issues

- Influenza viruses like H5N1 are highly contagious airborne pathogens that could potentially cause pandemic infection and death.

- Vaccines are critical for preventing disease but their development is hindered by the unpredictability of the emerging strain of virus, the fact that humans will likely not be immune to the virus, and that manufacturing capacity will be insufficient to meet worldwide demand.

- Inactivated H5N1 vaccines are poorly immunogenic and are inefficiently produced in chicken eggs.

- An aggressive effort is underway to develop new vaccine technologies that will maximize antigen activity and increase vaccine supply.

- New egg-independent vaccine antigens that appear effective in human clinical trials include cell-based virus production, recombinant protein and virus-like particles.

- Vaccine adjuvants augment adaptive immunity and are being used to broaden antibody responses against heterosubtypic strains of virus and to improve vaccine dose-sparing.

- Significant scientific and technological strides have been taken in preparing for pandemic influenza.

Table 1.

Immunogenicity response criteria for pandemic influenza vaccine licensure.

Parameter	US (FDA) acceptance criteria	European (CHMP) acceptance criteria
Geometric mean HI titer	No standard	No standard
Mean geometric increase (ratio day 42 GMT/day 0 GMT)	No standard	>2.5%
Seroconversion (HI) rate or significant increase in titer	Lower limit of 95% CI >40%	>40%
Microneutralization assay titer	No standard	No standard
Seroprotection rate (HI titer 40)	Lower limit of 95% CI >70%	>70%

CHMP: Committee for Medicinal Products for Human Use; GMT: Geometric mean titer; HI: Hemagglutination inhibition.

Table 2.

Immunogenicity profiles of avian H5N1 virus-based vaccines with and without adjuvants.

Manufacturer (trade name)	Antigen	HA dose (µg)	Seroconversion[†] without adjuvant (%)	Seroconversion with adjuvant (%)	Ref.
Sanofi Pasteur[‡]	Inactivated split virus	90[‡]	57		[28,72,73]
	A/Vietnam/1194/2004	45	41		
		30	52	67 (alum)[#]	
		15	24	86 (AF03)	
		7.5	13	89 (AF03)	
		3.8		81 (AF03)	
		1.9		72 (AF03)	
Novartis[§]	Inactivated subunit	45	29		[25,74,75]
	A/Vietnam/1203/2004	30	18	14 (alum)	
		15	24	7 (alum)	
		7.5	34	3 (alum)	
	Inactivated subunit	30	18		
	A/Vietnam/1203/2004	15	24	63 (MF59)	
		7.5	34	23–73 (MF59)	
		3.8		65 (MF59)	
GSK[§]	Inactivated split virus	30	41	85 (AS03)	[76,77]
	A/Vietnam/1194/2004	15	35	96 (AS03)	
		7.5	16	90 (AS03)	
		3.8	4	84–94 (AS03)	
CSL[¶]	Inactivated split virus	45		49 (alum)	[78]
	A/Vietnam/1194/2004	30		48 (alum)	
		15		35 (alum)	
		7.5		34 (alum)	
Baxter[§]	Vero cell-derived inactivated whole virion	15	68[††]		[11,14]
	(A/Vietnam/1203/2004)	7.5	62		
		3.8	41		

Data are from studies in adult subjects, employing the same clade of H5N1 virus antigen and administered following two immunizations either 21 or 28 days apart. Direct comparisons are not possible due to differences in reagents and methodologies to determine HI titers. Ranges are provided in instances where referenced studies contain the same antigen dose level.

[†]HA inhibition assays with titer 1:40 or fourfold increase in hemagglutination inhibition titer.

[‡]FDA approved.

[§]EU approved.

[¶]Australia approved.

[#]No significant difference between non-alum vaccine group.

[††]Calculated based on subjects achieving 1:20 using the microneutralization assay.

HA: Hemagglutinin; HI: Hemagglutination inhibition.

Table 3.

Immunogenicity profiles of recombinant avian H5N1 vaccine candidates with and without adjuvants.

Manufacturer (trade name/designation)	Vaccine antigen	HA dose (µg)	Seroconversion without adjuvant (%)	Seroconversion with adjuvant (%)	Ref.
Protein Sciences (Panblok, UMN-0501)	rHA (A/Hong Kong/156/1997)	90	52[†]		[51]
		45	28		
		15	17		
	rHA (A/Vietnam/1203/2004)	45	22	8 (alum)	[52]
		15	17	16 (alum)	
		5		20 (alum)	
	rHA (A/Indonesia/05/2005)	135	32		[58,79]
		45	15	82 (GLA-SE)	
		15		75 (GLA-SE)	
		7.5		66 (GLA-SE)	
		3.8		72 (GLA-SE)	
Fraunhofer (HAI-05)	rHA (A/Indonesia/05/2005)	90	10	5	[55]
		45		0	
		15		0	
VaxInnate (Vax161)	rHA (A/Indonesia/05/2005)	1.5–2.5		49[‡] (flagellin)	[58]
		1–2		Pending (flagellin)	
Novavax	VLP (HA, NA, M1) (A/Indonesia/05/2005)	90	61		[60,104]
		45	57–82[§]		
		15	40		
		3.8		86[§] (undisclosed)	
Medicago	VLP (HA) (A/Indonesia/05/2005)	45		37 (alum)	[63,64]
		30		57 (alum)	
		20		58–65 (alum)	
		10		25 (alum)	
		5		17 (alum)	

Data are from studies conducted in adult subjects, employing recombinant H5N1 antigen and administered following two immunizations either 21 or 28 days apart. Direct comparisons between studies are not possible due to differences in reagents and methodologies to determine hemagglutination inhibition titers.

[†] Microneutralization assay titer of 1:80.

[‡] Percent seroprotection.

[§] Unpublished results.

GLA-SE: Glucopyranosyl lipid adjuvant-stable emulsion; HA: Hemagglutinin; NA: Neuraminidase; rHA: Recombinant hemagglutination; VLP: Virus-like particle.