Progress towards a universal influenza vaccine

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Seasonal influenza is a common and highly transmissible disease, characterized by frequent and unpredictable mutations occurring in the viral envelope glycoproteins. Owing to this high variability, annual reformulation and immunization are required and still, the vaccine is not effective enough when there is an antigenic mismatch with circulating strains. A solution could come from the construction of a universal vaccine that would be based on highly conserved antigens and would be effective against many strains: some universal vaccine developers focus on the Matrix 2 protein, whereas others use additional conserved proteins, such as the nucleoprotein and Matrix 1, or even a range of peptides from these proteins and others to induce cross-strain immunity. This article aims to highlight recent significant advances in the development of a universal vaccine against influenza and focuses mainly on studies using the epitope-based approach that have also entered the clinical trial stage; it includes a brief summary of current vaccines against influenza as well as the ongoing efforts to develop a universal vaccine.

Influenza in general
Seasonal influenza is a very common disease that affects millions of people around the world. The most important characteristic of influenza, from an immunological perspective, is the rapid, unpredictable changes of the surface glycopolypeptides hemagglutinin (HA) and neuraminidase (NA). These changes occur mainly in the influenza type A strains, which are responsible for approximately 80% of human influenza infections. Influenza type B strains are divided into two antigenically distinct lineages – the Victoria and Yamagata lineages – and there is little or no cross-reactive protection between them: this means that good protection against the circulating virus relies on correctly predicting the prevalent influenza B lineage in any given season. Two influenza A viruses (H1N1 and H3N2) and one influenza B virus are included in the seasonal vaccine every year [1]. In a recent publication [2], Belshe claims that there is a clear need for a quadrivalent influenza vaccine containing representatives of both influenza B lineages in addition to the two influenza A viruses. These changes result from the virus’ RNA polymerases lacking a proof-reading function, allowing errors to be frequently introduced into the genome as nucleotide substitutions, some of which result in amino acid changes. These changes lead to the emergence of new strains that are the cause of most annual influenza epidemics [3–5].

Influenza infections in healthy adults are usually mild. However, the at-risk populations (i.e., the elderly, very young toddlers and people with chronic illnesses) suffer from complications and more severe symptoms, and hence are the groups that are recommended to be vaccinated by the health authorities, despite the known limitations of the current vaccines. However, this approach is changing in view of the pandemic strains that have appeared in recent years, which seem to be either highly pathogenic (avian strains) or highly infective (swine strains), leading to illnesses and enhanced morbidity in the healthy population, which has a lower chance of developing severe complications as a result of exposure to seasonal influenza. This fact, together with the economic losses associated with influenza infections (estimated to be up to US$80 billion per year in the USA [6]), is on one hand driving health authorities to promote vaccination programs for the entire population, while on the other hand, encouraging researchers and the pharmaceutical industry to improve the efficacy of current vaccines.

Current vaccines & limitations
At present, available commercial vaccines against influenza consist either of whole live-attenuated or of killed virus or of a mixture of their surface glycoproteins, HA and NA (trivalent split virion approach). When there is a close match between vaccine viruses and circulating viruses, the vaccine has been shown to prevent influenza in approximately 70–90% of healthy persons younger than 65 years. Among elderly persons and those persons with chronic medical conditions (e.g., asthma, diabetes or heart disease), the influenza vaccine has been demonstrated to be between 30 and 70% effective in
preventing hospitalization for pneumonia and influenza \[101,102,7\]. It is clear that these vaccines fail to induce complete, long-term and cross-strain immunity owing to their limitation regarding strain specificity. The frequent mutations of the viral RNA leads to changes in the HA and NA, which can give such mutant viruses a selective advantage in the immune population and, hence, a vaccine based on a specific combination of viruses (in the live-attenuated approach) or HA and NA (in the trivalent approach) will not be effective against other strains containing different HA/NA that may infect the population. This is the reason for the annual reformulation and vaccination required for influenza.

Owing to the antigenic changes (i.e., shift and drift, referring to major and minor changes, respectively), the commercial influenza vaccines are dependent on the annual forecasted virus strains for the following season, a prediction that may fail and affect the efficacy of those vaccines. To ensure that vaccines contain the most appropriate circulating strains, and thereby improve influenza control measures, in 1952 the WHO founded the Global Influenza Surveillance Network complex, comprised of more than 130 National Influenza Centers. This WHO system also ensures that vaccines have equivalent efficacy through the use of standardized potency testing reagents. Despite these efforts, the annual selection of the three strains (currently one H3N2, one H1N1 and one influenza B virus strain) for the trivalent vaccine is still an educated guess that is not always successful.

The conventional approaches for vaccine preparations also suffer from production-related shortcomings; most current vaccines are produced in hen eggs in a lengthy (6–8 months) and cumbersome process. The limitations associated with this production process were evident in 2004 when Chiron’s influenza vaccine manufacturing plant in the UK was found to have problems with bacterial contamination. As a result, Chiron’s license was suspended by the regulators, preventing them from supplying any influenza vaccine to the US market for the 2004–2005 influenza season, creating an instant and severe vaccine shortage just as the flu season began. Owing to the lengthy and therefore inflexible production process, it was impossible for other manufacturers to take up this shortfall and to produce the additional (≈50 million) doses in time. The compromised solution was to focus vaccination efforts on those people at greatest risk for influenza-related complications.

Similarly, the recent experience with the 2009 A/H1N1 swine flu pandemic has highlighted the limited ability of egg-based manufacturers to respond in time to unexpected demand, and to adjust their production quantities to changing levels of demand. The industry can produce approximately 500 million doses of seasonal vaccine per year \[103\] or divide this production capacity between seasonal and pandemic formulations. In case of a severe epidemic or a production problem, this may not be enough – indeed, at the beginning of the 2009 pandemic, it was thought that there would be a global shortage of swine flu vaccines, and vaccine manufacturers invested heavily in the expansion of their production capacity, producing approximately 800 million doses. However, as the pandemic progressed, it became evident that a vaccine excess would occur: now that the swine flu symptoms have waned and vaccine demand is diminished, many countries have found themselves with excess quantities of the vaccine \[104\].

Another shortcoming associated with the egg-based production of influenza vaccines is that the vaccines cannot be given to people suffering from hen egg allergies. Approximately 0.1% of the adult population and 2% of the young suffer from allergy to albumin \[105\], and hence, immunization with influenza vaccines that are produced in eggs is not recommended for them \[8,106\]. In reality, however, there are very few incidents with influenza vaccination in people with egg allergy \[9,10\]. Finally, it should be noted that, in the case of a pandemic that is the result of an avian influenza strain that is lethal to chickens, a sufficient supply of embryonated eggs could be compromised.

Nevertheless, and despite many efforts to develop alternative production processes, these decade-old technologies, the live-attenuated and trivalent split virus vaccines, dominate almost the entire influenza vaccine market today.

**The need for a universal influenza vaccine**

The outbreak of a new, highly infective pandemic A/H1N1 swine flu strain in 2009 emphasized the limitation of current vaccines and illustrated both the willingness of patients, physicians and health authorities to respond to emerging threats, as well as the need for overall pandemic preparedness.

A truly universal approach that protected against both antigenic drift and shift, and thereby protected against both seasonal and pandemic strains, could dramatically change the current
approach to influenza vaccination, reducing or even eliminating the need for seasonal vaccination [11]. In addition, a vaccine that could be produced in cell or bacterial cultures could also enable year-round production, allowing advance production and stockpiling, as well as facilitating immunization throughout the year. Given the new production methods (i.e., recombinant and cell culture) of the universal vaccines currently in development, such a vaccine would also eliminate the need for egg-based production and the many problems associated with it.

**Immune mechanisms mediating cross-protection**

Most mechanistic studies of cross-protective immunity were performed in mouse models; however, the principles were then confirmed in humans. There are some contraindications regarding the involved mechanisms and the discrepancies between preclinical and clinical studies, but these might be clarified in future studies.

These mechanistic studies concentrated on influenza A strains, while no cross-protection was seen with distantly related type B influenza viruses [12]. The animal studies showed protection by immunization with a live virus followed by a challenge with a divergent strain. For example, vaccination with the older H1N1 viruses, particularly A/FM/1/47, conferred protective immunity against the 2009 pandemic H1N1 virus, explaining the decreased susceptibility of the elderly to the 2009 H1N1 outbreak and the mild illness in the community caused by this virus in the recent pandemic [13–15].

Studies with gene knockout mice showed cross-protective immunity to be mediated mainly by T cells and to be dependent on the cytolytic effector molecule perforin. Reduced inflammation following infection with a heterotypic strain was associated with enhanced early recruitment of both CD4+ and CD8+ T cells, and with early influenza virus-specific cytotoxic T-cell responses targeting mainly the viral nucleoprotein (NP) [16]. The T-cell response afforded by an immune-stimulating complex-formulated virus was directed mainly against the HA and was accompanied by the secretion of cytokines such as IFN-γ [17] and TNF-α [15]. Studies in IFN-γ-knockout mice show that IFN-γ is not required for cross-protection [18]; however, this can be attributed to the abnormality of this model system since other studies have shown that this cytokine indeed plays a role when present [19].

The role for CD4+ T cells in cross-protection may be in supporting and enhancing cytotoxic T-lymphocyte responses and providing help for B-cell responses via cytokine secretion. CD4+ T cells specific for conserved viral antigens can potentiate subsequent antibody responses to the HA of a different virus encountered subsequently. Prior immunity to internal proteins has been shown to result in heightened or accelerated HA-specific antibody responses to virus of a different subtype [20,21].

Cross-protection can also be mediated by antibodies: passive transfer of antibodies against the conserved, surface-exposed matrix 2 ectodomain (M2e) has been shown to provide cross-protection against PR8 (H1N1) and A/Hong Kong/68xPR8 reassortant (H3N1) strains, but not against A/Hong Kong/97 (H5N1) strain, where survival after lethal challenge served as a parameter for protection [12] and direct immunization with M2e led to survival after lethal infection with X47 (H3N2) [21,22]. Cross-protective antibodies can also target a conserved epitope in the HA stem – this is possibly achieved by preventing HA-membrane fusion activity that results in protection against lethal infection [24,25]. The limitation of using these antibodies is the limited access of these antibodies to their target binding site in the living virus and their limitation to only Group 1 HA subtypes, consisting of H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 [26].

Within the respiratory tract, it seems that the humoral arm of the immune system plays a role in conferring cross-strain immunity and protection: co-administration of inactivated virus with cholera toxin as an adjuvant conferred complete heterosubtypic protection, without observed illness, even under conditions of CD4+ or CD8+ T-cell depletion. Analysis of immune correlates, by virus neutralization with antibodies, prior to challenge and postchallenge indicated that humoral immune responses with cross-neutralizing activity in lungs and in sera play a major role in conferring protective immunity against heterosubtypic challenge [27,28].

It has been known for decades that a mild influenza infection in animals provides protection against a subsequent, more severe challenge with a virus bearing different HAs and NAs (a heterosubtypic challenge) [29–32]. However, in humans, heterotypic immunity is not always found.

The unexpected emergence of pandemic H1N1 influenza gave us the opportunity to expand our understanding on immunological
memory to influenza and how previous encounters with seasonal strains influence our ability to respond to novel strains that are derived from antigenic changes. It has been shown that priming with seasonal viruses and vaccines induces cellular immunity, and that the memory CD4 cells can be immediately activated and recognize autologous cells infected with live H1N1 virus \[33,34\]. Cross-protective antibodies to H1N1 in humans were induced by the prime-boosting approach, showing the feasibility of the concept as a pandemic preparedness strategy \[35\].

One way to approach these challenges is the development of a universal vaccine formulation that promotes protective immunity against both influenza A and influenza B strains, and that will be effective against both seasonal and pandemic viruses.

The epitope-based approach

During the last three decades, tremendous advances in the understanding of immunology as well as biotechnology methods (i.e., genetic engineering, molecular biology and preparation of recombinant proteins) have enabled the emergence of new approaches towards the rational design of vaccines. Among them is the use of epitopes corresponding to immunogenic, conserved sequences of microbial proteins \[37\]. The epitope-based approach focuses on the minimal component that activates the lymphocyte: short peptides of eight to ten amino acids for activating T cells and longer regions of up to 20 amino acids for the B-cell epitopes \[38\]. Improved understanding of the molecular basis of antigen recognition and HLA-binding motifs has resulted in the development of rationally designed vaccines based on motifs predicted to bind to human class I or class II MHC molecules. Many studies have shown the immunological efficacy of peptide-based vaccines against infectious diseases in animal models \[39–42\], as well as in clinical studies in which the responses to peptide vaccines against various infectious diseases, including malaria \[43,44\], hepatitis B \[45\] and HIV \[46,47\], were demonstrated. However, there is one main obstacle associated with peptide vaccines, which is their low immunogenicity and, hence, there is a need for better adjuvants and carriers \[48–51\]. Another hurdle is the need for reliable and simple assays to measure the T-cell response.

Immunization with other viral antigens in addition to HA will broaden the immune response against influenza and will induce cross-strain immunity. It should be noted that such cross-protective immunity may not prevent a host from becoming infected, but can reduce viral replication, accelerate viral clearance and thus reduce the severity of disease \[52\]. Inclusion of the more slowly evolving NA and/or M2e proteins in a vaccine against influenza could reduce the vulnerability to antigenic changes, and conserved antigens from internal proteins NP and matrix 1 (M1), delivered to induce T-helper and cytotoxic T cells, could ensure the presence of activated T cells that facilitate clearance of influenza viruses. The peptide-based vaccines target defined regions within the target proteins or within pathogens’ proteins and selectively elevate humoral and/or cellular components of the immune system, generating more effective vaccines; this is especially demonstrated in cancer vaccines \[49–53,54\].

Epitope-based approach for a universal flu vaccine: benefits

Broad-spectrum protection by activating both arms of the immune system

An optimal influenza vaccine is a truly universal vaccine, targeting both influenza A and influenza B strains. Owing to the variations of the HA proteins of the various strains, this can be achieved either by using the conserved stem region of several HA proteins or by employing the epitope-based approach and using epitopes from both influenza A and B. An epitope-based vaccine is based on conserved peptides representing many influenza strains; ideally, it would target the B and T lymphocytes directly to achieve enhanced immunity against the virus. By selecting peptides that match the most prevalent HLA molecules in the population (i.e., both class I and class II), it is possible to induce strong T-cell immunity that, together with the humoral response, confers stronger protection against a broad spectrum of influenza viruses. Activating both arms of the immune system is especially advantageous against intracellular pathogens \[55,56\].
However, currently available influenza vaccines are approved based only on their ability to induce humoral immunity.

Long-term protection
A universal vaccine that provides multistrain protection would thereby confer multiseason coverage. In contrast to existing seasonal influenza vaccines, such a vaccine would be one and the same formulation to be used for several years, without the necessity to modify its composition every year.

Improves patient compliance
By breaking the linkage between the influenza vaccine and the specific strain of the virus that circulates in any season, a universal vaccine can be administered all year round, regardless of the timing of the flu season, and according to patient preferences and health authorities’ national or regional vaccination campaigns. A more systematic, organized year-round vaccination campaign would allow patients and health professionals to benefit from reduced workload around the flu seasons at the clinics.

Improved production
The annual production of influenza vaccine in the traditional process using fertilized hen eggs is a highly complex process, which requires careful coordination of both public health laboratories and vaccine manufacturers in order to provide the vaccines on time.

In the current procedure, the influenza virus is injected into the eggs and accumulates in the fluid surrounding the embryo. The embryo becomes infected so that the virus can multiply. Next, the virus is harvested and purified, chemically inactivated and used to produce the vaccine. On average, between one and two eggs are needed to produce one dose of seasonal vaccine. The entire production process lasts approximately 6 months.

An alternative way of producing flu vaccine is based on cell cultures, which, although new for influenza vaccines, is already employed to produce other viral vaccines (e.g., polio vaccine, measles–mumps–rubella vaccine and chickenpox vaccine). In this process, the virus is inserted into mammalian cells and multiplies within them. Next, the virus is harvested, purified and inactivated. Similar to fermentation, this process can be easily scaled up into huge bioreactors. Another advantage of cell culture production is that virus strains that sometimes do not grow at all in eggs can easily proliferate in mammalian cell culture and the resultant virus is more representative of the circulating wild-type virus than that grown in eggs.

The global capacity for vaccine manufacture in eggs or tissue culture is considerable, but the number of doses that can theoretically be produced in a pandemic context will only be sufficient for a small fraction of the world’s population, and even less if a high antigen content is required in cases of low immunogenic viruses or if more eggs are required to produce a single vaccine dose.

Another effective and faster approach to vaccine generation that bypasses the need for eggs is the construction of influenza virus-like particles (VLPs). VLPs contain repetitive high-density displays of viral surface proteins, which present conformational viral epitopes that can elicit strong T-cell and B-cell immune responses. VLPs are safe and have been produced from components of a wide variety of virus families including Parvoviridae (e.g., adeno-associated virus), Retroviridae (e.g., HIV) and Flaviviridae (e.g., HCV). For influenza, VLPs are purified from the supernatants of Spodoptera frugiperda Sf9 insect cells following infection with baculovirus vectors encoding an expression cassette made up of only three influenza virus structural proteins, HA, NA and M1.

VLPs, for example, those produced by Novavax Inc. (MD, USA) elicit antibodies that recognize a broader panel of antigenically distinct viral isolates compared with other vaccines in the hemagglutination-inhibition (HI) assay. However, the development of such a cell culture-based production process is still a relatively long and arduous process, especially in terms of its efficiency, standardization and validation.

When considering the peptide-based approach, the issue of production presents an advantage since a polyepitope product can be produced as a recombinant protein using standard fermentation processes. Biotechnological fermentation is already in use for the production of insulin, IL-2, IL-11, growth hormone and others. Briefly, bacteria (e.g., Escherichia coli) containing a plasmid with a coding sequence for the protein are grown in a short, simple and robust process. Next, the protein is purified and formulated as required. This process lasts approximately 6 weeks, enabling year-round production that is flexible and easily adapted to market demands. Unlike production in eggs or cell culture, the bacterial fermentation process can be rapidly expanded and scaled up in times of emergency, such as in the case of pandemics.
Avoidance of any potential infectious hazards
When employing the peptide-based approach, there is no need to grow the virus for the preparation of its peptides, thus avoiding the risk of including a living virus within the vaccine. The problem of growing viruses was evident during the preparations of vaccines against avian influenza – since this highly pathogenic virus is lethal to humans, its production in eggs was associated with high risk.

Epitope-based approach for a universal flu vaccine: requirements
Identification of epitopes
To support the rational design of a peptide-based vaccine, epitope-predicting algorithms have been developed to support empirical data on mechanisms of immunity and functionally relevant assays. The most conserved B- and T-cell epitopes within the influenza proteins were identified based on in silico and experimental data, showing their ability to bind the relevant and most prevalent HLA class I or II alleles in the population, and their potential to be used for vaccination [63]. Many of the epitopes identified in these studies are listed in the Immune Epitope Database [107] and are used in studies towards a universal vaccine [64]. A coverage of a large (90%) fraction of the human population can be achieved by focusing on three major HLA class I specificities (the A2, A3 and B7 super-types [super-type is defined as a family of HLA molecules sharing overlapping peptide specificity]) [65]. It is hard to predict the exact number of epitopes required, but combining T-cell epitopes specific to the prevalent HLAs together with B-cell epitopes that are not HLA dependent may provide wide coverage for the population. For ease of production, most of the peptide-based vaccines utilize recombinant production methods and, hence, are restricted to the use of linear epitopes that do not require specific refolding procedures during production in order to maintain their immunogenic forms.

Need for multiple epitopes
No single epitope can serve as a perfect vaccine candidate. An epitope may be shared by most but not all viruses, may be recognized by many but not all responders (HLAs) and may control symptoms and transmission to a helpful but incomplete extent. In view of the scarcity of conserved, protective peptide epitopes in the influenza virus, and their limitations, combining several of them in a single vaccine aims to improve the immunogenicity and strain coverage provided by the epitope-based vaccine.

Wide HLA coverage
A T-cell response to epitope-based vaccines is HLA dependent; the large degree of MHC polymorphism and the need for knowledge of HLA restrictions in the population to be vaccinated make it difficult to design a vaccine that will be effective for all. A vaccine intended for a broad population should include T-cell epitope(s) that will induce responses in the vast majority of individuals; this can be achieved by selecting several T-cell epitopes that are specific to the most prevalent HLA genotypes in the population [66].

Epitope-based approach for a universal flu vaccine: considerations
Low immunogenicity
Peptides are weak immunogens that are degraded quickly within the body, and hence cannot serve as vaccines unless incorporated into a carrier protein [65,67] or combined to form a polyepitope construct. Another approach to overcome their low immunogenicity is to prepare an artificial protein containing several epitopes, possibly with several repetitions of each peptide [68,69]. There are some publications demonstrating this, showing a way to define the optimal number of epitopes and the significance of their organization [70,71]. Another approach to overcome the low immunogenicity of peptides is their combination with an adjuvant, as shown in Table 1.

Pressure of mutations
Focusing immune pressure on a specific epitope carries the risk of selecting escape mutants. Such mutants have already been found in surface glycoproteins, such as the HA, allowing escape from antibody-mediated immunity, and from cytotoxic T-lymphocyte-mediated immunity [72]. Alterations in the HA were found in both its stem region and in its globular head [73,74], and escape mutants were also recognized to the NA glycoprotein [75]. In addition, mutation of residues adjacent to the epitope can create glycosylation sites, and the resulting glycosylation can block antibody binding, leading to escape. When using a mixture of target antigens as a universal vaccine, even if a mutation occurs in any one of them, the others will still be relevant and maintain the universal characteristics of the vaccine. It should be noted that escape mutants also result from vaccination with seasonal vaccines as demonstrated by Air and Laver [76], suggesting that influenza A
virus evolves by adjusting receptor-binding avidity via amino acid substitutions in response to variation in neutralizing antibody pressure.

**Inadequate correlate of protection**

For inactivated seasonal influenza vaccines, a serum HI titer of at least 40 is considered protective. As such, this parameter serves as a correlate for protection and for approval of influenza vaccines by the regulatory authorities. However, HI titer is not a good correlate for protection by live attenuated vaccines [77], and it is not relevant for universal vaccine candidates that are not based on HA. Although antibodies that inhibit viral NA also contribute to protection against disease, there is currently no routine assessment of NA inhibition titers. Some evidence has been reported for the correlation of T-cell responses with protective immunity in vaccinated elderly individuals [78] and in children but, currently, cellular immunity parameters (e.g., IFN-γ, which has known antiviral effects and showed correlation with protection in children [79]) are not recognized as measures that correlate with protection. When considering a peptide-based vaccine that is based on epitopes derived from the conserved viral proteins NP, M1 and M2, clearly correlates other than HI antibodies are needed.

**Companies pursuing a universal influenza vaccine**

There are several companies focusing on the development of a universal vaccine using different approaches, as summarized in Table 1. While all the approaches concentrate on conserved regions of the virus that are common to many strains, some include the whole antigen whereas others focus on conserved peptides (epitopes) within the viral proteins.

In terms of clinical advancement, BiondVax Pharmaceuticals (Ness Ziona, Israel) is the most advanced, conducting a Phase II trial with its Multimeric-001 vaccine. This vaccine is based on nine conserved epitopes from the HA, NP and M proteins that induce both humoral and cellular immunity. These epitopes are combined into a single recombinant protein expressed in *E. coli*. This vaccine has been tested in two Phase I/II trials in younger (18–49 years old) and older (55–75 years old) adults. In both trials, the vaccine was found to be safe and to induce both humoral and cellular immune responses [108]. Another vaccine candidate that is expected to enter Phase I trials is being developed by SEEK (London, UK). This vaccine contains six cytotoxic T-lymphocyte epitopes that are conserved among many influenza strains and can bind the HLA A*0201 [109]. This HLA is common (~40%) in several populations, including Caucasians, Japanese and Chinese [80]. FP01 is the lead candidate of Immune Targeting Systems (ITS, London, UK) to combat both seasonal and pandemic influenza using six long (35 amino acids) CD4+ and CD8+ conserved T-cell epitopes administered as synthetic fluoroconjugated nanoparticles: forming stable, immunogenic nanoparticles.

Several companies currently developing universal influenza vaccines are focusing on the M2e peptide: the M2 is an ion channel protein that permits viral uncoating. It is the target protein

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<th>Company</th>
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<th>Phase</th>
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<th>Technology</th>
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* A gene immunoadjuvant serves as an adjuvant (i.e., a DNA plasmid coding for a naturally occurring immune molecule that is co-delivered with the DNA vaccine). M2e: Matrix 2 ectodomain; NA: Not available in the ClinicalTrials.gov site [111]; NP: Nucleoprotein.

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Table 1. Universal influenza vaccines under development.
for the therapeutic drug amantadine and its methyl derivative rimantadine prevents further virus infection by blocking the M2 [81]. Vaccines based solely on the M2 derived from influenza A are not truly universal, since the M2 channel activity in influenza B strains is not affected by these vaccines.

The ectodomain of influenza A virus M2 protein (M2e) is conserved in both human and avian influenza A viruses, being present in nearly all strains detected to date, including highly pathogenic viruses that primarily infect birds and swine, and the current 2009 swine-origin H1N1 pandemic strain. It is composed of 24 amino acids and induces antibodies that can inhibit a broad spectrum of influenza A subtypes in vitro and in vivo. Immunization with M2e fails to induce neutralizing antibodies, but can slow the replication of certain influenza A viruses when included in the overlay of a plaque-titration assay, suggesting a mechanism of action for such a vaccine [82].

Although relatively conserved, 21 M2e variants have emerged in recent influenza A strains, with most of the mutations appearing in the middle part of the M2e domain, casting doubt on its ability to be a truly universal vaccine candidate. Monoclonal antibodies against the highly conserved epitope SLLTEVET (two to nine amino acids), which is common for both M1 and M2 proteins, potently inhibited the replication of influenza A virus H1 and H3 subtypes in Madin–Darby canine kidney cells. Two important amino acids in the M2e epitope, threonine at position five and the glutamic acid at position six, were identified as leading to antibody-escaping variants [83].

Since 1999, a number of studies have demonstrated protection against influenza A virus challenges in animal models using chemical or genetic M2 external domain (M2e) fusion constructs. One main limitation of this region is its low immunogenicity. However, this can be overcome by fusing it to a carrier, such as hepatitis B virus-derived VLPs or by administration with an adjuvant [84]. Another study showed that monoclonal antibodies to M2e that were isolated from human B cells could bind to the M2 protein displayed on virus particles and on virus-infected cells. Furthermore, these antibodies protected mice from a lethal influenza A virus challenge [85].

To further improve the efficacy of the M2e-based vaccine, it can be combined with another conserved protein. Dynavax’s (CA, USA) universal vaccine candidate is designed to offer protection against divergent strains by combining two highly conserved antigens, the NP and M2e, with their proprietary TLR9 agonist ‘immuno-stimulating sequence’. This immunostimulating sequence is a short, CpG-containing oligonucleotide that serves as a potent adjuvant for antibody and Th1 T-cell responses. In this combination, the NP provides cytotoxic T-cell protection, while M2e offers protective antibodies for protection against divergent strains. Dynavax have shown that this vaccine has the potential to boost the immune response and enable dose sparing, which could increase the quantity of standard flu vaccine available during a pandemic. Dynavax initiated a Phase I trial in late June 2010 to examine the safety and immunogenicity of this vaccine candidate in humans [10].

Universal vaccines represent a transformative technology and come with major advantages. However, as none of these universal vaccines are yet in advanced Phase III trials, it would appear that it will be a number of years before there is a universal influenza vaccine on the market. Moreover, significant regulatory questions remain to be answered by the authorities, related to defining new surrogate markers and the approval pathway for such a vaccine that is not HA based and may not therefore induce H1.

**Conclusion**

There is a clear and pressing need for the development of a universal vaccine against influenza, especially in view of the recent swine H1N1 pandemic. Several vaccine candidates based on varying approaches are currently being developed to achieve this goal, focusing on conserved regions of the virus. Although several such candidates use the single M2e epitope for vaccination, it seems that there is an advantage to including conserved regions from several viral proteins to enhance immunity to the wide divergence of influenza viruses in the different HLAs. There are still many questions and issues to be solved: how many different epitopes would be needed to cover the entire population? What antigens would the epitopes come from? What is the likelihood of finding protective epitopes covering the majority of HLA alleles in a given population? Would such a vaccine induce long-lasting protection across seasons? Would the composition of the epitopes have to be adjusted seasonally? How
would such an approach be viewed by regulatory and public health authorities? The research towards a universal vaccine is in different stages of preclinical and clinical trials that will show their potential to be used in the coming years.

Future perspective

Several vaccine candidates with cross-strain potential are currently under development. These include vaccines based on whole viral proteins, such as M2 and NP, that are comparatively conserved, and others that focus on conserved peptides (epitopes) from these proteins and others. The M2-based vaccines induce mainly humoral immunity, whereas those including NP and M1 induce cellular immunity as well. Future work should aim to identify the optimal combinations of NP, M2, M1 and perhaps other conserved antigens for the induction of protective immunity, preferably combining both humoral and cellular immunity. Those candidates that successfully show safety and immunogenicity in early phases of the clinical trials will be further tested in advanced trials and will have to show their protective effect in the population. In cases where low immunogenicity is found in humans, a better understanding of innate immunity will enable a rational re-formulation of the vaccine with new adjuvants that may improve its immunogenicity without causing significant side effects. More effective or user-friendly routes of administration can also be considered to improve the immunogenicity of the vaccine candidates.

In view of the high variability between influenza strains and within the human population, a single epitope or protein cannot serve as a perfect universal vaccine. An epitope may be shared by many but not all viruses, may be recognized by several but not all responders (HLAs) and may help to control symptoms but will not offer complete protection. For these reasons, the best vaccine candidates will probably be mixtures of target antigens. In the case of peptides, these will be combined with an adjuvant as used in some of the studies mentioned.

Universal vaccines designed to confer cross-protection do not usually contain the hypervariable regions of the HA and hence do not induce HI antibodies. In some of the cases, the protection is also mediated by T-cell immunity. In both cases, the vaccine lacks the surrogate marker for protection that is accepted by the regulatory authorities (i.e., HI antibodies). For the promising vaccine candidate that is the first to reach Phase III clinical trials, a field trial will be necessary to show the efficacy of the vaccine. However, it is expected that within such a trial, new surrogate markers for protection will be defined. These should be considered by the regulatory authorities that will have to expand the acceptance criteria and adopt them for the approval of new universal formulations.

Financial & competing interests disclosure

Tamar Ben-Yedidia author is the Chief Scientific Officer of BiondVax Pharmaceuticals, a company focused on the development of a universal vaccine against influenza. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Executive summary

* A truly universal vaccine formulation is one that will induce protective immunity against both influenza A and influenza B strains.
* For the design of a universal vaccine, conserved sequences are required that aim to cover the wide divergence of viruses. Optimally, such a vaccine should induce both humoral and cellular immunity.
* A peptide-based vaccine can be produced in a fast, safe and robust industrial process.
* Approaches to overcoming the low immunogenicity of peptides include immunization with polyepitopes and the use of adjuvanted formulations.
* When considering a universal vaccine that is based on conserved viral proteins, new correlates for protection other than hemagglutination-inhibition antibodies are needed.

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