Epitope-based vaccine against influenza

Tamar Ben-Yedidia and Ruth Arnon†

The currently available vaccines against influenza are viral strain specific and, hence, their efficacy is limited when the circulating strain is not the one included in them. We review herewith some of the more recently developed influenza vaccines and further describe our own data on the design of epitope-based broad-spectrum vaccine for human use. This vaccine is comprised of recombinant flagella that act as a carrier and adjuvant, expressing conserved epitopes of influenza proteins. These epitopes are common to the vast majority of influenza virus strains regardless of their antigenic drift and shifts. The vaccine, activating both the humoral and cellular arms of the immune response, induces long-lasting protection against many strains of the influenza virus. Consequently, it is expected to protect against future strains as well.


Influenza

Influenza is a highly infectious disease caused by frequently mutating influenza viruses. It spreads rapidly around the world in seasonal epidemics, affecting 10–20% of the total population. According to the WHO, 250,000–500,000 people worldwide die of seasonal influenza annually (epidemic outbreaks) [101]. In the USA alone, 20,000–90,000 people die annually of influenza and more than 110,000 people are hospitalized. Influenza is associated with pulmonary and cardiovascular complications leading to high morbidity and mortality rates, affecting mainly at-risk populations, such as toddlers, the elderly and individuals with chronic diseases.

There are three types of influenza viruses: A, B and C. Influenza A is responsible for approximately 80% of influenza disease in humans; influenza B viruses are accountable for an additional 20% of influenza infection, whereas influenza C viruses rarely infect humans. Influenza A viruses are the most common and are characterized by many substrains and species specificity. They are considered the major cause of widespread seasonal epidemics and pandemics (every 10–30 years) due to the frequent antigenic changes (drifts and shifts) of their surface proteins, hemagglutinin (HA) and neuraminidase (NA). Antigenic drifts are minor changes in the virus that occur continually over time, resulting in the appearance of new virus strains that may not be recognized by the body’s immune system. This is one of the main reasons why people can get the influenza repeatedly. In most years, the strains within the influenza vaccine are updated to keep up with the changes in the circulating influenza viruses.

The other type of change is a major change that happens occasionally and is called ‘antigenic shift.’ Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in new HA and/or NA proteins in influenza viruses that infect humans. Shift results in a new influenza A subtype. When shift happens, most people have little or no protection against the new virus. Influenza A viruses exhibit both kinds of changes whereas B viruses and probably C viruses change only by the more gradual process of antigenic drift [1]. Infection with unrecognized virus strains (due to such antigenic changes) may result from reduced immune response of the infected individual; the greater the change, the less effective is the body’s existing immune defense. Hence,
antigenic changes can trigger epidemics (drift) or even pandemics (shift) of influenza, such as the recent avian influenza pandemic threat [101].

The influenza pandemic is becoming one of the major concerns among health authorities due to increasing international travel, as well as overpopulation associated with extremely poor sanitary conditions for humans and livestock living together in some developing countries. As a result, there is a heightened risk of the emergence of new and more violent and resistant influenza virus strains, as well as increased human infection by animal virus strains, as observed since 1997 with the avian influenza.

It is difficult to predict when the next influenza pandemic will occur or how severe it will be. Health professionals are concerned that the continued spread of a highly pathogenic avian H5N1 virus across eastern Asia and other countries represents a significant threat to human health. The H5N1 virus has raised concerns regarding a potential human pandemic because it is highly virulent; it is spreading by migrating birds and can be transmitted from bird to human. A further change in this virus might result in a human-to-human transmission of the disease that could lead to a pandemic.

The currently available influenza vaccines are comprised of three virus strains (two strains of type A and one type B) that are selected on an annual basis. There are four types of influenza vaccines available on the marketplace:

- Whole virus vaccines – inactivated or live-attenuated virus
- Split virus vaccines (virus fragments)
- Subunit vaccines or purified antigens (in which the surface proteins HA and NA are purified from other virus components)
- Virosomal vaccines: synthetic virus-like particles with embedded HA and NA virus surface proteins

All these vaccine types are strain-specific and their efficacy relies heavily on inclusion of antigens (viruses or their proteins) similar to those that are likely to infect during the following influenza season. The influenza strains are selected yearly, based on the WHO and CDC’s predictions of the virus strains expected to be the most prevalent in the forthcoming season. Frequent changes in influenza viruses owing to antigenic drift or shift entail limited protection due to low correlation between the vaccines’ antigens and the current circulating wild type influenza virus. Commercially available strain-specific vaccines lead to a relatively poor clinical efficacy of approximately 40% when there is not a match between vaccine and circulating strains [2,3].

It should also be noted that the annual strain prediction/selection process makes it necessary for vaccines to be formulated on an annual basis only after prediction has been made, requiring vaccine manufacturers to undergo complicated, time-consuming and expensive annual production cycles. In turn, these production conditions pose substantial limitations on the ability to predict optimal quantities (often resulting in a shortage of doses) to vaccinate large populations in time, and to carry out global vaccination policies effectively. These cumulative limitations are the driving force for the development of novel vaccines.

**Novel approaches for influenza vaccines**

The novel vaccines under development aim at overcoming the shortcomings associated with current vaccines, including the limited efficacy resulting from their strain specificity, the limited production capacity as well as the hen egg allergy induced by current vaccines.

The current licensed vaccines are produced in eggs using technology that is more than 60 years old. In addition to being long and cumbersome, some strains of influenza viruses do not grow efficiently in eggs and this causes delays in the vaccine supply. Alternative approaches, in which the viral proteins are produced in cells, will be quicker and easier to make and might help create a more adequate supply of vaccines to fight the common seasonal influenza as well as a future pandemic. One such approach is the use of recombinant protein vaccines. These have been developed as a safer alternative to conventional vaccines and offer a number of advantages:

- They can be produced under safer and more controlled conditions
- Propagation of virus in eggs is not required
- The product is highly purified avoiding adverse reactions due to contaminating proteins
- Virus inactivation or extraction is not required, thus avoiding antigen denaturation by the organic compounds used for this purpose.

An example for this approach is the adenovirus-based recombinant HA expression that protected mice from lethal infection with H5N1 strains by inducing both humoral and cellular immune responses [4,5]. To further potentiate the recombinant proteins, they were administered in virus like particles and Novasome® adjuvant, which conferred protection to mice and ferrets against lethal H9N2 infection [6].

For example, FluBlok™ (Protein Sciences) consists of three rHA proteins derived from the influenza strains selected by the WHO and the CDC for each year’s vaccine. These proteins are produced in insect cells and formulated in phosphate-buffered saline without preservatives or adjuvants. Clinical trials have shown safety and efficacy in healthy adults and the elderly population [7]. A separate vaccine against the avian influenza can also be produced using the same technology. An additional advantage of this approach is that such a vaccine can be administered to individuals allergic to egg protein. In this context it should be noted that egg allergy is one of the most common food allergies in childhood; it is found in approximately 2% of young children, and approximately 1% of the adult population [102].

AlphaVax’s vaccine against influenza is another example of a novel approach in which alpha-virus vector expresses influenza HA and is used for immunization [8]. Recently, volunteers were immunized with an influenza A virus replicon-based influenza
vaccine that contained the HA gene from a single strain of influenza that had been shown effective in protecting animals against experimental influenza infection (ALPHAVAX, UNPUBLISHED DATA) [103].

For the production of pandemic vaccine, nonpathogenic strains prepared by reverse genetics can be employed. Growing them in a cell line acceptable for human vaccine production demonstrates the short time frame in which a reassortant virus can be derived, to facilitate vaccine production under quality controlled conditions [9]. This approach is also employed by Baxter, which produces its Influent™ seasonal vaccine in mammalian (Vero) cells. This technology provides a highly pure vaccine, no risk of residual egg proteins, and improved cost-effectiveness. This vaccine has been approved for use in The Netherlands [104].

The route of administration is another factor addressed by developing novel vaccines. The currently approved influenza vaccines are administered mostly via the intramuscular route. New-generation vaccines that are administered intranasally offer an alternative for inducing protective immunity. Efficacy of such a vaccine was demonstrated by FluNsure™ (GlaxoSmithKline/ID Biomedical), an intranasally administered trivalent recombinant subunit (purified protein conjugate) strain/season-dependent influenza vaccine based on Proteosome™ delivery/adjuvant technology. Successful Phase I and II clinical trials have been completed [10].

All these approaches may improve the efficacy of influenza vaccine. However, there is an obvious need for new vaccines with long-term, multistrain protection and improved immunogenicity, with fewer side effects [11]. An ideal vaccine against influenza should include the following characteristics:

- Ability to confer multistrain protection
- Capacity to enhance both humoral and cellular immune responses to enable an effective elimination of the invading virus
- Ease of administration (mucosal delivery rather than injection)
- Safety (no induction of allergic responses or other side effects)
- Ease of production (shortening of the current 6–8 month production period)

Such a novel concept being applied to the development of new influenza vaccines is the epitope-based approach. Several companies including BiondVax, Acambis, VaxInnate and Cytos, are currently at the development stage of such vaccines (TABLE 1). As described in more detail below, BiondVax Pharmaceuticals is employing several conserved influenza epitopes expressed within bacterial flagella that serve both as a carrier and as an adjuvant [12]. The company performed a Phase I clinical trial during 2007. Each of the other companies mentioned above utilizes a single epitope of the M2 viral protein, for a vaccine against influenza. As the extracellular part of M2 protein is highly conserved in all known human influenza A strains, a vaccine based on this protein may protect against all human influenza A strains, which would represent a major advantage over current vaccine strategies. The major drawback is that the coverage is limited to influenza A and the restriction of the cytotoxic T lymphocyte (CTL) epitope included in it (amino acids 7–15) to HLA B44 only [13]. Moreover, if the M2 epitope undergoes mutation, the vaccine will lose its potency against the mutated virus [14,15].

In addition to these vaccines, many companies are in the process of devising vaccines for influenza that are intended to overcome particular shortcomings of the existing vaccines, as reported in [16].

The epitope-based approach: background

The identification of specific epitopes derived from infectious pathogens and tumors has significantly advanced the development of peptide-based vaccines. 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. This was supported by technological achievements that

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**Table 1. Influenza vaccines under development.**

<table>
<thead>
<tr>
<th>Indication</th>
<th>Company (vaccine)</th>
<th>Type</th>
<th>Stage of development</th>
</tr>
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<tbody>
<tr>
<td>Seasonal</td>
<td>Protein Sciences</td>
<td>Recombinant HA in insect cells</td>
<td>Phase IIb</td>
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<tr>
<td></td>
<td>(FluBlok™)</td>
<td>Recombinant HA in viral vector</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Seasonal</td>
<td>AlphaVax</td>
<td>Reverse genetic viruses in Vero cells</td>
<td>Marketed</td>
</tr>
<tr>
<td>Seasonal</td>
<td>Baxter (Influent™)</td>
<td>Intranasal trivalent recombinant subunit</td>
<td>Marketed</td>
</tr>
<tr>
<td>Seasonal</td>
<td>GSK/ID Biomedical</td>
<td>Multiple epitope</td>
<td>Phase I</td>
</tr>
<tr>
<td>Universal</td>
<td>BiondVax</td>
<td>M2e epitope</td>
<td>Phase I</td>
</tr>
<tr>
<td>Universal</td>
<td>Acambis</td>
<td>M2e epitope and HA</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Universal</td>
<td>VaxInnate</td>
<td>M2e epitope</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Universal</td>
<td>Cytos</td>
<td>M2e epitope</td>
<td>Preclinical</td>
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HA: Hemagglutinin

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further encouraged the development of this approach, including the use of computer algorithms and transgenic mice that enable a rapid screening of vaccine candidates. Indeed, many studies showed the immunological efficacy of peptide-based vaccines against infectious diseases in animal models [17], as well as in clinical studies, which demonstrated the responses to peptide vaccines against infectious diseases, including malaria [18,19], hepatitis B [20] and HIV [21,22]. In some of these cases, immunization with the entire pathogen or even infection by it may not provide sufficient protection, since the immune response they elicit is not towards protective epitopes. The use of synthetic peptides in vaccines offers practical advantages, such as inclusion of specific protective epitopes and their exposure to the immune system, exclusion of suppressive epitopes, relative ease of construction and production, chemical stability and an avoidance of any infectious or autoimmune potential hazard. An additional aspect to be considered is the similarity between the epitope sequence and any sequence of human proteins to avoid autoimmune responses. This should and can be avoided or kept to a minimum, ensuring that the E score is higher than $10^{-4}$ (E score or expected value describes the likelihood that a sequence with a similar score will occur in the database by chance. The smaller the E value, the more significant the alignment) [23–25].

Peptides may also allow better manipulation of the immune response through the use of epitopes designed for stimulating particular subsets of lymphocytes, leading to selective B- and T-cell responses. These considerations might be of value in designing novel anticancer vaccines as well. The B-cell epitopes mainly induce antibody production, particularly antibodies. The T-cell epitopes induce cellular response and cytokine secretion, as well as cytotoxic T cells. The initial studies conducted in our own laboratory on the immunological aspects of influenza dealt with a peptide-based vaccine in which a single conserved B-cell epitope from the influenza HA was evaluated for its reactivity and efficacy in mice [36]. This epitope is located close to the fusion site of the virus to the host cells’ membrane [27,28], which is conserved among many H3N2 strains, as concluded from sequence comparison [105] and from the results of Webster et al. [29]. Immunization with this single epitope partially protected mice from a lethal challenge with influenza H3N2 virus. This partial protection was enhanced by the addition of two T-cell epitopes from the inner nucleoprotein (NP) to the vaccine formulation, thus improving the efficacy of vaccination by inducing the cellular arm of the immune system [30]. It should be noted that when considering the addition of T-cell epitopes, the issue of MHC specificity is crucial.

A potential problem in the development of CTL epitope-based vaccines is the large degree of MHC polymorphism and the need for understanding of HLA restrictions in the population to be vaccinated. However, it is now known that HLA class I molecules can be divided into several families or supertypes based on similar peptide-binding repertoires [31]. A vaccine intended for a broad population should include T-cell epitopes that will induce responses in the vast majority of people; this can be achieved by selecting several T-cell epitopes that are specific to the prevalent HLA genotypes in the population, for example, the most prevalent HLA class I phenotypes include the HLA A2 or A24. Hence, ideally, epitopes that can be recognized and presented by these molecules should be included in a vaccine.

The epitope-based concept for vaccine, in which a combination of B- and T-cell epitopes are required to confer protection against viral infection, was utilized by Steward et al. in a study on Respiratory syncytial virus (RSV), where virus-specific CTL or neutralizing antibodies were induced by immunization with a cocktail of synthetic peptides. Following immunization with B- and T-cell epitopes and challenge infection, a 190-fold reduction in RSV titer was observed in the lungs of immunized mice [32].

In the case of influenza, different approaches were considered in our study for the presentation of peptides to the immune system, including the use of protein conjugate or proteosomes, live recombinant Salmonella and, eventually, the use of the recombinant flagella, which has been the most effective approach [30,33,34]. The flagella, which comprise polymeric flagellin, are highly immunogenic and encompass additional characteristics as detailed in the following section. The flagella-based vaccine was tested successfully in several animal models, including young mice, old mice (approximately 24 months of age), ‘humanized’ mice (irradiated mice transplanted with PBLS) and transgenic mice that expresses the human HLA A2.1. The results obtained in these experiments illustrate the efficacy of the vaccine against different strains of influenza virus including the H5N1 avian strain.

The following section describes influenza epitopes that were utilized in epitope-based vaccines, as well as the flagellin that acts as a carrier and adjuvant for these epitopes.

### The influenza epitopes

The influenza epitopes included in the proposed vaccines are all ‘conserved’; they do not undergo antigenic changes and are therefore shared by many influenza strains. The specific selection and combination of conserved epitopes enable the elicitation of an effective immune response by both arms of the human immune system (humoral and cellular). Recently, the Immune Epitope Database and Analysis Resources (IEDB) [106] was established, a useful resource for epitope-related data. This enables the identification and analysis of epitopes mainly from the HA and NP of influenza, based on a wide literature review [35]. Wang et al. developed a technology to identify potential CTL epitopes for vaccination or diagnostics use; they identified CTL epitopes that are HLA-I restricted based on bioinformatics tools for prediction of antigen processing and presentation. These, together with a biochemical assay for IFN-γ, found 13 peptides that are highly conserved among human influenza A pathogens, including avian influenza isolates [36].

One suitable conserved influenza epitope is the extracellular peptide M2e of the integral M2 protein, which is conserved in all influenza A strains. It was used as a potential broad-spectrum immunogen in a mouse model for influenza infection including H1, H5, H6 and H9 strains [37]. This epitope induces antibody production that could passively induce protective immunity in
In addition it contains a CTL epitope specific to HLA B44 [13]. When fused to hepatitis B virus core (HBC) it induced complete protection in mice against a lethal influenza challenge. Increasing the copy number of M2e inserted significantly enhanced the immune response and reduced the number of vaccinations required for complete protection against a lethal challenge with influenza A virus. Overall, increased resistance to influenza challenge in the immunized mice correlated with an enhanced Th1-type M2e-specific antibody response induced by vaccination [14].

The epitopes included in the vaccine studied in our own laboratory are a B-cell epitope, Th epitopes and CTL epitopes derived from the HA and NP, respectively. It was shown that such a combination significantly enhances the protective effect of vaccination against either sublethal or lethal challenge with the influenza H3N2 virus. Whereas the B-cell epitope alone conferred only a partial protection (<20%), immunization with the B-cell epitope together with the CTL epitope resulted in approximately 60% survival, while the combination of B-cell epitope together with CTL and a Th epitope protected all the mice (100%) from a lethal challenge.

These conserved epitopes can activate the immune system in a long-lasting and effective manner against known and future strains of the influenza virus [30].

In contrast to B-cell epitopes, the response to T-cell epitopes is MHC restricted. Hence, in designing an influenza vaccine for human use, the HLA specificity of the T-cell epitopes is a major consideration in their selection as detailed above.

Bearing in mind these qualifications, cumulative results, in various vaccination studies have shown that synthetic peptides could be employed for vaccines successfully [39,40], providing protection against influenza infection.

Flagellin: a carrier encompassing adjuvant characteristics

The concept of utilizing flagella as a carrier is well documented in scientific literature. In many studies, attenuated Salmonella strains were also used to express and present foreign epitopes from various pathogenic proteins such as F1 antigen of Yersinia pestis, cholera toxin, malaria circumsporozoite protein, hepatitis B surface antigen, tetanus toxin and streptococcal M protein. Immunization with the whole recombinant bacterium induced a specific immune response directed against these foreign antigens [41–44]. The intensive response to the bacterium and more specifically to the flagella is mediated by Toll-like receptors (TLRs).

The TLR family recognizes bacterial components such as lipopolysaccharide (TLR4), heat-shock protein 60 (TLR1), CpG oligodeoxynucleotides (ODN; TLR9), as well as flagellin, which is associated specifically with TLR5. Stimulation through these receptors leads to a cascade of events that result in dendritic cell and natural killer (NK) cell activation followed by cytokine secretion, thereby linking innate and adaptive immunity [45,46]. Consequently, the incorporation of TLR ligands into vaccines could result in more potent and efficacious vaccines.

Huleatt et al. reported that immunization of mice with the recombinant flagellin fused to several proteins resulted in potent antigen-specific T- and B-cell responses that were equal to or better than responses induced by the same proteins emulsified in complete Freund’s adjuvant. These included rapid and consistent antibody responses, as well as the development of protective CD8 T-cell responses upon challenge with virulent Listeria monocytogenes [47]. The contribution of flagella to the adjuvant effect stems also from the prolonged exposure of the immune system to the peptide when it is presented on the flagella. Degradation of peptides in the body occurs within 30 min, whereas flagella can be detected in the blood up to 12 h postintramuscular administration [UNPUBLISHED DATA].

It should be noted that pre-existing anti-flagellin antibodies had no significant effect on the adjuvant activity of flagellin [41]. Similarly, pre-exposure to the Salmonella administered orally did not lead to carrier suppression and, hence, did not affect the protective response of a recombinant flagellin-based vaccine expressing a foreign antigen [48]. Furthermore, as concluded from our own data [UNPUBLISHED] and those of others, the flagella preparation is safe as it originates from nonvirulent Salmonella bacteria (vaccine strain) [49]. In addition it does not induce allergic responses [50]. These characteristics support the use of flagella as adequate carriers and adjuvants. The flagellin system was employed to potentiate DNA vaccination with the influenza NP that served as the specific antigen. Mice given dermal injections of FliC expression vector together with a vector encoding the influenza A virus NP were protected against lethal influenza A virus infection, showing that DNA-encoded TLR agonists by mammalian cells greatly enhance and broaden immune responses [51].

Efficacy of an epitope-based vaccine against influenza

Different formulations of the epitope-based influenza vaccine were evaluated in animal models throughout the years, in which murine epitopes and human epitopes from the influenza virus were expressed within flagellin. Their efficacy was tested by intranasal, subcutaneous and intramuscular administrations. Several animal models were employed in the various experiments to show the efficacy of the vaccine. Thus, the epitope-based vaccine against influenza, which contains three murine epitopes, has been shown to be highly effective in protecting both young and aged mice despite the reduced immune responses in the aged mice [52]. The next step was to test the efficacy of the epitope-based vaccine in a human–mouse radiation chimera model. This model enables testing of human immune responses in mice by engraftment of human peripheral blood mononuclear cells (PBMCs) to mice. The mice are irradiated prior to transplantation, with the aim of destroying their own immune system, and eventually the human cells serve as the functioning immune system in the chimera [53]. Significant human humoral as well as cellular responses can be generated by immunization of the humanized mice with foreign antigens [54,55]. In this model system, the transplanted mice were immunized with a mixture of four influenza epitopes: a B-cell epitope and a Th epitope from the HA,
together with two CTL epitopes from the NP. A cross-strain protection against the H1N1, H2N2 and H3N2 strains of influenza virus was demonstrated following a single immunization with an epitope-based vaccine consisting of these four epitopes [12]. In another model, transgenic mice were used to demonstrate the efficacy of the epitope-based vaccine. In this model, the D b7β2 microglobulin (β2m)-null mice, transgenic for a recombinant HLA-A2.1/D b7β2 microglobulin single chain (HHD mice), were employed. These mice combine classical HLA transgenesis with selective destruction of murine H-2 and show only HLA-A2.1-restricted responses. These mice serve as an animal model for research on systems involving cellular immunity such as cancer, autoimmunity and vaccination [56–60]. This model is suitable for evaluation of the protective capacity of the vaccine towards H5N1 avian influenza challenge.

**Description of the immune response towards the vaccine**

The aforementioned recombinant epitope-based vaccine induces both humoral and cellular immune responses that together lead to efficient protection and reduction of viral load in mice challenged with different strains of the influenza virus. The humoral response comprises antibodies that react with the peptide included in the vaccine but also with the intact virus. This was demonstrated in sera from humanized mice immunized with epitopes from the HA and an epitope from the NP that contained human antiviral antibodies [12,61].

The cellular immune parameters that were observed following immunization with the vaccine include proliferation responses, cytokine secretion and NK cell activation (UNPUBLISHED DATA). As for the specificity, splenocytes as well as lymph node cells, which were recovered from transgenic mice immunized once with the epitope-based vaccine containing a single NP epitope, demonstrated a significant proliferation in response to *in vitro* stimulation not only with the immunizing peptide but also with the intact H3N2 virus. The proliferation was associated with IFN-γ secretion by these cells in response to the peptides, indicating the induction of a Th1-type response.

NK lysis also contributes to the antiviral response, as it was shown that infected cells are more sensitive and are lysed more readily than noninfected cells [62]. It was previously accepted that NK is a nonspecific lymphocyte response that attacks and kills cancer cells and cells infected by microorganisms, without recognition of a specific antigen on it. However, it was demonstrated recently that the sequence of the peptide presented by HLA class I molecules can modulate target cell recognition and lysis [63]. Hence, it is speculated that the recognition of target cells by NK cells is more specific than previously thought. A similarity between peptide motifs of HLA-A2 binders and HLA-G (expressed on NK cells) binders was also demonstrated [64]. Therefore, in addition to the nonspecific mechanism of NK activation, peptides specific to HLA-A2 can elicit further specific elevation of NK activity (lysis).

While these observations do not provide proof of a specific mechanism of action, they are indicative of the role played by both humoral and cellular immune responses in the protective effect of the multiepitope-based vaccine. The contribution of TLR-induced innate immunity to the adaptive response is also supported by the previous findings of Takeshita et al., showing that TLR adaptor molecules can bridge innate and adaptive immunity and potentiate the effects of DNA vaccines against influenza virus infection [65].

**Summary & conclusion**

The cumulative data regarding the epitope-based influenza vaccine in general, and the specific preclinical data with the recombinant epitope-based vaccine expressed in flagella, demonstrate that the vaccine was effective in mice when administered intranasally and also when injected. As such, it could potentially induce both local (respiratory tract) and systemic peripheral immune responses. The vaccine was effective, without the addition of an external adjuvant, due to the capacity of flagella to serve both as a carrier for the influenza epitopes as well as a general inducer of the immune system.

The following data from the literature and our own results support the adjuvant characteristics of flagella:

- Like other microbial components it acts as an adjuvant, inducing local inflammation, signaling macrophages or dendritic cells to become more effective antigen-presenting cells, and inducing the production of inflammatory cytokines and potent local inflammatory responses;
- Recombinant flagella enable prolonged exposure of the inserted epitopes to the immune system, resulting in its activation;
- Flagella bind TLR5, causing a cascade reaction initiated by secretion of IL-12. IL-12 stimulates T lymphocytes, and then stimulates NK cells to release IFN-γ, thus promoting a Th1 response.

Immunization with the epitope-based vaccine resulted in protection against different strains of the virus including the highly pathogenic H5N1 avian influenza strain. Despite variations in their outer proteins, the infectious virus was effectively cleared from the lungs and a significant survival advantage was observed in the immunized mice after a lethal dose challenge. The successful results obtained in the humanized and transgenic mouse models lead us to believe that such a vaccine will be effective in humans as well.

**Expert commentary & five-year view**

Influenza is a major public health concern: it occurs in recurrent epidemics that start abruptly, spread rapidly and are frequently distributed worldwide. Usually the influenza infection is a mild disease, responsible for many millions of infected individuals, causing an economic burden. However, in sensitive segments of the population, such as infants and the elderly, mortality is high, leading to tens of thousands of deaths annually. The influenza virus undergoes frequent and unpredictable changes of the surface glycoproteins HA and NA, which allows it to escape the immune system. Some of these changes may result in a highly virulent strain, such as H5N1 avian influenza, with the potential for
causing a pandemic. Furthermore, these antigenic variations enable the virus to escape the immune system and reduce the effectiveness of vaccines. The currently available vaccines are of several types, namely whole virus vaccines, subunit vaccines and live-attenuated influenza virus vaccines; they are all strain specific and their efficacy is limited. Hence, there is an unmet need and, as a consequence, an intensive effort to develop more effective vaccines and novel approaches are being considered, including the development of recombinant vaccines, employing different vectors and cell cultures. An alternative approach is the use of epitope-based vaccines as described in this review. The main expected benefit of epitope-based vaccines is that they may allow immunization with a minimal structure of well-defined antigen. Appropriate epitopes may stimulate protective immunity, while avoiding potential adverse effects. Furthermore, the use of conserved epitopes may circumvent the limitation of strain specificity and may offer a universal influenza vaccine. Similar advantages could be offered by vaccines based on recombinant conserved influenza proteins such as the M2 and NP. Hence, the development of effective broad-spectrum influenza vaccines has now entered an exciting stage.

We envisage the following developments that may occur in the next 5 years:

- Development of reliable procedures for scaling up the production of the currently available vaccines, either by using cell culture instead of egg-grown vaccines or by HA expressed in appropriate vectors. This will prevent shortage of vaccine doses;
- Development of specific vaccines for newly emerging influenza strains, including avian influenza. Such vaccines will still suffer from the limitation of strain specificity, but will provide a solution to infection by highly virulent strains;
- Development of more efficient adjuvants that will be approved for human use and enable immunization with smaller quantities of the antigen as well as the use of small immunogens such as peptides.
- Development of a universal influenza vaccine that will overcome or circumvent the problems of strain specificity. Such a vaccine will be based on either conserved viral proteins or on conserved protective epitopes that induce both humoral and cellular immunity;
- Development of live vaccines based on attenuated strains of influenza virus. While one such vaccine is already approved, additional ones may follow suit;
- Finally, the combination of the most suitable strain and/or recombinant product and an adequate adjuvant or vehicle may lead to the development of an efficient and safe nasal vaccine.

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Financial & competing interests disclosure

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Key issues

- The currently licensed influenza vaccines comprise three strains of the virus (selected by the WHO) grown in eggs. They suffer from cumbersome production procedures, sometimes leading to shortages in vaccine doses, as well as from strain specificity that may lead to reduced efficacy.
- Novel approaches to influenza vaccine include the use of cell culture for viral growth; the use of recombinant viral proteins and the development of peptide-or epitope-based vaccines.
- The use of synthetic peptides in vaccines offers practical advantages, such as the inclusion of specific predictive epitopes and their exposure to the immune system; exclusion of suppressive epitopes and avoidance of infectious or autoimmune potential hazard. Another advantage is ease of production and chemical stability.
- Epitope-based vaccines offer the advantage of inclusion of both B-cell epitopes eliciting antibody production, T-cell epitopes inducing Th response and cytotoxic T lymphocyte epitopes inducing cytotoxic T-cell activity.
- Epitope-based influenza vaccine may comprise conserved epitopes that lead to protective immunity against several strains. Indeed, inclusion of four such conserved B- and T-cell epitopes, expressed within an appropriate carrier – flagellin – led to an effective broad-spectrum anti-influenza response in both young and aged mice, as well as in humanized mice.
- Recent data indicate that a similar vaccine, based on six conserved epitopes, induced both humoral and cellular immunity in mice, resulting in protection against several influenza strains including the highly pathogenic avian strain H5N1. This combination may be effective as a human vaccine as well.
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Papers of special note have been highlighted as:
• of interest
** of considerable interest


• Comprehensive review summarizing all clinical trials performed with influenza vaccines during the years 1966–2006.


• Demonstrates a novel approach to vaccine production that includes both virus manipulation and advanced growth of the virus in Vero cells that leads to a candidate vaccine against H5N1 virus.


• Explains the epitope-based approach and its ability to confer cross-strain protection in an advanced animal model.


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Affiliations

- Tamar Ben-Yedidia, PhD
  Director of R&D, BiondVax Pharmaceuticals Ltd., Ness Ziona, Israel
  Tel.: +972 8940 1898
  Fax: +972 8930 2531
  benyedidia@biondvax.com

- Ruth Arnon, deg
  Professor of Immunology, Weizmann Institute of Science, Rehovot, Israel
  Tel.: +972 8934 4018
  Fax: +972 8946 9712
  ruth.arnon@weizmann.ac.il