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Advances in influenza vaccination

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Abstract

Influenza virus infections yearly cause high morbidity and mortality burdens in humans, and the development of a new influenza pandemic continues to threaten mankind as a Damoclean sword. Influenza vaccines have been produced by using egg-based virus growth and passaging techniques that were developed more than 60 years ago, following the identification of influenza A virus as an etiological agent of seasonal influenza. These vaccines aimed mainly at eliciting neutralizing antibodies targeting antigenically variable regions of the hemagglutinin (HA) protein, which requires regular updates to match circulating seasonal influenza A and B virus strains. Given the relatively limited protection induced by current seasonal influenza vaccines, a more universal influenza vaccine that would protect against more—if not all—influenza viruses is among the largest unmet medical needs of the 21st century. New insights into correlates of protection from influenza and into broad B- and T-cell protective anti-influenza immune responses offer promising avenues for innovative vaccine development as well as manufacturing strategies or platforms, leading to the development of a new generation of vaccines. These aim at the rapid and massive production of influenza vaccines that provide broad protective and long-lasting immunity. Recent advances in influenza vaccine research demonstrate the feasibility of a wide range of approaches and call for the initiation of preclinical proof-of-principle studies followed by clinical trials in humans.

Introduction

Seasonal influenza virus infections yearly cause three to five million hospitalizations, resulting in 250,000 to 500,000 deaths worldwide [1]. Current vaccines against seasonal influenza viruses offer protection from infection and disease caused by seasonal influenza viruses closely related to those represented in the vaccines. Yet they fail to provide broadly protective and long-lasting immunity and they provide little or no protection against so-called drift variants (see below) or zoonotic influenza viruses that may be at the origin of rare but devastating pandemics. Novel insights into the correlates of protection against influenza virus infection and disease and into broadly protective B- and T-cell responses have been gained in recent years. Together with the development of novel and innovative influenza vaccine manufacturing strategies and platforms, they offer promising avenues toward the generation of more universal influenza vaccines.

Seasonal influenza is caused by influenza A and B viruses, both belonging to the *Orthomyxoviridae* family [2]. Whereas influenza B viruses are principally human pathogens, diverse influenza A virus (IAV) lineages are maintained in a wide range of animal species, including wild water birds, poultry, domestic swine, dogs, and horses. Several of these animal IAVs can cause zoonotic infections, with little or no onward transmission between humans. Their further adaptation to humans may lead to the development of a pandemic IAV, sweeping through the human population. Four IAV pandemics have occurred in the past 100 years, resulting in more than

50 million deaths [3]. Following the pandemic they have caused, pandemic viruses continue to circulate in the human population as seasonal IAVs, after replacing one of the previously circulating seasonal strains. Seasonal IAVs may therefore be considered descendants of pandemic viruses. They continuously evolve to escape from antibody-mediated immunity in the human population by accumulating mutations leading to so-called antigenic drift. These changes, which occur predominantly in IAV surface glycoproteins—in particular the HA protein—thus circumvent the neutralization of antigenic drift variants by pre-existing antibodies. Because of this continuous IAV evolution, regular updates of IAV vaccine strains are required to match circulating IAV strains. A more universal influenza vaccine that would not need these periodic updates, but would give broad and long-lasting immunity, is one of the largest unmet medical needs of the 21st century. In the present article, we will review the latest advances in our understanding of the path that may eventually lead to a more universal vaccine against influenza.

Correlates of protection

HA-specific neutralizing antibodies are recognized as a major correlate of protection against IAV infection and disease [4,5] (Fig. 1). These antibodies are induced upon IAV infection and vaccination with current seasonal IAV vaccines. Their induction is used as a primary correlate of efficacy of classic non-live vaccines. They are typically directed to epitopes in or in close proximity to the HA receptor binding site, located in the globular head of trimeric HA proteins [6]. By preventing the actual binding of the HA proteins to their cellular receptors, these antibodies efficiently neutralize IAVs. Both serum immunoglobulin (Ig)Gs and secretory or mucosal IgAs are efficient at inhibiting virus attachment and entry and are induced upon vaccination with inactivated and live-attenuated vaccines, respectively. At sufficiently high titers, these antibodies can provide adequate immunity, effectively protecting against infection or re-infection.

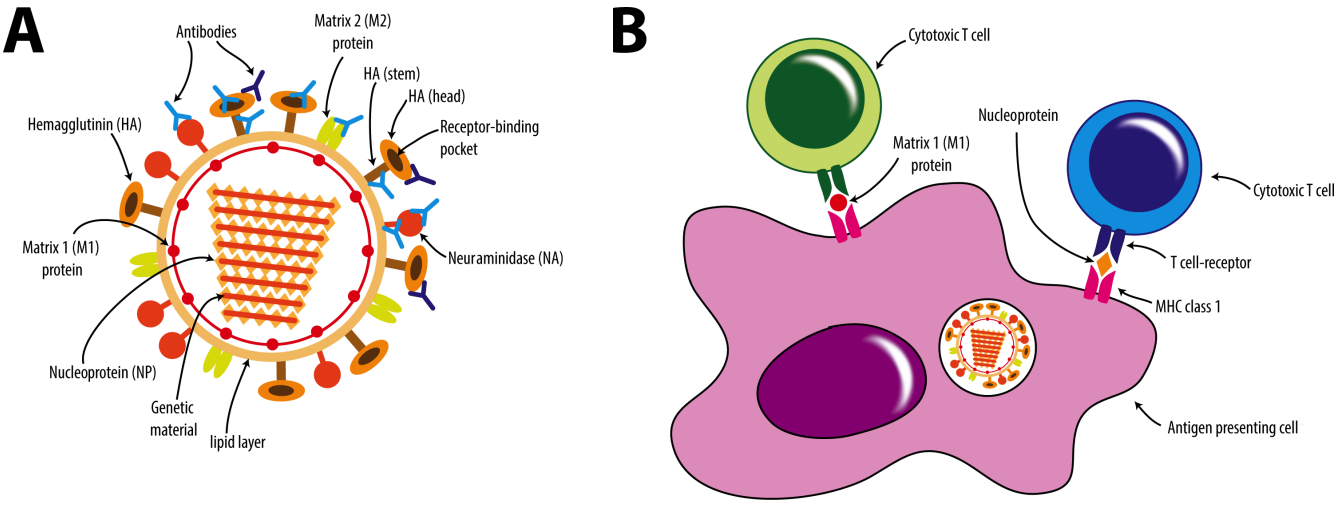
As mutations in HA antigenic sites are responsible for IAV antigenic drift [6], resulting in antibody-escape variants that can evade pre-existing immunity [4,5], current seasonal IAV vaccines need regular updates in order to induce neutralizing antibodies that match circulating IAV strains. Furthermore, HA-specific neutralizing antibodies offer no protection against infection with distantly related IAVs or IAVs of different HA subtypes. Highly pathogenic avian influenza virus (HPAIV) H5N1 and low pathogenic avian influenza virus (LPAIV) H7N9 are currently at the origin of large numbers of sporadic zoonotic infections with little or no sustained transmission between humans [7,8]. Should such viruses acquire the trait of efficient transmissibility among humans, they may develop into novel pandemic IAVs. Neutralizing antibodies against today's seasonal IAVs of the H1 and H3 subtypes would offer little protection against such candidate pandemic IAVs. Pandemic preparedness plans should therefore include the development of pre-pandemic vaccines against zoonotic IAVs that may eventually evolve into candidate pandemic IAVs [9,10]. Given the level of antigenic drift in these zoonotic IAVs, as for seasonal IAV vaccines, such pre-pandemic vaccines may eventually offer too little protection against the newly emerging pandemic IAVs. To increase the immunogenicity of currently available vaccines and obtain increased and broadened antibody responses, the use of adjuvants can be considered an integral tool of pandemic preparedness plans [11].

Although virus neutralization is considered the gold standard in terms of correlates of protection from IAV infection or influenza, antibodies that have no or poor neutralizing activity may also confer a certain degree of protection [12]. Protective antibodies directed to the other IAV surface proteins, like the neuraminidase (NA) protein and the ecto-domain of the matrix 2 protein (M2), have widened the scope of potential IAV correlates of protection [4,5] (Fig. 1). Because of the more conserved nature of the epitopes involved, they hint at the possibility of inducing broader immune responses, cross-reactive to a range of antigenically distant IAV strains.

Protective effects of NA-specific antibodies have been demonstrated in animal models since the late 1960s (see references in [4]) and more recently in seroepidemiological studies in humans [13-15]. Recently, monoclonal antibodies targeting the conserved region of the NA protein were shown to provide heterosubtypic protection in mice [16]. Because the NA protein is involved in virus release from infected cells, NA-specific antibodies may not provide sterilizing immunity but may limit infection and disease severity. Specific antibodies against the M2 ion-channel protein have also shown potential for broad protection in animal models [17-30]. Although such antibodies are not neutralizing and appear to be produced in limited quantity upon infection, they may contribute to antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells and thereby limit disease severity [24]. Seroprevalence of antibodies targeting the M2 protein was recently shown to increase with age in humans [31]. Moreover, the 2009 pandemic IAV H1N1 elicited a recall serum antibody response against the M2 protein in some individuals.

The potential protective role of cellular immune responses against IAVs has been increasingly recognized in recent years (reviewed in [32]). Cellular immune

Figure 1. Antibody and cytotoxic T-cell epitopes on influenza A virus proteins



(A) Antibody epitopes are principally expressed on influenza A virus surface proteins: hemagglunitin (HA), neuraminidase (NA), and ecto-domain of matrix 2 (M2) ion-channel proteins. Epitopes located in the stem of the HA and in the NA and M2 proteins are typically conserved, inducing broadly neutralizing antibodies (light blue antibodies). Epitopes located in the globular head of the HA are typically variable, leading to antigenic drift (targeted by dark blue antibodies); exceptions may include conserved epitopes inside the receptor-binding pocket. **(B)** Cytotoxic T-cell epitopes are principally expressed on influenza A virus internal proteins, notably the nucleoprotein (NP) and matrix 1 (M1) protein. They are processed and presented by antigen-presenting cells via major histocompatibility complex (MHC) class I molecules.

correlates of protection may be particularly relevant for novel antigenic variants or pandemic IAVs, when pre-existing humoral immunity provides little protection. Although they do not prevent re-infection, cytotoxic T lymphocytes (CTLs) effectively limit infection and disease severity (reviewed in [32]). Because CTL epitopes are typically conserved, CTL responses also have the potential to be broadly reactive, including against IAVs of different subtypes. Evidence for cellular immune correlates of protection in humans was recently indicated by strong inverse correlations between pre-existing $CD8^+$ T lymphocyte populations and disease severity during the influenza pandemic of 2009 [33].

Broadly protective immune responses

Because of the antigenic variability of the globular head of the HA protein, the immune protection conferred by neutralizing antibodies is typically highly specific to circulating IAV strains and is short-lived. The evidence for other correlates of protection, mediated by antibodies directed to more conserved proteins or parts thereof and by cellular immune responses directed to conserved epitopes, raises hope for the development of broadly protective influenza vaccines.

The potential for broadly reactive immune responses against IAVs is further supported by the production of broadly neutralizing antibodies directed to the HA stem or other relatively conserved regions of the HA protein (Fig. 1), by plasma cells of individuals infected with or vaccinated against IAVs (reviewed in [12]). Although the first broadly neutralizing and protective antibody against IAV was discovered in the early 1990s in mice [34-36], major advances in the recognition of such antibodies in humans have occurred in the past six years. Broadly neutralizing antibodies are directed to epitopes of either the HA stem or the HA globular head [37]. Those directed to the HA stem target conserved epitopes and are generally broadly cross-reactive, recognizing IAVs of different lineages and subtypes. Antibodies recognizing IAVs belonging to the first IAV phylogenetic group (comprising H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, and H17) are induced in many individuals infected with or vaccinated against IAVs but typically remain subdominant (reviewed in [12]). The HA globular head is immunodominant and more immunogenic than the HA stem in general, regardless of IAV strain or subtype. The subdominant nature of broadly neutralizing antibody responses may result from limited accessibility of HA stem epitopes closely packed on the viral membrane. Antibodies recognizing IAVs belonging to the second IAV phylogenetic group (comprising H3, H4, H7, H10, H14, and H15) appear more rarely in humans. Antibodies recognizing the stem region of the HA proteins of IAVs of both phylogenetic groups as well as antibodies recognizing the HA stem of both IAVs and influenza B viruses

have recently been reported [38,39]. Broadly neutralizing antibodies directed to the globular head may target the relatively conserved region of the receptor-binding pocket [12,37]. However, despite being strongly neutralizing, these antibodies have usually a more limited breadth of reactivity and can allow viral escape.

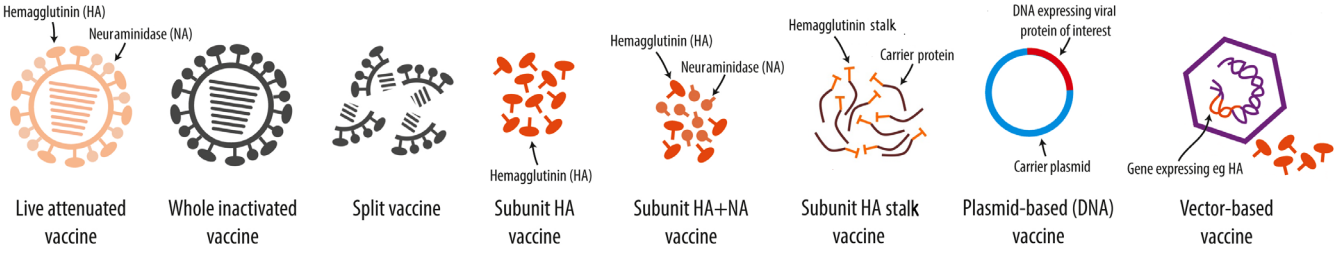
Because the HA inhibition assay currently used to measure the human immune response to IAV does not detect stem-specific antibodies, little is known about the degree of protection conferred by broadly neutralizing antibodies [12]. Other assays are available to detect such antibodies and call for further research. Subdominant broadly neutralizing antibody responses can become dominant after heterologous boosting [5]. These antibodies are known to efficiently bind to the less tightly packed HA proteins expressed on the membrane of infected cells. Recently, binding of these antibodies to Fc receptors on effector cells, such as monocytes, macrophages, or NK cells, was shown to activate ADCC and to provide protection in mice [40]. It suggests that protection conferred by broadly neutralizing antibodies may occur after viral entry, by recognition of HA stem epitopes expressed on infected cells. At high doses, however, these antibodies also mediate neutralization of viral binding, fusion, and entry [40]. It was recently demonstrated that the majority of HA trimeric proteins on intact 2009 pandemic influenza virions are available to bind broadly neutralizing antibodies [41]. Thus, protection conferred by broadly neutralizing antibodies may occur via both virus neutralization and ADCC.

$CD8^+$ CTLs are largely directed to epitopes within conserved internal proteins, such as nucleoprotein (NP) and matrix (M) proteins [32] (Fig. 1). These epitopes are shared within and across IAV subtypes, contributing to broad reactive responses. Although CTLs do not prevent infection, they reduce virus replication and disease severity, also in the absence of a detectable antibody response (reviewed in [32]). Such protective effect may be particularly relevant for heterosubtypic strains, such as antigenic variants or pandemic IAVs, against which pre-existing humoral immunity offers little protection. Protective heterosubtypic immunity has mainly been demonstrated in animal models and is indicated to play a role by epidemiological studies in humans during past and most recent pandemics [32,33]. Further evidence for protective effects is indicated by selective pressure exerted by cellular immune responses that can lead to immune escape variants with amino-acid changes in CTL epitopes [32]. Some CTL epitopes are immunodominant, complicating the analysis of CTL responses induced by infection or vaccination. Efficient induction of CTL responses requires endogenous antigen processing, their binding to major histocompatibility complex (MHC) class I molecules, and presentation by antigen-presenting cells (APCs) or infected cells. The human leucocyte antigen (HLA) haplotype determines the epitope on presentation and thus the magnitude of CTL responses. Immunodominance, HLA restriction, and antigen delivery to APC cytosol need to be addressed by T cell-based vaccine formulations to induce broad protective CTL responses [32]. Innovative vaccine platforms increasingly aim at inducing such responses, complementing induced humoral immunity.

Innovative vaccine platforms

Current vaccine formulations target three or four seasonal influenza virus strains (IAVs H1N1 and H3N2) and one or two (since 2013) influenza B virus strains [4,5,11,42]. Most conventional vaccines are subunit, split, or whole inactivated preparations and are administered intramuscularly (Fig. 2). They induce virus neutralizing serum antibody responses against the globular head of the HA protein, when matching circulating strains. Adjuvants are increasingly used to enhance and broaden immune responses induced by inactivated vaccines. They modulate the immune response, improve the immunogenicity of the vaccine, and allow lowering of antigen loads. MF59

Figure 2. Range of current and new vaccine presentations against influenza A virus



Current vaccines against influenza A viruses are principally live attenuated, whole inactivated, split virion, and subunit vaccines. Among the new generation of vaccines against influenza A viruses are subunit vaccines with carrier proteins acting as adjuvant (e.g. flagellin) and DNA plasmid and viral-vectored vaccines, which target conserved regions of surface proteins or conserved internal proteins or both.

and AS03 are oil-in-water emulsions currently used in seasonal and pandemic vaccine formulations [11]. A number of other adjuvants are in clinical evaluation. Live attenuated vaccines (Fig. 2), administered topically in the respiratory tract, induce good mucosal antibody responses against the globular head of the HA protein matching circulating strains, as well as CTL responses, following uptake and processing by APCs. CTL responses induced by these vaccines were shown to provide a certain degree of heterosubtypic protection in mice [43,44]. Live attenuated vaccines have been approved for marketing and use in the United States and Russia [4] and, since 2011, in Europe [45].

Despite advances in the field, most vaccine formulations continue to be produced by rather antiquated techniques that have been in use for over 60 years and that involve the growth and passaging of the vaccine strains in embryonated chicken eggs [42]. Production and subsequent formulation can take several months and rely on the availability of embryonated chicken eggs. Increasing demands for influenza vaccines, including the need for rapid production of pandemic influenza vaccines when a pandemic emerges, urgently require the development of new egg-independent manufacturing platforms. The first recombinant egg-free influenza vaccine, produced in an insect cell line, was approved by the US Food and Drug Administration in January 2013 and is available for the influenza season of 2013-2014, demonstrating the feasibility of the concept [46].

Although debate and controversies are waging on influenza vaccine efficacy and effectiveness, current influenza vaccines provide beneficial protection against seasonal influenza virus infection and disease, especially for the elderly [47]. The degree of protection varies per age group and per influenza season, depending in particular on the strength and status of individual host immune responses and underlying conditions and on the level of match between influenza vaccines and circulating strains [48]. Meta-analyses of randomized controlled trials and observational studies to assess influenza vaccine efficacy and effectiveness are a challenging task, as many confounding factors may yield erroneous conclusions. Although current vaccines do not offer complete protection, they reduce the risk of IAV infection and influenza-associated disease and death, including in high-risk groups, such as young children and the elderly [47]. Given the highly specific and rather short-lived immunity they induce, they raise hope in view of the significant improvements new and innovative vaccine platforms may offer in the near future.

The next generation of influenza vaccines (Fig. 2), produced in egg-independent manufacturing platforms, aim at inducing more broadly reactive, long-lasting immunity against a wide range of influenza virus types and subtypes [4,5,11,42]. The selection of antigens as well as the antigen delivery system used plays essential roles in enhancing, broadening, and prolonging influenza virus-specific immune responses. Conserved surface antigens, such as epitopes in the NA and M2 proteins and in the stem region of the HA protein, are interesting candidate antigens for the induction of broad protective humoral immune responses against IAVs. Both M2-based and stem-HA-based vaccines have now been developed and tested in animal models [19-30,49-58]. In a phase I clinical trial, M2-based vaccines have proved relatively safe and immunogenic in humans, although efficacy has yet to be evaluated [59]. Adverse effects were observed following injection of high doses, potentially mediated by cytokine release. The expression of M2 antigens in vectored vaccines, co-expressed with the NP protein, or in combination with an inactivated vaccine, also demonstrated heterosubtypic protective effects in mice [28,30].

Stem-HA-based vaccines would currently be attractive targets for the development of a more universal vaccine and are under active research [42]. Their efficacy has been shown to be promising in mice, ferrets, and non-human primates. Large portions of the HA protein—including the long alpha helix of the HA protein of IAVs belonging to the second phylogenetic group [49,50], the HA2 portion of the protein [51], "headless" HA constructs missing the immunodominant globular head of the HA protein [52,53], and whole consensus HA proteins [54,55]—have been used as conserved domains. Globular head constructs as well as chimeric HA constructs that express both stem and globular head regions have also been developed and proved efficacious in mice [56,60]. These respective constructs were delivered to experimental animal models in association with a carrier protein, expressed on transfected cells, as DNA primes, or via a viral vector. These vaccination schemes typically elicited broadly neutralizing antibodies in the animal models. Priming with DNA plasmids of a 2009 pandemic influenza virus H1N1 followed by boosting with matched inactivated vaccine also induced broadly neutralizing antibodies in animal models, including non-human primates [57], and showed promising results in a phase I clinical trial in humans [61]. Most recently, self-assembling influenza nanoparticles expressing HA trimeric proteins elicited broadly neutralizing antibodies targeting both the HA stem and the receptor binding pocket and protected ferrets against distantly related IAVs [58].

In addition, a range of T cell-based vaccines have been developed and tested in both animal models and phase I

clinical trials in humans, offering additional avenues for the induction of heterosubtypic immunity [32,42]. Targeted antigens are principally conserved internal proteins, including NP and M proteins typically expressed in combination with HA proteins either in vaccine vectors, as DNA plasmids, or in particulate formulations, such as immune stimulating complexes (ISCOMs) and virosomes. Promising clinical trials in humans have involved a Modified Vaccinia virus Ankara (MVA)-based vaccine targeting influenza virus NP and M1 proteins [62] and an epitope-based vaccine made of an *Escherichia coli* expressed recombinant protein encoding for HA, NP, and M1 epitopes [63].

The diversity of the approaches used in the above-mentioned studies demonstrates the wide range of options leading to avenues exploring the opportunities of developing a universal influenza vaccine. This calls for more preclinical proof-of-principle studies, the successful completion of which should be followed by clinical trials in humans.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; HA, hemagglutinin; HLA, human leucocyte antigen; IAV, influenza A virus; Ig, immunoglobulin; M, matrix protein; M1, matrix 1 protein; M2, matrix 2 protein; NK, natural killer; NP, nucleoprotein.

Disclosures

Albert D.M.E. Osterhaus is head of the Department of Virology of Erasmus MC Rotterdam and part-time Chief Scientific Officer of Viroclinics-Biosciences BV, a spin-off Contract Research Organization (CRO) of Erasmus MC that collaborates with pharmaceutical companies. He also provides expert advice to various public and international organizations involved in the area of general human and veterinary health. For purposes of transparency and to avoid possible conflicts of interest, he discloses all his interests in matters related, directly or indirectly, to his position as head of the Department of Virology of Erasmus MC at www.virology.nl. Guus F. Rimmelzwaan is advisor to Viroclinics-Biosciences BV. Leslie A. Reperant declares that she has no disclosures.

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