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Review Article

Universal Influenza Vaccines - A Short Review

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Abstract

Since the development of the first vaccine against influenza virus in 1940s, considerable progress has been made in preventing this devastating disease. Despite of the encouraging advances, influenza vaccine research and development still remain challenging mainly due to the hurdle of antigenic drift and shift of viral immunogens. The current seasonal influenza vaccines need to be reformulated annually and provide only limited protection in the case of mismatch. The reoccurrence of seasonal epidemics and occasional outbreak of pandemics have urged the development of novel influenza vaccines capable of eliciting broad cross-protective immune responses. In this scenario, antigens composed of conserved regions shared among various influenza subtypes have raised great hopes for such vaccines. Delicate antigen design and integrated molecular adjuvants in combination with improved delivery and controlled release strategies present the promising directions towards universal influenza vaccine development. The present review summarizes the recent progress in such endeavors. Influenza matrix protein 2 and stalk domain of the hemagglutinin draw general interests for improved antigen design. Additionally, epitopes that are recognized by cytotoxic T lymphocytes (CTLs) become attractive targets for antigen design. On the other hand, multiple delivery platforms including virus-like particles, microneedles and nanoclusters could be employed to facilitate the optimization of an affordable universal influenza vaccine.

INTRODUCTION

Influenza virus is one of the most important respiratory pathogens with significant medical and economic burdens [1-3]. It belongs to genera A, B and C of the family *Orthomyxoviridae* with type A viruses being the primary pathogens claimed for seasonal and pandemic influenza outbreaks [4]. Type A viruses can be further divided into different subtypes based on the serotypes of their main surface antigens hemagglutinin (HA) and neuraminidase (NA) [5]. So far, 17 HA and 9 NA subtypes have been identified. Phylogenetically, 16 HA subtypes are categorized into two groups (H1, H2, H5, H6, H8, H9, H11, H12, H13 as well as H16 in group 1, and H3, H4, H7, H10, H14 as well as H15 in group 2) [6]. Historically, H1 (H1N1), H2 (H2N2) and H3 (H3N2) have caused influenza pandemics in humans and resulted in millions of death [7]. At present, H1N1 and H3N2 viruses are causing epidemic infections in humans and constitute the main components of seasonal influenza vaccines. However, the efficacy of current influenza vaccine is limited due to the antigenic drift. Further, the occasional antigenic shift of HA and NA may cause the emergence of new influenza pandemics. In addition, non-human influenza viruses may acquire the capacity for transmission in humans as well [8]. With the frequent infection by highly pathogenic avian influenza A (HPAI) H5N1 in humans in recent years, and the most recent human infection by a novel avian influenza virus (H7N9) in China [9,10], this concern has become more urgent. Although current influenza vaccines are effective in battling closely matched viruses, the major hurdles are the need to produce new vaccines every season, the uncertainty in choice of the correct strains, and the inability to prevent a new influenza pandemic. Improved vaccines inducing broadly protective immune responses against multiple type A influenza viruses, namely universal influenza vaccines, are needed, not only for seasonal but also for pandemic influenza prevention. Conserved epitopes are potential immunogens for such vaccines [11,12]. Combined strategies including improved design of antigens, integrated molecular adjuvants, new antigen delivery techniques and vaccination regimens should be considered together to overcome current obstacles in the development of a universal influenza vaccine [6,13-17].

ENHANCEMENT OF TRADITIONAL INFLUENZA VACCINE APPROACHES FOR BROAD CROSS-PROTECTION

Several approaches have been improved to induce broad

cross-protection based on current vaccine strategies. Co-administration of adjuvants is a classic but effective approach. Typically influenza vaccines do not induce CD8+ cytotoxic T lymphocyte (CTL) responses which are required to control viral pathogens. By using adjuvants, co-administrated vaccines are able to induce CTL responses that target highly conserved epitopes among different viruses. Therefore, co-administrated adjuvant presents a potential approach for inducing broad cross-protection. Several mechanisms may be involved in adjuvant-induced T cell responses [18-20]. Some adjuvants, such as alum and emulsions, can trap antigens at the injection site and increase recruitment and activation of antigen presenting cells (APCs) [21]. The others are essential ligands for pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), and are highly effective in inducing potent adaptive immune response [22]. For instance, the TLR-5 ligand flagellin has been modified into different forms to enhance influenza immunity for broad protection [17,23-26]. This protein can be expressed in a membrane-anchored form and be incorporated into influenza virus-like particles (VLPs) which have been reported to confer heterosubtypic protection [23,24]. In a fusion protein form with the conserved extracellular domain of the influenza matrix protein 2 (M2e), flagellin was shown to induce high levels of M2e-specific antibodies and confer complete protection against a heterologous viral challenge [17]. In addition to adjuvants, several newly developed vaccine delivery platforms have been employed to enhance the immune response and cross-protection. Of them, VLPs are one of the most striking platforms [27-29]. By mimicking the organization and conformation of native viruses but lacking the replicative genomic information, VLPs can be produced in heterologous expression systems on large scales, thus can be safer and cheaper vaccine candidates [30,31]. Because of the self-assembly feature of VLPs, targeted viral antigens form multimeric complexes displaying a high density of epitopes. The size and conformation of these particles are similar to intact native virions [24,32-35] . Moreover, VLPs can enter both MHC class I and class II antigen processing pathways in APCs, eliciting both humoral and cellular immune responses [35-37].With the development of nanotechnology, microneedles and nanoparticles have been used as new platforms for influenza vaccine delivery for enhanced immune protection. Inactivated virus, VLPs and subunit vaccines were coated on microneedles and induced improved protective immunity by skin vaccination [38-40]. Co-immunization with A/Puerto Rico/8/1934 (A/PR8, H1N1) HA DNA vaccines and inactivated virus by coating on a microneedle patch conferred immune protection against lethal A/PR8 and pandemic 2009 H1N1 challenge in mice [15]. Being assembled into nano size with controlled antigen release, nanoparticles exhibit adjuvant effects and stimulate APCs upon binding and/or internalization [41-43], and have been employed to deliver influenza vaccine for enhanced immune protection [44-46]. However, in many cases the amount of antigen loaded into the nanoparticle is low due to the presence of polymer core, and the process by which the particle is made can damage or unfold the antigen [41]. Newly developed nanoparticles bring the hope to overcome these limitations. For instance, novel nanoclusters assembled directly from influenza HA and/or M2e with no need of encapsulating agent was shown to maximize antigenic protein load. The gentle fabrication conditions allow the antigen to maintain their native form. These nanoclusters were found to induce broadly cross-protection (Wang et. al, unpublished data). Recently, self-assembling influenza nanoparticle vaccines have also been established by fusing viral HA to ferritin, a protein that naturally forms nanoparticles composed of 24 identical polypeptides [47,48]. This self-assembling nanoparticle vaccine elicited neutralizing antibodies to two highly conserved vulnerable HA structures that are targets of universal vaccines: the stem and the receptor binding site on the head [49,50]. Resulting antibodies neutralized H1N1 viruses from 1934 to 2007 and protected ferrets from an unmatched 2007 H1N1 virus challenge. Self-assembling nanoparticles improve the potency and breadth of influenza immunity and indicate a potential platform for development of universal influenza vaccines.

M2E, A FAVORABLE EPITOPE FOR A UNIVERSAL INFLUENZA VACCINE

The influenza genome is composed of eight segments which encode 11 viral proteins including the major surface glycoproteins HA and NA [51,52]. These viral proteins endow targets for influenza vaccines that induce protection against similar viruses [53]. Influenza matrix protein 2 (M2) is an integral transmembrane protein. Although only a few copies are expressed on the surface of influenza A virions, relatively dense presentation of this protein was found on the surfaces of infected host cells [54]. This protein consists of 97 amino acids, including 24 amino acid residues at the N-terminus which form the ectodomain of M2 (M2e) [55]. M2 exists as a homotetramer formed by two disulfide-linked dimers which assemble into an ion channel, and plays an important role in uncoating the virus during viral entry [56-58]. Since M2e is highly conserved among influenza A viruses, it has been considered as a promising target for inducing cross protection against different influenza A viruse subtypes [59,60]. Studies have shown that M2e-specific antibodies can reduce the plaque size [61,62]. Passive immunization with these antibodies reduced virus titers in the lungs of mice infected with influenza A viruses [63,64]. However, M2e specific antibodies can rarely be induced by a natural influenza infection or seasonal vaccination [65,66]. Several groups attempted to overcome the low immunogenicity of M2e using various platforms such as fusing M2e protein with carrier molecules including keyhole limpet hemocyanin (KLH), hepatitis B virus core (HBc), multiple antigenic peptides (MAP) or fusing four repeats of M2e to a membrane anchor from influenza virus HA [17,67-69]. In all these studies, M2e-specific immune responses were induced but the protective effect to different virus was weak. Partial protection or severe sickness was observed in virus challenge experiments with immunized laboratory animals. One possible reason is that minor variation of M2e sequences from different viruses still exists although this protein is considered to be relative conserved, which may contribute to the different protective efficacy of M2e vaccines against various viral challenge [70]. One approach to overcome this limitation is to integrate multiple M2e repeats from different viruses into one vaccine platform. A membrane-anchored form of M2e fusion protein containing multiple M2e sequences has been incorporated into VLPs and induced M2e-specific antibodies reactive to different influenza viruses, conferring improved cross-protection against various subtypes of influenza virus including A/California/2009 (H1N1)

or A/Philippines/82 (H3N2) [16]. Another reason for the weak protection of M2e vaccine candidates investigated in the past is that most of these M2e were presented in a non tetrameric form [71-73], or without an appropriate vaccine delivery platform even though a tetrameric M2e was employed [74]. In a recent study, structure-stabilized M2e tetramers were expressed and purified from a baculovirus-derived insect cell protein expression system, and were assembled into highly immunogenic nanoclusters. The resultant nanoclusters induced high level of M2e-specific humoral and cellular immune response, conferring complete protection against lethal challenges with either A/California/2009 (H1N1) or A/Philippines/82 (H3N2) virus (Wang et. al, unpublished data). The progress in coming up with enhanced antigen design, integrated adjuvants, novel delivery and controlled release strategies (such as VLPs, microneedles or nanoclusters) suggest that M2e has great potential for development of broadly protective influenza A virus vaccines.

CONSERVED EPITOPES DERIVED FROM HA

The HA protein plays pivotal roles in influenza virology and is highly immuogesnic [75,76]. This envelop glycoprotein is anchored to the virus membrane as spikes consisting of two subunits, HA1 and HA2, up on the cleavage of the immature protein HA0 [77-79]. The architecture of HA can be dissected into the membrane-distal head (contains the most majority of HA1) and membrane-proximal stalk (comprises the N- and C-terminal portion of HA1 and the complete HA2) [80,81]. The HA1 head mediates binding of virion to host cell receptor and is essential for attachment of virus to target cells while HA2 promotes the fusion between viral and host endosomal membrane and virus entry [76]. HA2 contains a 23 amino acid long fusion peptide in its N-terminus followed by two α helices, A-helix and CD-helix [49,82,83]. At neutral pH, the fusion peptide is buried inside of the molecule. After the virus is taken up into endosome where HA is exposed to low pH, these motifs undergo dramatic conformational rearrangements which trigger the extrusion of the fusion peptide from interior of the molecule to the near end of the endosomal membrane [84,85]. Conventional neutralizing antibodies against HA are known to bind to host receptor binding domain within HA1 that are subjected to antigenic drift and are highly variable among different subtypes, which helps to explain the restricted protection of these antibodies against specific virus subtypes [86,87]. In comparison, the sequence of HA stalk is highly conserved across all influenza subtypes, which provides this domain the potential as a conserved epitope candidate for universal influenza vaccine design [12,88]. This rationale is further supported by the recent achievement in discovering several broadly neutralizing antibodies specifically against the HA stalk domain [49,50,89-91]. The identification of CR6261, a broadly neutralizing antibody against most group 1 influenza virus (including H1, H5, H9 and some H2 subtypes), has revealed the presence of a conserved epitope located in the A-helix of HA2 [49]. Meanwhile, a newly discovered monoclonal antibody CR8020 with broad neutralizing activity exclusively against group 2 HAs has been shown to protect mice against the lethal dose challenge of H3N2 as well as H7N7 [91]. The epitope against CR8020 consists of the C-terminal portion of the fusion peptide and surrounding residues. By screening large number of human peripheral blood plasma cells, Davide Corti and colleagues isolated monoclonal antibodies that recognized the HA of all 16 subtypes and neutralized both group 1 and group 2 influenza A viruses [89]. It is suggested that these antibodies recognize the fusion peptide as well as A-helix of HA stalk domain which are the regions bound by CR8020 and CR6261, respectively [90,91]. Taken together, these studies provide valuable information for potential cross-protective epitopes that researchers can consider to work with. To fulfill the ultimate goal of obtaining a universal influenza vaccine that can elicit broad-cross immunity, efforts have been made to optimize the way that epitopes are presented to the host immune system. Various approaches have been employed including the generation of virus like particles (VLPs), nano-grade particles, and recombinant carrier proteins. In an attempt to produce a HA-stalk-oriented immunogen for broad cross immunity, a truncated HA that lacks the global head (residues 52-277 of HA1) from A/Puerto Rico/8/1934 (PR8, H1N1 subtype) was created and could be stably incorporated into VLPs when co-transfected with HIV Gag-based construct in mammalian cells. The resultant VLP vaccine elicited antibodies that were cross-reactive among group 1 HA subtypes and provided protection against lethal homologous challenge although some weight loss was observed in mice [6]. It was proposed that this headless HA is in its neutral-pH conformation which resembles what it looks like in the native form of full length HA such that the elicited antibodies may exert their neutralizing function by inhibiting the transition of HA2 from neutral pH to low pH conformation and further inhibiting the fusion process. There are other studies suggesting the soluble HA2 in the absence of HA1 actually exists in the low-pH conformation, which may not be favorable for eliciting antibodies that are neutralizing [92]. In order to obtain soluble stalk domain that is in native state (neutral-pH conformation), Gayathri Bommakanti and other researchers designed a novel stalk construct (in the context of A/Hong Kong/1968, HK68, H3N2) containing 1-172 aa of HA2 connected to the portions of HA1 (both its N– and C-terminal regions) that constitute part of the stalk domain [93]. By using protein design and minimization methods, the parts of HA1 that interact with HA2 were included in the designed molecule and two hydrophobic residues in HA2 were mutated to charged residues in order to destabilize the low-pH conformation. This stalk construct was expressed in *Escherichia. coli (E. coli)* system and refolded from inclusion body to its neutral-pH conformation. This highly immunogenic construct exhibited binding ability to a previously identified broad neutralizing antibody 12D1

and protected mice from lethal homologous challenge. This research provides us an example for delicate antigen design based on careful structural and biophysical analyses. As described above, the emerging nanoparticle approach opens another promising avenue for production of multivalent antigen to boost the host immune response. Potential self-assembled nanoparticles as highly effective platform for vaccine design can be a powerful candidate for universal influenza vaccine when combined with the possible cross protective nature of HA stalk domains. Linear epitopes in the form of relatively short peptides in HA can be genetically coupled to certain protein carrier that may function as adjuvant for better vaccine efficacy. One recent example of such approach showed its competence in conferring broad protection against influenza subtypes from both group 1

and group 2 [94]. In this study, the epitope consisting of amino acid 76-130 of HA2 from H3 subtype (HK68), the binding target for neutralizing antibody 12D1, was coupled to the keyhole limpet hemocyanin (KLH) for enhanced antigenicity. This conjugate vaccine elicited antisera reactive to a board range of HAs from H1, H2, H3, H5 and H7 subtypes. It also protected mice from lethal challenge of not only H3, but also H5 and H1 subtypes. It is intriguing to find that the full length HA2, as compared to this conjugate containing only a small fraction of HA2, exhibited actually weaker antigenicity as it induced antisera reactive only to subtypes limited to group 2 influenza viruses. This may imply that inclusion of only the linear epitope by itself may help to elicit a more focused anti-stalk domain response, which is in line with the high protein sequence conservation of this region among both group 1 and group 2 influenza viruses. Another promising carrier protein is bacterial flagellin protein. In the context of fusion protein design, the central variable region of flagellin could be substituted by desired antigen, such as the global head of HA, without affecting its TLR-5 binding activity [95]. This recombinant fusion protein strategy has also proven to be successful in inducing anti-influenza immunity [96,97]. However, so far not much work has been reported for flagellin being used in stalk domain-based vaccine design which might be an attractive approach for universal influenza vaccine production.

T-CELL BASED EPITOPES

B-cell mediated humoral antibody immunity against influenza essentially protects the host from virus infection. As mentioned above, the conserved M2e and HA epitopes are in hope to elicit broad neutralization and protection. As another equally critical immune response against influenza virus, the cytotoxic T lymphocytes (CTLs) mediate cellular immunity by recognizing viral peptides presented on virus-infected epithelial cells [98,99]. These immune-responses are essential for inhibiting virus spread and clearance of infected cells. Epitopes that are recognized by CTLs reside on various influenza proteins including viral nucleoprotein (NP), matrix protein M1, RNA polymerase subunits PB1 and PB2 [100]. The value of combined B and T cell conserved epitopes as enhanced vaccine candidate has been recognized for years [101,102]. Recently, a first-in-human trial of a novel vaccine containing conserved linear epitopes from HA, NP and M1 proteins of both influenza type A and B strains has been carried out [103]. It was found that this vaccine was capable to induce both humoral and cellular immunity and was potentially cross-strain immunogenic. However, due to the small scale of experiment population and moderate elevation of immune responses in vaccinated group, further studies and evaluation need to be performed.

CONCLUSIONS

Universal influenza vaccines, as a concept, have been raised for a long time. There is an urgent need to provide broader, universal protection against a broad spectrum of influenza viruses. A lot of attempts have been made in these years; however, not so much progress has been gained. Recently, with the combination of conserved region of influenza virus such as M2e or HA stalk domains with efficient delivery strategies such as VLPs, microneedles or nanoparticles, the light of a universal vaccine finally shows up at the end of a long dark tunnel.

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