Review

Vaccine design of hemagglutinin glycoprotein against influenza

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Influenza viruses continue to cause annual epidemics and pose the threat of a deadly global pandemic. Vaccination has remained the best approach for prevention and control of influenza infection. However, current influenza vaccines are only effective against closely-matched circulating strains, and therefore must be updated and administered every year. In this review, we discuss recent developments in the search for better influenza vaccines, especially using the major virus surface glycoprotein hemagglutinins (HAs). Understanding how glycans on HAs affect the immune response and knowledge of how broadly neutralizing antibodies are induced will pave the way for a cross-protective influenza vaccine that does not require frequent updates or annual immunizations.

Introduction to influenza

Every winter, flu season claims the lives of a few hundred thousand people worldwide [1]. Influenza viruses cause a highly contagious respiratory disease that is transmitted via respiratory droplets from one person to another through cough or sneeze of the infected individuals [2]. Annual influenza epidemics are estimated to cause a global disease burden of 1 billion infections and between 3 and 5 million severe cases of illness [1]. The three historical influenza pandemics in humans include the 1918 pandemic flu (H1N1 Spanish flu) that caused between 50 and 100 million deaths (of mostly young adults), the 1957 H2N2 Asian flu that caused 2 million deaths, and the 1968 H3N2 Hong Kong flu that caused 1 million deaths [3].

Influenza viruses are members of the RNA virus *Orthomyxoviridae* family. Their genome contains eight negative-stranded RNA segments that encode 11 viral proteins, including the major surface glycoproteins (see Glossary), hemagglutinin (HA) and neuraminidase (NA) [1]. Three types of influenza virus, A, B and C, are classified based on their antigenic differences in two viral proteins, the nucleoprotein and the matrix protein. Influenza A and B viruses are prevalent and responsible for the seasonal flu [1]. Influenza A viruses can be further categorized into subtypes based on antigenic differences in HA and NA. There are a total of 16 different HA subtypes and nine NA subtypes, however only three subtypes of HA (H1, H2 and H3) and two subtypes of NA (N1 and N2) have been circulating in humans [1]. Variations of the globally circulating strains are mostly caused by antigenic drifts, which are the mutations on the surface glycoproteins HA and NA. Occasionally, when a host is coinfected by two or more different influenza viruses, reassortment of the eight gene segments can take place and lead to drastic changes in antigenicity, namely antigenic shift. The reassorted influenza viruses can be difficult to recognize by the human immune system and cause pandemics as seen in 1957 (H2N2), 1968 (H3N2) and 2009 (H1N1).

Since late 2003, the highly pathogenic avian H5N1 virus has caused global outbreaks and raised great concern that further changes in the virus may facilitate the transmission across the species barrier and become a deadly pandemic [4]. In April of 2009, the outbreak of a novel human H1N1 influenza virus was identified in Mexico and the new virus was transmitted around the world so quickly that the World Health Organization (WHO) declared a pandemic alert of phase six (human-to-human transmission in two different WHO regions) in June [5,6]. This 2009 swine-origin influenza A (H1N1) virus occurred through complicated events of gene reassortments of avian, human and swine influenza viruses, and had caused a global pandemic with a death toll of at least 13 000 people as of December 2010 [7] (http://www.who.int/csr/don/2010_01_15/en/index.html). In contrast to seasonal influenza viruses that caused mortality in children and the elderly, the 2009 pandemic influenza A (H1N1) virus caused severe illness and death primarily in adult age groups [8]. Influenza pandemics occur when the major virus surface glycoprotein HA is not recognized, to some extent or entirely, by the immune system, and the virus efficiently transmits from human to human.

HA glycoprotein

HA is the most abundant glycoprotein on the surface of influenza viruses. The glycosylation of HA plays an important role in the life cycle of influenza viruses. The conserved glycosylation sites are required for the structural

Glossary

Glycoproteins: proteins with glycans covalently linked to amino acid residues on polypeptides co- or post-translationally. Protein glycosylations mainly occur at the amide nitrogen of asparagine (N-linked glycosylation) and at the side chain of serine or threonine (O-linked glycosylation). More than 50% of human proteins are glycoproteins that play important roles in a large variety of biological functions. The glycoproteins HA and neuraminidase of influenza viruses possess N-linked glycosylation.

Hemagglutinin (HA): is the most abundant membrane integral glycoprotein on the influenza viral surface, and there are 300–350 HA molecules on the surface of one virion [81]. The HA proteins are responsible for the interaction between the virus and the infected cells, and the fusion between viral and host endosomal membranes that allows the viral gene to enter the host cell.

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integrity and stability in the process of HA biosynthesis and the formation of intact virions [9,10]. For example, in the HA sequences of H5N1 strains, the conserved glycosylation sites are located on amino acid residues 27, 39, 170, 181, 302 and 500. These six conserved glycosylation sites can also be found in the corresponding HA sequences of H1N1 and H3N2 viruses [11]. HA interacts with sialylated glycan (sialoside) receptors in the respiratory tract and/or intestine of the host, which allows the virus to enter the cell [12]. HA from avian influenza such as H5N1 predominantly binds to α2,3 sialosides that are more typical of avian cells whereas HA from human influenza such as H1N1 or H3N2 preferentially binds to α2,6 sialosides that are commonly found on human cells [13,14]. HA glycoprotein forms a homotrimer with a large extracellular domain comprised of a globular head and a stem structure [15]. Both regions possess N-linked glycosylation [16]. Variations in the structure and number of the glycans on the head region have been shown among different influenza A viruses, whereas the stem glycans are more conserved. The functions and properties of HA were affected by glycans at specific sites [17,18]. The glycans at the HA antigenic epitopes interfere with the access of antibodies and may result in antigenic drift of influenza virus [19]. The proteolytic activation of HA, from the precursor form HA0 to the mature form HA1 and HA2, is critical for fusion activity, and it can be modulated by glycans near the cleavage site [20,21]. The conserved glycans at the stem region are also required for fusion activity [17]. HA is considered the most important antigen of influenza virus that can induce neutralizing antibodies against the influenza virus infection [22,23]. However, changes in the amino acid sequence of HA in influenza virus can often affect the effectiveness of vaccination from previous seasons. As a result, seasonal influenza vaccines need to be updated and administered every year. The amino acid changes sometimes result in alterations in the numbers and positions of glycosylations on HA, and the glycans on HA have been shown not only to affect the binding specificity to hosts, but also to play an important role in evading the immune response [24,25]. A recent analysis has demonstrated that glycosylation generally shields HA from antibody-mediated neutralization, and the fitness costs in accommodating glycans limit virus escape from HA hyperglycosylations [26].

Current vaccines and drawbacks

There are two forms of current trivalent influenza vaccines, inactivated vaccines and live attenuated vaccines, which are administered by intramuscular injections and intranasal sprays, respectively. Both are proven to be effective in preventing influenza infection and its illness. Trivalent seasonal influenza vaccines are made with embryonated chicken eggs to contain three reference virus strains that are identified by WHO as circulating in the forthcoming influenza season on a yearly basis, that is two influenza A viruses, H1N1 and H3N2, and one influenza B strain [27]. The WHO-recommended reference virus strains are hybrid viruses created by reassortment of HA and NA genes from the circulating strains and other gene segments from a laboratory strain (A/Puerto Rico/8/1934) that have been adapted to propagate well in eggs [1]. Therefore, the complex-type glycans are retained on surface glycoproteins of the virus (Figure 1a). This process is time consuming and not always successful. Occasionally, as seen in the case of 2009 pandemic H1N1 vaccine productions, low yields of virus or low levels of HA proteins were observed. It is estimated that six months are required for the manufacture of seasonal influenza vaccines to go from receiving the reference strains, through virus propagation, purification, inactivation and, lastly, to production. Inactivated vaccines are estimated to be 70–90% effective in healthy individuals and 50–70% in people with underlying chronic health conditions [28–30]. In comparison with inactivated vaccines, live attenuated vaccines are delivered intranasally, inducing higher mucosal IgA responses, and are considered to provide immunity against infections from other subtypic strains [31–34]. However, because the vaccines contain live viruses, which are adapted only to temperatures below 25 °C, recipients are limited to individuals in the age range 5–49 years [35]. Pregnant women and immunocompromised individuals are excluded, and typical side effects include rhinorrhea, sore throat and flu-like symptoms [36]. A problem with current egg-based vaccine production is that when the new pandemic strain virus unexpectedly appears, the global manufacturing capacity does not always meet the vaccine demands because of the limited quantities of embryonated chicken eggs. It is also a concern that if avian influenza has crossed the species barrier to become a pandemic, the supply of eggs can be a serious problem. Furthermore, seasonal influenza vaccines only provide limited protection against antigenically closely related viruses, display lower protection against heterologous subtype viruses [37] and can cause side effects such as muscle soreness at injection sites, low-grade fever or body aches, and cannot be given to people who have egg allergies. Another method to produce influenza viruses for vaccine development is based on cell culture. Though the cell culture process may have less undesirable side effects, it is less cost effective. Future influenza vaccines need to overcome the aforementioned limitations of the current ones, to be safe and more effective, and must be efficiently manufactured in large quantities [38]. In this review, we focus on recent studies of influenza vaccine design using influenza glycoprotein HAs (Figure 1 and Table 1).

High mannose-type glycosylated HA and virus-like particles (VLPs) as flu vaccines

Cell culture production of influenza vaccines is advantageous over traditional egg-based procedures because it is more efficient (weeks versus months), it does not require pathogenic viruses in the process, and it does not contain egg proteins that can cause allergy. Recombinant HA proteins containing heterogeneous complex-type glycans can be produced from human cells and used as influenza vaccines [39–41] (Figure 1a). Moreover, baculovirus-based insect cell cultures have been used to produce recombinant HA proteins and recombinant noninfectious VLPs as vaccines [42–44] (Table 1). The heterogeneous N-glycans expressed from insect cells are mainly in the glycoform of $GlcNAc_2Fuc_1Man_3$ [45] (Figure 1b). The insect cell-expressed HA protein vaccine named FluBlok is comprised

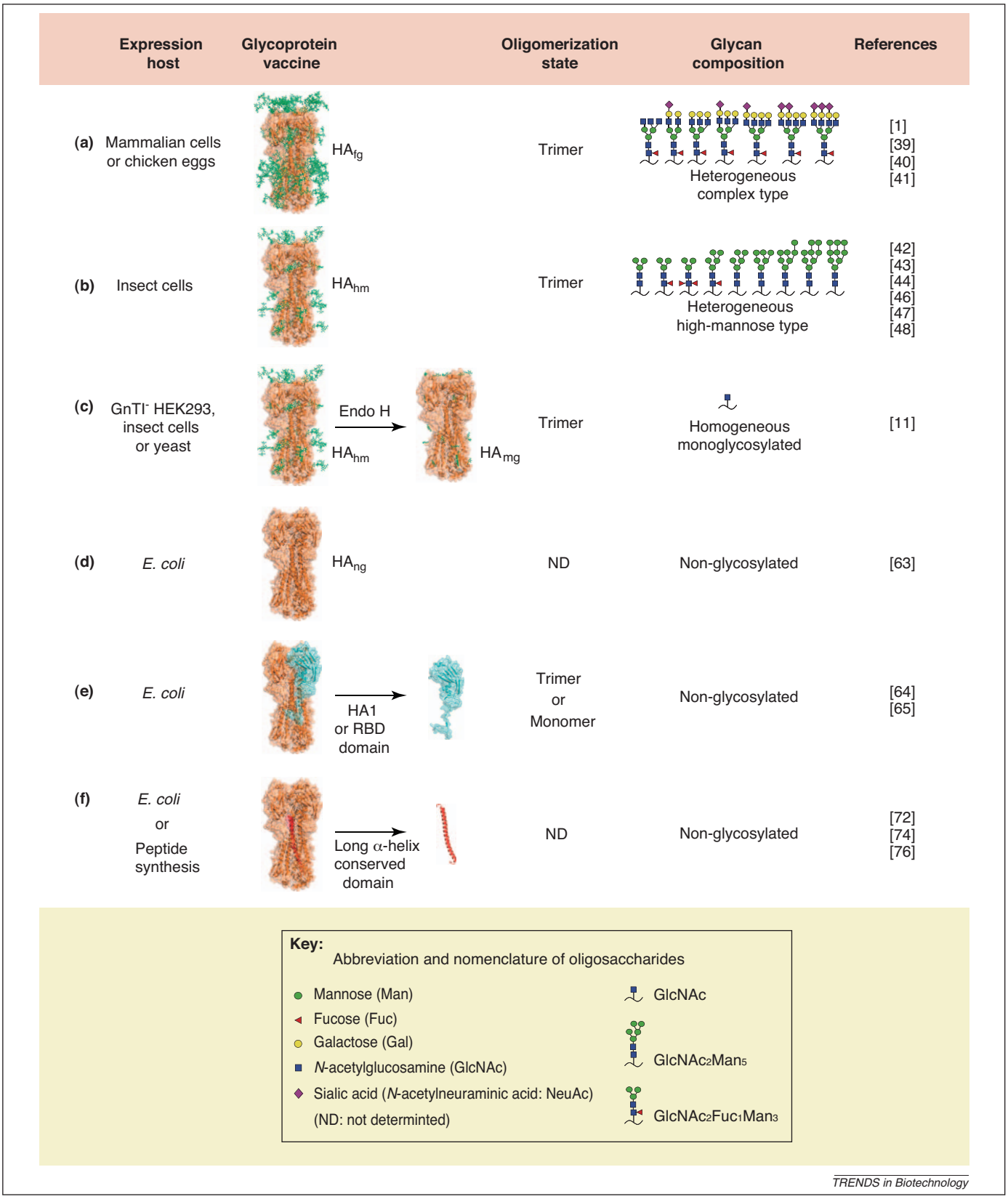

Figure 1. Development of new influenza vaccines containing glycoprotein hemagglutinins (HAs). The approach of using the major surface glycoprotein of influenza, HA, either in whole or in part, as a new influenza vaccine is categorized by expression hosts, oligomerization states and glycan compositions. **(a)** Fully glycosylated HA (HA_{fg}) is trimeric and contains heterogeneous complex-type glycans that are similar to live viruses. **(b)** Trimeric HA with heterogeneous high mannose-type glycans (HA_{hm}) can be obtained from insect cell culture. **(c)** Trimeric HA with homogeneous single GlcNAc residues attached (HA_{mg}) can be obtained by expression in GnTI-deficient human cells ($GnTI^-$ HEK293), insect or yeast cells and treated with endoglycosidase Endo H. **(d)** HA with no glycosylations (HA_{ng}) can be obtained by *Escherichia coli* expression. Refolding is often necessary to obtain soluble HA. **(e)** Domains of HA without glycosylations can be obtained by *E. coli* expression. **(f)** Conserved domains or peptides can be obtained by *E. coli* expression or chemical synthesis. Structures are created with Protein Data Bank ID code 3LZG [82] and the N-linked glycans are displayed in green. All N-linked glycans are added manually and energy minimized by program CNS [83]; graphics are generated by program PyMOL (www.pymol.org).

Table 1. New approaches and technologies of broadly protective HA-based influenza vaccine

Vaccines	Vaccine strain	Animal model	Mode of immunization	Adjuvants	Vaccine efficacy	Refs
Recombinant proteins						
HEK293 cells expressed HA and treated with Endo H to form the trimer of HA_{mg}	Consensus HA (H5N1)	Mice	Intramuscular	Aluminium hydroxide	Induced high titer of antibodies against a lethal challenge with H5N1 virus and crossreacted with heterosubtypic viruses	[11]
E. coli expressed HA2 conserved peptide (75–98)	A/Philippines/2/1982 (H3N2)	Mice	Intramuscular	CpG7909	Mice were 80% protected against a lethal challenge with A/HK/68 H3N2 virus	[72]
Chemical synthetic long α-helical (LAH) peptide	A/Hong Kong/1/1968 (H3N2), HA2 stalk domain (76–106) coupled with (KLH)	Mice	Subcutaneous	Complete Freund's and incomplete Freund's	Mice were protected against a homologous H3N2 influenza virus and conferred a partial protection against a H5N1 virus challenge in mice model	[74]
VLPs						
VLPs were made with recombinant baculovirus expressing the genes for, HA, NA and M1	A/Fujian/411/2002 (H3N2)	Mice or Ferrets	Intramuscular	None	Elicited antibodies that recognized a broader panel of antigenically distinct H3N2	[42]
	A/Indonesia/05/2005 (H5N1)	Mice	Intranasal or intramuscular	None	100% survival rate in a lethal challenge with (A/Viet Nam/1203/2004 (clade 1) or A/Indonesia/05/2005 (clade 2)	[43]
	A/South Carolina/1/1918 HA(H1N1)	Mice	Intranasal	None	Mice were protected against a lethal challenge with both the1918 virus and the H5N1 virus.	[44]
			Intramuscular	None	Mice were protected against a homologous virus challenge 1918 virus	[44]
	A/South Carolina/1/1918 HA(H1N1)	Ferrets	Intranasal	None	100% survival rate in a lethal challenge with the H5N1 virus.	[44]
			Intramuscular	None	50% survival rate in a lethal challenge with the H5N1 virus.	[44]
PR8-based Headless DNA vaccine plus VLPs vaccine	A/Puerto Rico/8/1934 (H1N1) (PR8)	Mice	Electroporation plus intraperitoneal	Freund's complete adjuvant	Mice vaccinated with the headless PR8 HA showed protection against PR8 virus challenge and greater activity of antibodies against heterologous H2 and H5 strains in ELISA assay	[76]
DNA vaccine						
Consensus H5N1/HA DNA vaccine (pCHA5)	Consensus HA (H5N1)	Mice	Electroporation	None	The consensus HA vaccine could induce broad protection against divergent H5N1 inuenza viruses	[79,80]

of trivalent full length HA proteins from two influenza A viruses H1N1 and H3N2 and an influenza B strain, as in the current egg-based inactivated virus flu shots [46,47]. The HA proteins produced from insect cells carry the heterogeneous high mannose-type glycans on each glycosylation site and retain native HA protein structure [48] (Figure 1b). A single dose of FluBlok contains a threefold higher dose of HA proteins than traditional trivalent inactivated vaccine; it was shown in a clinical trial to be immunogenic in more than 3000 adults older than 18 years [46,47,49]. Although FluBlok has been demonstrated to be effective in producing an immune response against influenza virus, a US FDA committee expressed concern about the safety of the vaccine being licensed in the USA in November 2009 [50]. A recent study using soluble trimeric H5 HA expressed from an *N*-acetylglucosaminyltransferase I-deficient ($GnTI^-$) strain human embryonic kidney cell culture as a vaccine has shown protection against H5N1 infection in mice and chickens [51]. Alternatively, noninfectious VLPs have been considered as influenza vaccine candidates. The VLPs morphologically resemble their live virus counterparts and are thought to be easily recognized by antigen-presenting cells and processed to produce an immune response [52–54]. An influenza VLP vaccine has been developed using insect cell cultures to contain functional viral coat proteins in their natural membrane-bound state, which consist of HA and matrix protein (M1) with or without NA, and thereby mimic the process of native virus stimulation of the immune response. The development of VLPs has been studied in various subtypes, including H1N1, H3N2, H5N1, H5N3 and H9N2, which are capable of inducing an immune response against homologous or heterologous strains of influenza viruses [55] (Table 1).

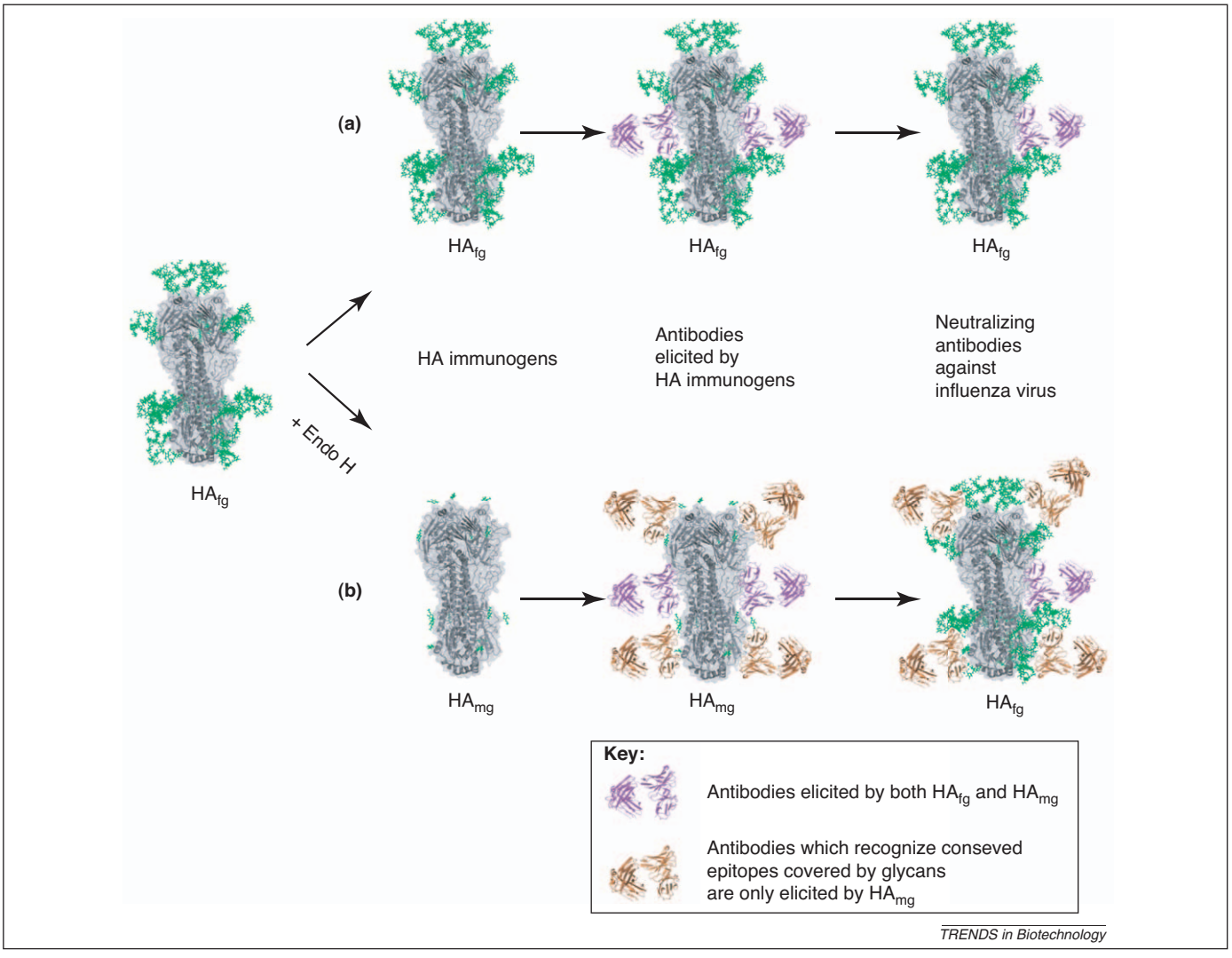

Figure 2. A hypothesis for the monoglycosylated hemagglutinin (HA_{mg}) as a better influenza vaccine than fully glycosylated HA (HA_{fg}). The efficacy of influenza vaccine depends on its ability to induce neutralizing antibodies. **(a)** The complex-type glycan shields possessed by HA_{fg} limit the protein surface area to be exposed for antibody recognition. Glycans are shown in green, HA protein in gray and antibody Fab domain in brown. The restricted exposed surface contains the B cell epitopes that are critical for the generation of neutralizing antibodies. Therefore, vaccination with HA_{fg} is considered to generate limited neutralizing antibodies that can only recognize HAs from closely-matched strains. **(b)** The HA_{mg} contains only a single GlcNAc sugar residue on each glycosylation site and maintains the structural integrity as seen in HA_{fg}. The removal of glycans increases the exposed surface area for antibody recognition. Therefore, vaccination with HA_{mg} generates more broadly neutralizing antibodies because the newly exposed sequences near glycosylation sites are more conserved across different influenza strains. HA and Fab structures are extracted from Protein Data Bank ID code 3GBM and rearranged here to illustrate the hypothetical interaction between antibodies and HA proteins. They do not imply any specific interaction as seen between the stem region of HA proteins and monoclonal antibody CR6261 [70].

Monoglycosylated HA (HA_{mg})

Glycan shields of HA have been shown to contribute to poor immune response [56–58]. By trimming down the size of glycans with enzymes, HA_{mg} has been demonstrated to be a better vaccine than fully glycosylated HA in a mouse model [11]. HA_{mg} is produced from a suspension cell culture of $GnTI^-$ human embryonic kidney cells [59], and the resulting N-glycan of HA, $GlcNAc_2Man_5$, is then treated with the recombinant endoglycosidase Endo H, which can be readily overexpressed and purified from *Escherichia coli* culture, to produce a single *N*-acetylglucosamine (GlcNAc) glycan residue [11] (Figure 1c). HA_{mg} retains a single GlcNAc on each glycosylation site and forms a trimer as the native conformation, which can be easily analyzed by MS and size-exclusion chromatography to characterize the glycan composition and the protein trimeric homogeneity in solution, respectively. Studies have shown that the core trisaccharides or monosaccharide (GlcNAc) of the N-linked glycan is primarily responsible for protein folding and stabilization, supported by the fact that the core trisaccharide is highly conserved in eukaryotes ranging from yeast to human [60,61]. This observation was applied to the preparation of HA_{mg}. HA_{mg} can be produced in a timely manner because such vaccine can be produced not only from human cell culture but also via insect cell culture or yeast fermentation under well-controlled conditions. In comparison with fully glycosylated HA (HA_{fg}), HA_{mg} has been shown to be more effective as a vaccine because it can expose conserved epitopes, which are ordinarily hidden by large glycans, to elicit an immune response that recognizes HA variants in higher titer [11] (Figure 2). The effects of various degrees of glycosylation on HA have been systematically analyzed regarding host receptor binding and immune response [11,39,62]. Trimming down the N-glycans on HA from avian influenza H5N1 can successively increase binding to α2,3 sialosides on a glycan microarray. According to circular dichroism analysis, the secondary structure of HA protein is not altered upon removal of the glycan shields. Stronger binding to sialosides also implies that HA with less glycans attached can have better interaction with antigen-presenting cells when used as a vaccine (J.-R. Chen *et al.*, unpublished). Antibodies raised against HA protein bearing only a single N-linked GlcNAc at each glycosylation site show better neutralization activity against influenza subtypes than elicited by HA_{fg} [11] (Figure 2b and Table 1). Moreover, our recent results showed that the protein vaccines of HA_{mg} designed from a seasonal influenza A/Brisbane/59/2007 (H1N1) can provide cross-protective immunity against infections from other H1N1 strains, including A/WSN/33/1933, A/Puerto Rico/8/1934, and A/California/07/2009 in mice and ferrets (J.-R. Chen *et al.*, unpublished).

Non-glycosylated HA expressed from *E. coli* as flu vaccine

HA proteins expressed in *E. coli* do not contain glycans and, theoretically, are more immunogenic than glycosylated HA proteins. The HA proteins prepared from *E. coli* are the most economical and can be rapidly produced in a large quantity and purified in a few weeks. However, in *E. coli,* HA proteins commonly express in the form of insoluble aggregates that require a refolding process to obtain homogenous antigens. The process includes solubilization of the insoluble HA proteins in denaturing agents such as guanidine hydrochloride and a subsequent dialysis to remove the denaturing agents to obtain properly folded HA proteins. Extra care is also needed to ensure that the level of bacterial endotoxin that can be carried over from bacterial production is below the acceptable value for a human vaccine. A recent result showed that three dosages of the bacteria-purified H5 HA (15 μg), formulated with adjuvant alum, can induce HA antibody levels (HA inhibition$\geq$40) in mice against epidemic and pandemic influenza H5N1 viruses [63] (Figure 1d). A vaccine containing the properly folded and trimeric globular domain of HA from A/California/07/2009 H1N1 virus can be obtained from *E. coli*, and the proteins elicit a potent neutralizing antibody response in ferrets against virus challenge [64]. Similarly, the expression of the HA receptor-binding domain (RBD) from *E. coli* has been studied: its monomeric form can be obtained after the refolding process and was used as a vaccine to elicit an immune response against the H1N1 virus infection in a ferret model [65] (Figure 1e). Structural integrity of the HA proteins throughout the refolding process cannot be easily controlled. The vaccine containing only the HA1 head domain can have the disadvantage of losing the HA2 highly conserved domain of the stem region to induce broadly neutralizing antibodies.

Consideration of glycoforms on HA vaccine design

Glycans of HA on viruses have been shown not only to affect the binding specificity to hosts but to play an important role in evading the immune response [57,58,66]. Therefore, it is rational to design a HA glycoprotein vaccine with less glycans attached while maintaining the protein structural integrity for the proper presentation of useful epitopes for an effective immune response. Pandemic influenza viruses historically have fewer potential sites for N-linked glycosylation on the globular head of HAs upon emergence from the avian reservoir. Gain of glycosylation sites on the globular head of HA has been proposed as a mechanism for influenza A viruses to facilitate immune evasion during adaptation to humans [57,58].

During the past four decades, the prevalent influenza H3N2 virus strains have gradually increased additional potential sites for glycosylation within the globular head domain of the HA proteins [58]. The increase of glycosylations on HA decreased the virulence of the H3N2 viruses and host immunity in mice [58]. Highly glycosylated H3N2 virus elicits poor antibody response and interrupts host antibody recognition of the HA. In a recent study, a host primed with highly glycosylated H3N2 virus was vulnerable to subsequent infection with H3N2 virus with less glycosylation; this outcome may be caused by a mismatched adaptive immune response that is characterized by poor antibody production but robust T cell response [57]. The authors implied that the lack of two glycosylation sites on the globular head region of HAs in comparison with prior seasonal H1N1 influenza is the reason why during the 2009 H1N1 pandemic, severe illnesses were observed in adults with poor preexisting immunity and robust T cell responses [57]. Overall, the highly glycosylated viruses are

poor immunogens for the host to elicit neutralizing antibodies and this implies that less glycans should be considered as an important factor for the development of new influenza vaccines (Figure 2).

Conserved domain

Conserved domains near the stem region of HAs have been proposed to induce cross-reactive and neutralizing antibodies [67,68]. Two recent crystal structure studies of broadly neutralizing monoclonal antibodies against influenza, CR6261 in complex with H1 and H5 HAs and F10 in complex with H5 HA, revealed a conserved domain that is located in the stem region of HA in the junction of HA1 and HA2, which contains the key epitopes mainly in the fusion peptide and along the α-helix region in HA2 [69–71]. Both CR6261 and F10 monoclonal antibodies can specifically bind to the stem region of HAs and prevent critical fusion of viral and endosomal membranes in the influenza life cycle by blocking the low pH induced conformational change. The amino acid residues from these regions are highly conserved across different subtypic HAs and served as the basis in designing a conserved domain, which when used as a vaccine, can induce broadly neutralizing antibodies [72–74] (Figure 1f). Another monoclonal antibody, S139/1, was found to have broad neutralization reactivity against H1, H2, H3 and H13 subtypes of influenza A viruses, and the conserved epitope was found to be located on the globular head of HA adjacent to the receptor-binding domain [75]. When the peptide containing residues 75–98 of HA2 from H3 HA, either by *E. coli* expression or chemical synthesis, was used as a vaccine, protection against homologous H3N2 infection could be observed, however it failed to protect a heterosubtypic H1N1 A/Puerto Rico/8/1934 challenge in a mouse model [72] (Table 1). The long α-helical (LAH) peptide of HA2 stalk domain containing residues 76–106 has been coupled with a carrier protein Keyhole limpet hemocyanin (KLH) and used as a vaccine [74]. The LAH–KLH vaccine showed protection against a homologous H3N2 influenza and conferred partial protection against a H5N1 virus challenge in mice [74] (Table 1). In another vaccine study, a PR8-based 'headless HA' vaccine has been designed on the surface of VLPs to present the conserved stalk region, which elicits cross-reactive HA antibodies and confers protection against a lethal PR8 virus challenge in mice [76] (Table 1). A recent study showed that broadly neutralizing antibodies against conserved epitopes in the HA2 stalk region can be identified from B cells of 2009 pandemic H1N1 infected individuals [77]. Whether this phenomenon correlates with the fact that the receptor binding site of HA protein from 2009 pandemic H1N1 virus lacks two glycosylation sites in comparison with HA proteins from previous seasonal H1N1 influenza viruses will need further investigation.

Concluding remarks

Ideally, influenza vaccines should prevent infections from a wide range of influenza viruses, which are known to constantly undergo antigenetic drifts and shifts. Despite great efforts in the search for a universal influenza vaccine that can provide long-lasting and cross-subtypic protection, we are still left with the traditional egg-based annual flu shots, which provide only satisfactory defense, therefore, the pursuit continues. However, studies from the past two decades have identified a number of broadly neutralizing antibodies such as F10, CR6261 and C179 [67,69,71]. They are identified from human B cells (F10 and CR6261) or vaccinated mice (C179) and shown to bind to the stem region of HA near the HA2 fusion peptide [69,70]. These purified antibodies can neutralize different influenza subtypes *in vitro* and are protective against inter-subtypic influenza infections when passively immunized in mice [69,71]. Searches for escape virus mutants under pressure of these neutralizing antibodies have been performed and only one escape H5N1 virus has been reported from under the selection of neutralizing antibody CR6261 with a mutation on histidine 111 on HA2 helix [71]. Other studies aimed at generating escape mutants from the neutralizing antibodies that target the HA2 conserved epitope of viruses have failed even after multiple passages of viruses in the presence of neutralizing antibodies [69,78]. The fact that it is difficult to isolate escape mutants indicates the effectiveness of these broadly neutralizing antibodies. The "conserved epitope" recognized by these antibodies, in size, is however rather limited to be explored in comparison with the whole hemagglutinin glycoprotein molecule. Therefore, how to design a vaccine from the conserved domain that can induce broadly neutralizing antibodies such as F10, CR6261 and C179 to a protective level remains a difficult task, and nevertheless one of the main focuses in the development of universal influenza vaccines [22,76].

Along the same line of thinking, HA DNA and protein vaccines designed from a 'consensus sequence' have been shown to induce cross-strain protection against H5N1 virus infections from different clades [11,79,80]. A consensus sequence of DNA or proteins is the most commonly found nucleotide or amino acid sequence at every given position after performing multiple sequence alignments. Conserved regions or motifs can often be identified among

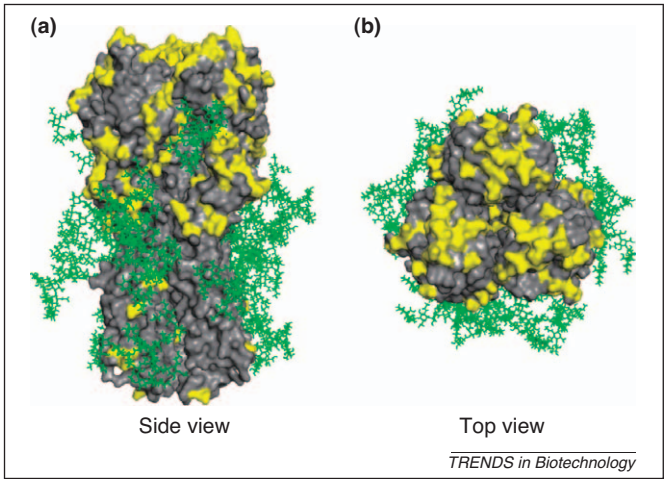

Figure 3. Comparison of the surface antigenic differences between hemagglutinin (HA) proteins from 2008 seasonal H1N1 (Bris/07) and 2009 pandemic H1N1 (Cal/09) viruses. **(a)** The side view and **(b)** top view of the HA trimeric structures represent the surface regions and their differences between Cal/09 and Bris/07. Identical amino acid residues between the two proteins are colored in gray, different residues colored in yellow and the glycans of HA from Cal/09 colored in green. Structures are created with Protein Data Bank ID code 3LZG [82].

protein homologs and indicative of similarities in three-dimensional structure and/or functions. When multiple sequence alignments are performed on HAs from various strains, the resulting consensus sequence can represent conserved epitopes or regions that are common for induction of antibody response or recognition. The most conserved amino acid sequences are found in the protein interior of HA (Figure 3). By contrast, the surface residues of HA, which should be recognized by neutralizing antibodies, are less well conserved. The variations of amino acid residues at the surface of HA are correlated with antigenic drifts and shifts and annual updates and renewals of current flu shots. However, among the surface amino acid residues, the sequences near the glycosylation sites of HA are found to be relatively more conserved [11,26]. The preparation of a HA_{mg} vaccine can potentially expose these more conserved sequences that are ordinarily covered by large glycans for antibody recognition. Therefore, it is enticing to design a broad-spectrum influenza vaccine that combines the concept of using consensus sequences of HA from various strains and the removal of most N-glycans, as in the design of HA_{mg}, to present the most conserved epitopes at glycosylation sites for induction of more broadly neutralizing antibodies.

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