

Influenza virus neuraminidase (NA): a target for antivirals and vaccines

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Abstract Influenza, the most common infectious disease, poses a great threat to human health because of its highly contagious nature and fast transmissibility, often leading to high morbidity and mortality. Effective vaccination strategies may aid in the prevention and control of recurring epidemics and pandemics associated with this infectious disease. However, antigenic shifts and drifts are major concerns with influenza virus, requiring effective global monitoring and updating of vaccines. Current vaccines are standardized primarily based on the amount of hemagglutinin, a major surface antigen, which chiefly constitutes these preparations along with the varying amounts of neuraminidase (NA). Anti-influenza drugs targeting the active site of NA have been in use for more than a decade now. However, NA has not been approved as an effective antigenic component of the influenza vaccine because of standardization issues. Although some studies have suggested that NA antibodies are able to reduce the severity of the disease and induce a long-term and cross-protective immunity, a few major scientific issues need to be addressed prior to launching NA-based vaccines. Interestingly, an increasing number of studies have shown NA to be a promising target for future influenza vaccines. This review is an attempt to consolidate studies that reflect the strength of NA as a suitable vaccine target. The studies

discussed in this article highlight NA as a potential influenza vaccine candidate and support taking the process of developing NA vaccines to the next stage.

Introduction

Influenza viruses belong to the *Orthomyxoviridae* family of viruses. The genome of influenza viruses consists of negative-sense RNA segments encoding viral proteins. The envelope of the virus possesses two major surface glycoproteins, namely, hemagglutinin (HA) and neuraminidase (NA) [1]. Influenza A viruses are subtyped based on HA and NA, with recent studies having identified two new subtypes of HA and NA making a total of 18 subtypes of HA (H1–18) and 11 subtypes of NA [2–4]. HA is a highly antigenic trimeric membrane glycoprotein that is essential for receptor-binding and for fusion of the virus and the host membrane [5].

Influenza viruses are known to cause highly contagious respiratory illness in humans, which can lead to potentially fatal outcomes. Epidemics of influenza can be traced back to the 5th century BC, when it was described as “Cough of Perinthus” by Hippocrates [6, 7]. The world has witnessed three influenza virus pandemics in the 20th century (1918, 1957 and 1968) and one pandemic in the 21st century (2009). The 1918 influenza pandemic (Spanish flu) was the most fatal one to date, exceeding even the death toll recorded during World War I. An estimated 50 million people died due to the 1918 Spanish flu. In April 2009, a new influenza virus, influenza A(H1N1)pdm09 virus, emerged and spread globally [8], affecting around 43 to 89 million people worldwide [8, 9]. According to the World Health Organization (WHO), influenza epidemics occur annually and are estimated to result in 3 to 5 million cases

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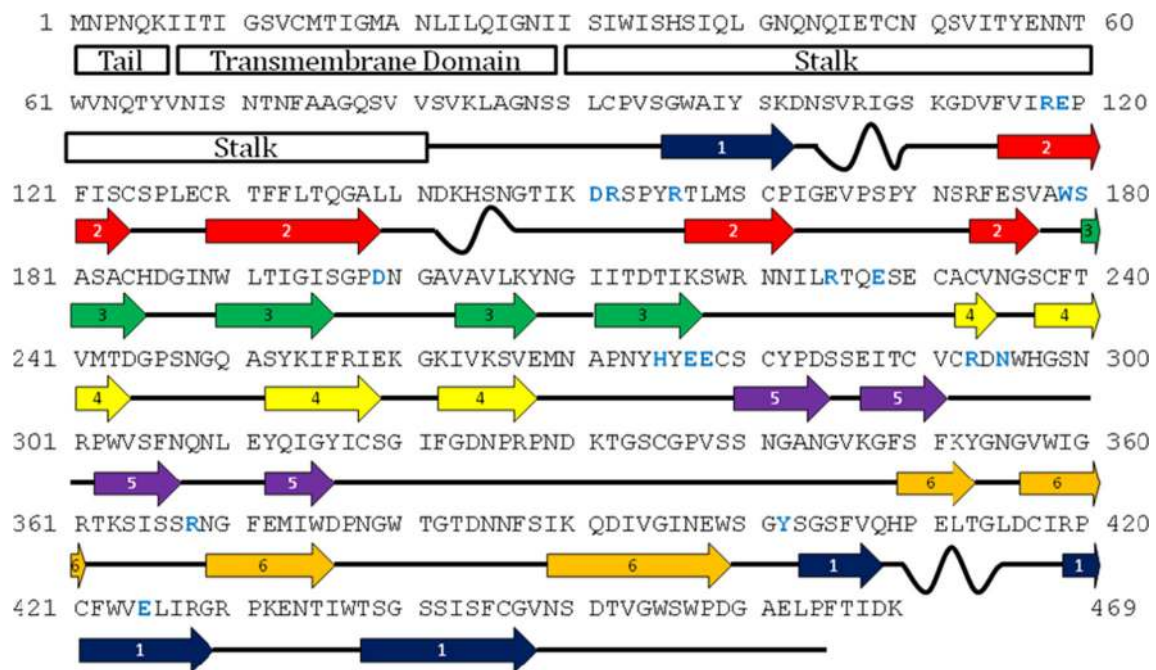


Fig. 1 Sequence of influenza virus neuraminidase. Amino acids that form the cytoplasmic tail, transmembrane domain, stalk and globular head are shown. Secondary structures based on Protein Data Bank (PDB) ID: 4B7Q are shown, and six propeller β -sheets (➡) are coloured differently. Helices (⤿) and loops (—) are also shown.

of severe illness, and approximately 250,000 to 500,000 deaths worldwide [10]. Influenza virus alters its antigenicity dynamically by accumulating mutations in the surface glycoproteins, HA and NA. Minor changes in the protein structure, called antigenic drift, occur frequently, resulting in small outbreaks, whereas major changes called antigenic shift occur by reassortment of different influenza A virus subtypes, especially those infecting animals and humans [11]. Such mutations result in the emergence of new strains that cause pandemics, posing more and more challenges in the development of newer drugs and vaccines [12]. Antigenic variability and immunogenicity of HA have been well documented, but studies about NA are sparse. This review focuses on the various studies of NA protein structure, its function, immunogenicity and the level of immunity it induces. Subsequently, the review emphasizes the need to consider NA as a component of influenza vaccines rather than just as a target for antivirals.

Neuraminidase (NA) protein: structure and function

NA is a mushroom-shaped tetrameric transmembrane glycoprotein made of four identical subunits. NA is the second major surface glycoprotein present in influenza A and B viruses, but it is absent in influenza C virus [1]. NA protein is

The six 4-stranded antiparallel β -sheets, arranged like the blades of a propeller, are marked and coloured in magenta, red, green, yellow, blue and orange. Drug-binding sites are highlighted and coloured in blue (colour figure online)

made of four main regions: a cytoplasmic tail domain (six amino acids), a hydrophobic transmembrane domain (7-29 amino acids), a stalk (~ 50 amino acids) and a globular head containing the enzyme active site (19 amino acids) (Fig. 1) [13]. The structure of each NA subunit (~ 469 amino acids) consists of six topologically identical 4-stranded antiparallel β -sheets arranged like the blades of a propeller (Fig. 2). The enzyme active site (19 amino acids) is located at the centre of each subunit. The active site is a deep pocket made of amino acids that are conserved in all strains of influenza virus. Antibody-binding sites are located on the surface loops that surround the enzyme active site [14, 15].

The function of NA is to cleave the terminal sialic acid residues present on cell surfaces and progeny virions, facilitating release of the virus from infected cells and thus playing an important role in release and spread of progeny virions. Some studies have also demonstrated NA's role in facilitating hemagglutinin-mediated fusion and virus invasion in the ciliated epithelium of human airways [16].

Neuraminidase inhibitors

The emergence of resistance to the adamantane group of influenza antivirals targeting the M2 gene diverted research interest towards NA inhibitors, which then surfaced as the next important class of antivirals for the treatment and

Fig. 2 Three-dimensional structure of influenza neuraminidase protein. The 3D structure of NA protein is depicted as a cartoon. The six 4-stranded antiparallel β -sheets in each tetrameric subunit are shown and coloured as in Figure 1. Helices and loops are coloured in cyan. Oseltamivir is shown as a sphere in the catalytic site of one of the subunits. PDB ID: 3TI6 (<http://www.pdb.org>) was used for generating figures [17]. The figure was rendered using Pymol (<http://www.pymol.org>) (colour figure online)

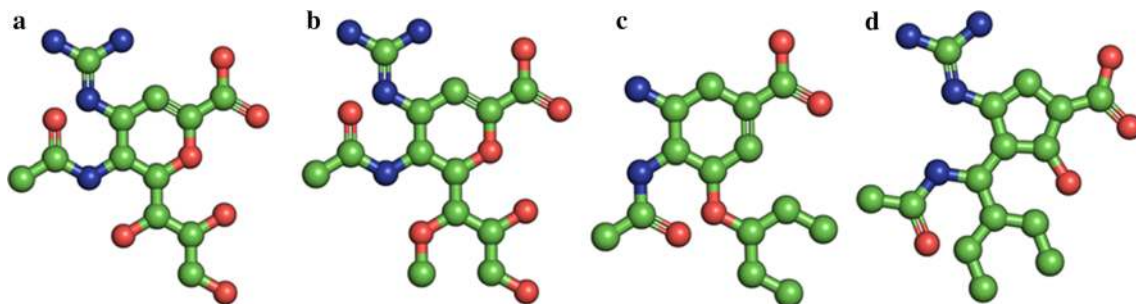
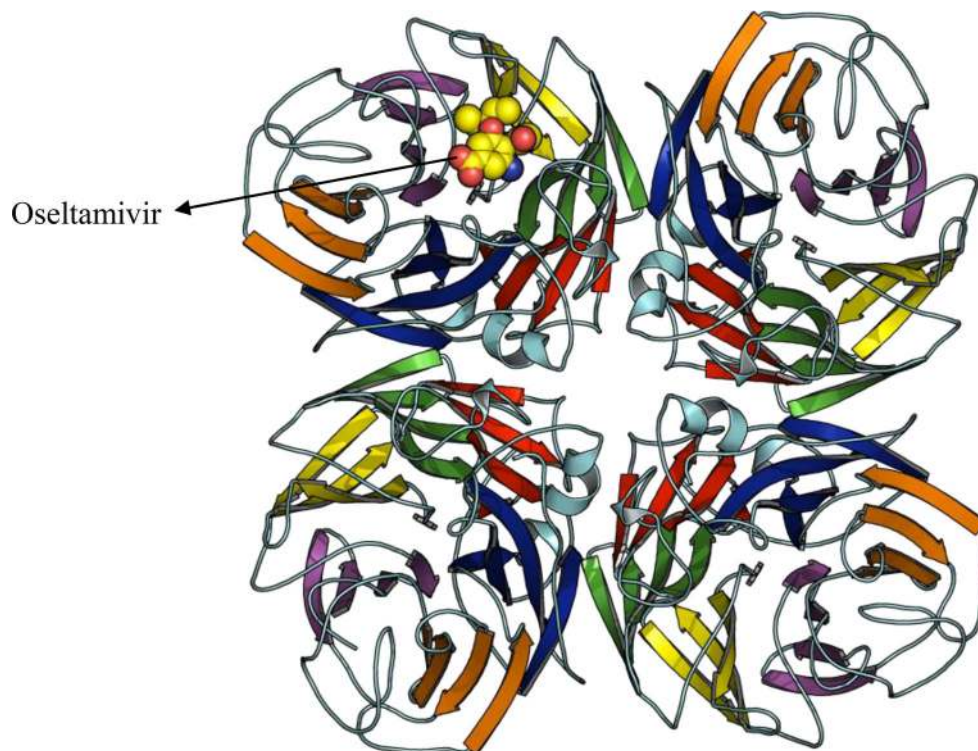


Fig. 3 Chemical structure of (a) zanamivir (PubChem CID: 60855), (b) laninamivir (PubChem CID: 502272), (c) oseltamivir carboxylate (PubChem CID: 449381), and (d) peramivir (PubChem CID:

154234), shown as ball-and-stick models and coloured in green (carbon), red (oxygen) and nitrogen (blue). For the sake of clarity, hydrogen atoms are not shown (colour figure online)

prevention of influenza [18]. NA inhibitors block the enzyme active site of NA, thereby preventing the release and spread of virions [19]. The Centres for Disease Control (CDC) recommends the use of three influenza antivirals, which include oseltamivir (Tamiflu), zanamivir (Relenza) and peramivir (Rapivab) [20]. Another antiviral, laninamivir (Inavir), has been approved for use in Japan since 2010 [21]. Zanamivir (Relenza) was the first neuraminidase inhibitor to be synthesized based on the transition state analog DANA (deoxy dehydro *N*-acetyl neuramic acid) and the crystal structure complex of neuraminidase and sialic acid. Relenza has a guanido group substituted at the C4-OH of DANA and suffers from the limitation of not being orally administrable. To overcome this limitation, oseltamivir (Tamiflu) was

designed [19, 22]. Tamiflu is orally administered as a pro-drug (oseltamivir phosphate), which is then converted to the active form (oseltamivir carboxylate) by the hepatic enzyme convertase. Peramivir and laninamivir were developed subsequently (Fig. 3) [19, 21].

Studies have shown that residues that are involved in the catalytic function of NA are conserved among all of the NA subtypes of influenza A and B viruses and thus serve as the major targets for antivirals. The residues (N2 numbering) that constitute the active site and have direct contact with inhibitors such as oseltamivir and zanamivir are Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371 and Tyr406. The geometry of the catalytic site is stabilized by residues Glu119, Arg156, Trp178, Ser179, Asp/Asn 198,

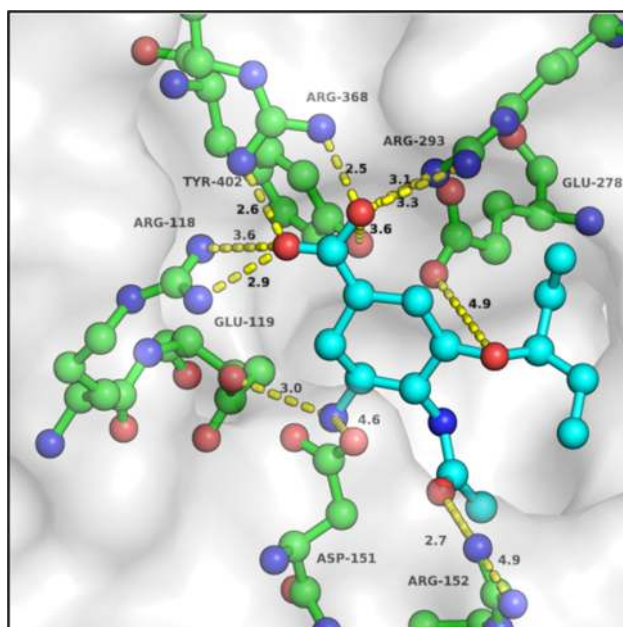


Fig. 4 Active site of influenza virus neuraminidase in complex with oseltamivir. The active site is represented in a surface view. Amino acid residues of the catalytic site (PDB:3TI6) that interact with the drug are shown as a ball-and-stick representation in green. The oseltamivir substrate is shown as a ball-and-stick representation in cyan. The H-bonds are represented by dotted lines, and the corresponding distances are labeled (colour figure online)

Ile222, Glu227, His274, Glu277, Asn 294 and Glu425 [23] (Fig. 4). Mutations in the catalytic site and framework residues can lead to drug resistance. According to a global update on the susceptibility of influenza virus to NA inhibitors during the 2013–2014 season, ~98 % of circulating viruses were found to be sensitive to all four influenza antivirals, and only 2 % showed resistance to at least one drug, mainly oseltamivir. A large cluster of NA-inhibitor-resistant influenza A(H1N1)pdm09 viruses with the H275Y substitution were detected in Japan and other countries such as China and the USA [24]. Antiviral surveillance reports from the National Institute of Infectious Diseases, Japan, during the 2013–2014 influenza season showed ~4.1 % resistance to both oseltamivir and zanamivir [25]. The number of resistant viruses is likely to increase in the future due to the increasing use of NA inhibitors in recent times. There is therefore an urgent need to develop newer drugs and vaccines that can impart long-lasting and broader range of protection against influenza.

NA antigenic domains

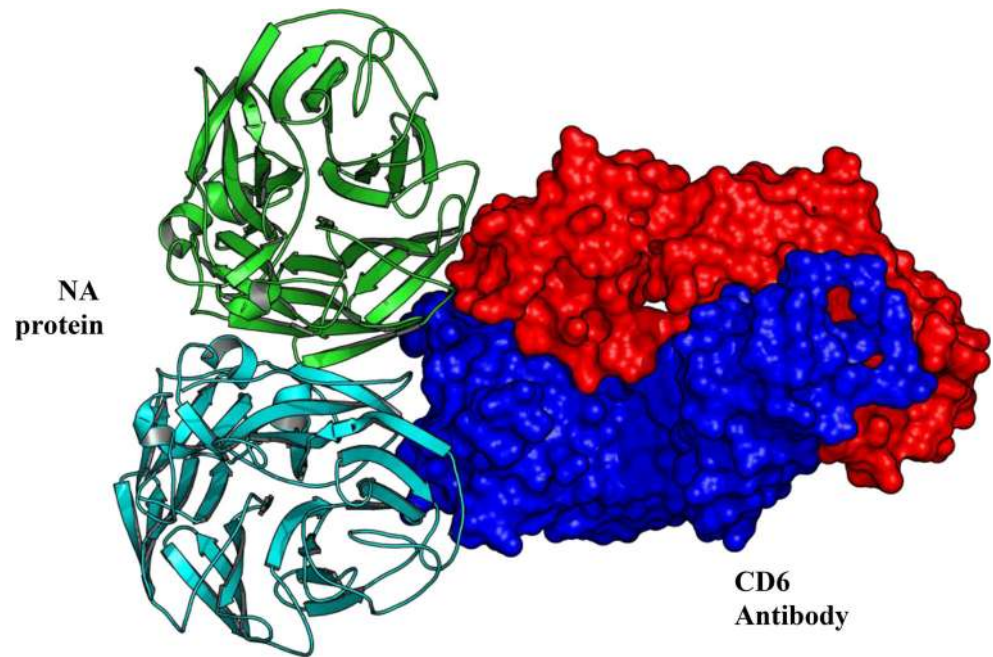
The crystal structures of four NA-specific antibodies have been determined to date [26–29]. The first crystal structure of NA antigen-antibody complex was of neuraminidase N9

and monoclonal antibody NC41 [29]. Antigenic epitopes of N9 were discontinuous and comprised 19 residues with large surface areas buried by the interaction. Subsequently, another antibody, N10, was synthesized with almost 80 % overlap of the binding site with NC41 [26]. In 2006, the crystal structure of N2 in complex with Mem5 was determined at a resolution of 2.1 Å and exhibited novel patterns of water-mediated H-bond interactions that stabilized the complex [27]. Residues at 198, 199, 220, and 221 contributing to the antigenic domain were found to be mutated in the viruses isolated after 1998, confirming that Mem5 binds to an epidemiologically important antigenic site [27, 30]. Recently, Wan et al. determined the crystal structure of antibody CD6 in complex with NA of influenza A(H1N1)pdm09 virus, revealing a unique antigenic epitope with the light-chain complementarity-determining regions (LCDRs) binding to one NA subunit, heavy-chain CDRs (HCDRs) 1 and 2 binding to the other subunit, and HCDR3 binding to both subunits. HCR1 interacts with Pro93, Val94, Ser95, Trp358, Trp375, Ser388, Ile389, Asn449, Ser450 and Asp451. HCR2 interacts with Asn355, Pro377 and Asn378 of the same subunit. HCR3 interacts with Asn449, Ser450, Asp451 of one subunit and Ile216, Lys262, Ile263, Val264, Lys265 and Ser266 of the other NA subunit. LCR interacts with residues Trp219, Arg220, Gln250, Ala251, Ser252, Lys254, Ser266, Val267, Glu268 and Asn270 (PDB:4QNP, Fig. 5) [28]. More crystal structures of NA antigen-antibody complex should be made available, as the information will be useful in designing NA-based vaccines.

Evolution of the neuraminidase gene

Variations in the membrane proteins (HA and NA) make it necessary to update vaccine strains frequently and pose challenges for the design of effective vaccines [12]. A study on the evolution of the hemagglutinin and neuraminidase genes in the Province of Quebec (Canada) during three influenza seasons (1997–2000) showed that amino acid substitutions in NA occur at a slower rate (0.45–1 %) than in HA (1–2 %) [31]. Sandbulte et al. showed that antigenic drift in NA is slower and dissimilar to the antigenic drift of HA among H1N1 and H3N2 seasonal influenza vaccine viruses [32]. A recent study on the complete genome sequences of influenza A(H3N2) viruses circulating between 1968 and 2011 has documented that amino acid substitutions in the HA1 subunit occurred at a maximum rate of 14.9×10^{-3} /site/year compared to 9.1×10^{-3} /site/year substitution rate in NA [33]. An antigenic analysis of crystallized antibody CD6 in complex with influenza A(H1N1)pdm09 virus NA revealed that the epitope is mostly conserved, even after several years of circulation,

Fig. 5 NA protein of influenza A(H1N1)pdm09 virus in complex with antibody CD6. The NA-CD6 model based on PDB ID: 4QNP is shown. NA is shown in cartoon and coloured in green (chain A) and cyan (chain B). Antibody heavy and light chains are shown as surface representations and are coloured in blue and red, respectively. The antibody binds to the lateral surface of two NA subunits [28] (colour figure online)



with mutations of some residues occurring at lower rates [28]. All of these studies emphasize that evolution of NA is slow-paced, which renders NA antibodies capable of providing long-term protection.

Role of NA antibodies

NA-specific antibodies may not be effective in preventing infection but do inhibit the spread of the virus, thereby reducing the severity of disease [34]. Epidemiological evidence suggests that the reduced impact of the 1968 Hong Kong flu (H3N2) virus could be attributed to the presence of pre-existing NA antibodies produced against the Asian flu (H2N2) virus [35]. Past studies have demonstrated no difference in the immunogenicity of HA and NA glycoproteins. Therefore, if a balanced formulation or supplementation of conventional trivalent vaccines with purified N1 and N2 is used, it will induce a more balanced response, avoiding the usual HA-skewed response that occurs towards the available vaccines [36, 37]. It has also been postulated that the antigenic competition between HA and NA results in greater B- and T-cell priming to HA antigen, presumably due to the larger number of HA proteins found on the virion surface and its amplified presentation to the antigen-recognition cells of the immune system [38]. Purified preparations of vaccines containing influenza A virus N2 neuraminidase have been proven to be immunogenic and non-toxic in humans [39]. Couch et al. showed that naturally occurring influenza NA-inhibiting (NI) antibody is an independent predictor of immunity, even in the presence of HA-inhibiting antibody [40].

The role of NA antibodies has been demonstrated by different vaccines, such as an affinity-chromatography-purified NA vaccine [41], plasmid DNA encoding NA [42, 43], a mammalian expression system [44], baculovirus-derived recombinant NA protein [45, 46] and virus-like particle (VLP)-based vaccines co-expressing NA with HA and/or M proteins [47, 48]. Several approaches have been used to estimate the antibodies against NA, such as the traditional thiobarbituric acid assay. However, the use of an enzyme-linked lectin assay (ELLA) is considered more practical and accurate, as the results are subtype specific and reproducible [49].

Heterologous protection

Numerous studies have demonstrated that vaccines containing NA protein provide both homologous and heterologous protection against influenza viruses. Wu et al. developed H5M2eN1 VLPs that induced high titers of NA antibodies and conferred homologous (H5N1 virus) and heterologous (H1N1virus) immunity [50]. Some studies have also shown that seasonal influenza vaccines can induce partial immunity against highly pathogenic H5N1 [51]. NA-VLPs expressing NA and M of H1N1 influenza virus conferred 100 % immunity against infection by homologous H1N1 virus as well as heterologous H3N2 virus [52]. Doyle et al. showed that monoclonal antibodies against a conserved epitope of NA (amino acid 222-230) inhibited all nine subtypes (N1-N9), providing evidence for consideration of NA in universal influenza vaccine development [53].

Current influenza vaccines

The CDC's Advisory Committee on Immunization Practices (ACIP) recommends the use of live attenuated influenza vaccine (LAIV), inactivated influenza vaccine (IIV) and recombinant influenza vaccine (RIV) to prevent influenza [54]. Six new influenza vaccines have been approved since 2012, which include 1) Fluarix, a quadrivalent IIV; 2) Flumist, a quadrivalent LAIV; 3) Flulaval, a quadrivalent IIV; 4) Fluzone, a quadrivalent IIV; 5) Flucelvax, a cell-culture-based trivalent IIV; 6) and Flublok, a trivalent recombinant HA vaccine [54]. These current vaccines are standardized mainly on the basis of the HA content [55], with varying amounts of other proteins, such as NA. The immune response to vaccination is estimated by measuring antibodies against HA using a hemagglutination inhibition assay (HAI), as these antibodies against HA are best characterized in terms of their ability to provide protection against the circulating strains of influenza virus [56]. WHO conducts meetings twice a year, in February and September, to recommend the vaccine strains for the influenza season in the Northern and Southern hemisphere, respectively [57]. During the 2014-2015 influenza season, virus activity was moderately high especially in the United States (late 2014 and beginning of 2015), due to the circulation of an antigenically distinct influenza A(H3N2) virus. Vaccination offered reduced protection, as the vaccine strain showed only 48 % similarity to the drifted strain [58]. HAI assay of drifted viruses with ferret antisera showed reduced inhibition due to amino acid changes in the HA protein, which led to changes in the WHO vaccine strain recommendations for the 2015-2016 season [59]. Such observations pointed at standardizing the amounts of NA along with HA protein in influenza vaccine formulations in order to compensate for the variations due to HA, to reduce and better contain the severity of influenza epidemics.

A study was conducted by Couch et al. to evaluate the anti-HA and anti-NA responses to six trivalent inactivated vaccines (TIVs). The findings revealed that all TIVs with similar HA content induced similar levels of HA antibodies in healthy adults, but the vaccines did not induce similar NA responses, since they contained varying amounts of NA. The levels of NA antibodies induced by LAIVs were lower than those induced by TIV [60]. The stability of neuraminidase in inactivated influenza vaccine showed that NA immunogenicity is consistent throughout the shelf life of the vaccines [61]. Due to the lack of appropriate analytical methods, guidelines for the standardization of NA have not been developed [62]. Recent studies have demonstrated that mass spectrometric techniques such as LC-MS and LC/MS/MS can be used to quantify HA and NA antigens in various vaccine preparations simultaneously [63, 64].

Potential of NA in influenza antiviral and vaccine development

The NA protein has been a well-established and important target for the treatment of influenza, but approved NA vaccines are not a reality yet. All the above-mentioned studies suggest that NA is a potential target for future influenza vaccines, but this does not mean that current vaccines should be replaced with NA vaccines. NA antibodies are infection permissive and only reduce disease severity, whereas HA antibodies prevent infection [36]. Thus, vaccines containing standardized amounts of both HA and NA glycoproteins are needed to provide complete protection against influenza. Studies have demonstrated that the HA:NA ratios are different for different subtypes, and also different strains within a subtype [62]. Hence, the strain-specific NA content and HA:NA ratio in such vaccines need to be standardized. At present there is no approved analytical method to standardize the NA content in vaccines. The best approach could be alternative vaccines that induce NA antibody production in sufficient amounts, such as VLP-based or recombinant vaccines. VLP-based vaccines stimulate T and B cells and induce better immune responses to both HA and NA antigens [65–67]. Such vaccines are immunologically similar to live vaccines but are safer because there is no nasal shedding or reversion to a virulent form [68]. Flublok, a recombinant HA vaccine, was approved by the FDA in the year 2013, which draws our attention to the point that if similar vaccines containing NA are approved, they might induce consistent amounts of antibodies against NA [69, 70]. For a broader spectrum of immunity, a combination of both vaccines may be the best choice. However, a question that still remains unanswered: will the use of these types of vaccines impose further selection pressure on influenza viruses?

Conclusion

There is evidence that NA is immunogenic and that NA antibodies provide a heterologous and broader-range immunity. Studies have shown that antigenic variation occurs at a slower rate in NA than in HA, and thus, NA antibodies can induce long-term immunity. Decades of research on NA antibodies, especially on their role in immunity and detection methods, will facilitate the development of new vaccines that can be evaluated for both HA and NA content and potency. Such vaccines may emerge as universal vaccines, offering protection against newer influenza viruses.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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