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SUPPLEMENT ARTICLE

Neuraminidase, the Forgotten Surface Antigen, Emerges as an Influenza Vaccine Target for Broadened Protection

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For 50 years it has been known that antibodies to neuraminidase (NA) protect against infection during seasonal and pandemic influenza outbreaks. However, NA is largely ignored in the formulation and standardization of our current influenza vaccines. There are a number of factors that contributed to this antigen being forgotten, including the lack of an easily performed test to measure NA antibody. With the availability of that test, it has been possible to show its independent contribution to protection in various situations. The challenge now is to make it possible to include known amounts of NA in investigational vaccines or to routinely measure NA content in licensed vaccines. Vaccines containing optimal amounts of NA may be particularly useful when there are antigenic changes, either drift or shift, in the hemagglutinin because NA immunity offers broad protection. It is now time to remember the NA as we work toward improved influenza vaccines.

Keywords. influenza; neuraminidase; vaccine; antibody.

HISTORICAL PERSPECTIVE ON NEURAMINIDASE AS A VACCINE ANTIGEN

Early studies of influenza recognized an activity that destroyed cellular receptors, thereby eluting virus from red blood cells [1]. Sixty years ago, the activity was identified as an enzyme and named neuraminidase (NA) because of its ability to release *N*-acetyl neuraminic acid from erythrocytes and mucins [2]. It could be separated from other viral proteins after detergent disruption [3], facilitating studies of its biochemical activity that provided a foundation for understanding the role of NA in the virus life cycle. It was later identified to be antigenic, eliciting specific antibodies.

In the 1960s, NA was shown to be distinct from the hemagglutinin (HA) and to evolve independently. This was clearly documented in the 1968 Hong Kong influenza A(H3N2) pandemic that involved a shift in the HA but not the NA which remained similar to that of previous influenza A(H2N2) viruses [4]. The contribution of the NA to broadened protection during the pandemic was confirmed in a contemporary serologic study, which showed that individuals with higher N2 titers were less likely to be infected [5, 6]. Why then has the contribution of the NA to broad protection received only intermittent attention during the subsequent years? The assay to quantify functional antibody affecting enzymatic activity was difficult to perform safely and reproducibly, and had limited throughput, so immunogenicity studies usually did not evaluate antibody responses to NA. In addition, the amount of NA in various viruses varied, was not easily measured, and was often unstable, resulting in reports of NA immunogenicity that were often in conflict. As a result, it was concluded in 1998 that it was unwise for licensed influenza vaccines to have specifications for NA content [7].

In many ways, it was also the strong role of HA in driving protection, as long as the vaccine virus was closely matched to the circulating strain, that led to ignoring the possible contribution of other factors in immunity. That role was demonstrated in the first trials of influenza vaccine in 1943 and continued to monopolize thinking even in the regulatory community where new vaccines could be licensed in Europe simply on demonstration of production of HA antibodies. An exception to this was the work of Kilbourne and coinvestigators who doggedly pursued an understanding of NA as a potential vaccine antigen [8–12]. The basic concept was that antibodies against NA would not prevent infection, providing "permissive" immunity that would allow asymptomatic infection and production of a better response to the infecting virus than would vaccination with an inactivated, split virus that induces HA-specific antibodies [13].

The NA vaccine development program recognized that recall responses to HA would easily predominate following exposure to a seasonal influenza virus, providing little opportunity to establish a robust response against NA. For this reason, clinical studies were performed with vaccines comprised of reassortants with an "irrelevant" HA, usually from an equine virus [13]. Later, purified NA was used [11]. A fundamental flaw in these

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clinical studies was that the end points to support the permissive infection approach (eg, prevention of symptoms following exposure), were not distinct from measures of vaccine failure. Some animal studies were performed by the group to examine the benefit of supplementing a standard inactivated vaccine with purified NA [9, 14], presaging some of the approaches currently being considered to generate long-lasting, broadened antibody responses. In the course of these studies, it was demonstrated that vaccination with purified NA was safe and produced 4-fold seroconversion at doses ≥7.7 μg in healthy adults [11].

BREADTH OF NA IMMUNITY

Separate studies of the antigenic drift of NA demonstrate antigenic changes in HA and NA are independent [15, 16]. NA immunity is therefore highly likely to provide a level of protection when drift in HA occurs. An extreme example of this phenomenon was the 1968 pandemic where antibodies to the prior H2N2 viruses contributed to protection against H3N2 infection.

Antigenic drift of HA is usually observed as low reactivity of a reference ferret antiserum against a circulating strain as well as the reciprocal, that is ferret antiserum raised against the circulating virus inhibits the reference virus poorly. In contrast, studies of antigenic change in NA showcase a phenomenon of "one-way" drift [16], with antiserum against one virus having reduced inhibition of NA activity, but the reciprocal continuing to inhibit both viruses well. The asymmetry in loss of antibody reactivity when there are antigenic changes coincides with an observation of NA providing broad protection against heterologous viruses within the same subtype, suggesting some NA epitopes are relatively conserved. The idea of NA offering broad protection is not new, having been observed in early studies of immunity induced by NA of the N2 subtype [12, 13, 17] and more recent studies of N1 subtype [18, 19]. It is likely that antibodies specific for conserved epitopes in "seasonal" H1N1 viruses that preceded the 2009 pandemic contributed to the relatively low incidence of influenza in the elderly [20].

The molecular basis for "one-way" drift and heterologous protection became evident when epitope mapping identified a number of conserved epitopes on NA that are recognized by mouse and human monoclonal antibodies [21–23]. The location of these epitopes on the lateral edge of each monomer, and in one case an epitope that spans neighboring monomers, explains why the antibodies inhibit enzyme activity to some degree, but usually do not inhibit cleavage of small substrates. Monoclonal antibodies that bind conserved epitopes often need higher concentrations to inhibit enzyme or formation of plaques; consequently, higher antibody amounts are needed to prevent disease in mice than strain-specific antibodies, which usually inhibit enzyme activity at low concentrations [22–26].

An unusual monoclonal antibody that binds all influenza A NA subtypes is specific for a conserved linear epitope [27]. Compared to other NA-specific monoclonal antibodies, it requires much more antibody to inhibit NA both in vitro and in vivo. Unpublished studies with monoclonal antibodies that inhibit NA poorly in vitro show these antibodies are protective in immunologically competent mice, but not in mice deficient in complement (C) or innate immune mechanisms, suggesting that C or Fc receptor (FcR)-mediated cellular cytotoxicity mechanisms may afford some protection against severe disease. The mechanism of antibody action, that is whether FcR-mediated or direct inhibition of enzyme activity, antibody avidity, and fine specificity of NA-binding antibodies are likely to determine which antibodies are most effective in reducing virus replication.

Would antibodies against N1 be sufficient to protect individuals against severe H5N1 infection, should it become pandemic? Mouse and ferret studies show that there is indeed protection against lethal heterologous virus challenge; however, NA-specific antibody-mediated protection against challenge with a wild-type highly pathogenic H5N1 virus is not as complete as protection against attenuated strains [22, 28] and in some cases protection is not observed [29]. Monoclonal antibodies that bind to strain-specific epitopes are usually more effective in vivo than those binding conserved epitopes [30], suggesting that, like HA, inclusion of an NA that is antigenically similar to that of the circulating virus would provide greatest protection. While a homologous antigen may be best, the protection afforded by a heterologous vaccine at the beginning of a pandemic is likely better than no immunity and could prime the response to an antigenically matched vaccine when it becomes available. Further studies to identify NA-specific antibody titers that afford heterologous protection are needed as these will help determine the dose to target in NA-containing vaccines claiming induction of universal protection.

HOW ARE VACCINE NA CONTENT AND RESPONSES TO NA MEASURED?

NA immunogenicity is determined by the amount of protein in its native, tetrameric form [31]. This form is active and therefore the potency of NA in monovalent vaccines could be measured by enzyme units. However, this is not suitable for measuring NA content of seasonal vaccines that contain multiple NA subtypes and therefore capture enzyme-linked immunosorbent assays (ELISAs) have been developed using subtype-specific monoclonal antibodies to evaluate NA content. The amount of NA in seasonal influenza vaccines measured by the capture ELISA assay corresponds well with its immunogenicity [32, 33], suggesting that this type of assay can be used for routine determination of NA potency in trivalent and quadrivalent influenza vaccines.

To ensure the most effective NA-specific antibodies are induced following vaccination, it is important to measure responses in assays that reflect their functional properties.

Antibodies that inhibit NA activity were originally quantified by measuring reduced amounts of sialic acid released from fetuin after incubation with serum/virus mixtures [12]. The assay is cumbersome to perform, requires hazardous chemicals, and, while it has been miniaturized to improve throughput [34], is not practical for routine use in large serology studies. Lambré et al [35] developed an alternative method that quantified the amount of galactose, the penultimate sugar of glycoconjugates, that became exposed after digestion of fetuin bound to an ELISA plate. This assay improved throughput immensely, using the specificity of peanut agglutinin (PNA) for galactose as the basis for quantification by incubation with PNA-peroxidase conjugate followed by addition of a chromogenic peroxidase substrate. This enzyme-linked lectin assay (ELLA) has been optimized for routine serology [36] and used in many studies to measure neuraminidase inhibition (NI) titers following seasonal influenza vaccination [37–40].

Whole virions are generally used in ELLA as the source of antigen, providing stable NA activity that is in the "natural" form and orientation. A caveat of all assays that measure NI titers with whole influenza virus is that antibodies that bind HA can nonspecifically inhibit NA activity by steric hindrance of the enzyme's active site or limiting access of NA to its sialylated substrate [41]. For this reason, reassortant viruses that express a novel HA subtype are generally used as antigen. These can be constructed by reverse genetics to include an HA from an avian influenza virus [34]. Unfortunately, this cannot be done in all laboratories because additional safety requirements often need to be met to comply with regulations imposed by Departments of Agriculture and Health and Human Services. Alternate sources of antigen are therefore needed. Some investigators have used recombinant NA [42], virus-like particles [43], pseudotyped viruses [44], or detergent-disrupted wild-type virions [37] in ELLA, but additional studies are needed to define the stability of these antigens and comparability of results.

ELISA can be a suitable alternative to ELLA [45] and is advantageous because it allows large numbers of sera to be analyzed. To obtain antibody titers that are likely to correspond with titers measured in a functional assay, it is imperative to use the native form of NA as antigen in ELISAs. Whole virus can be used to coat the plates as NA in this form is usually stable. However, because most sera to be tested will contain HA and nucleoprotein antibodies (either resulting from vaccination or prior natural infection), antibody titers measured in ELISAs with whole virus are usually reported as virus, but not NA, specific. Given the advances in expressing multimeric proteins, recombinant NA is used more commonly to measure antibodies that are NA specific. A cell-based ELISA in which NA is transiently expressed offers an alternative approach to laboratories unable to obtain recombinant NA for serologic studies [22].

APPROACHES TO CLINICAL TESTING OF NA-CONTAINING VACCINES

The NA dose needed to induce a protective antibody titer has not been established. Titers associated with protection against challenge with viruses containing the homologous and heterologous NAs need to be identified for each influenza NA type and subtype. Until this is achieved, it will not be possible to determine the optimal amount of NA to be incorporated into developmental vaccines.

Careful planning of clinical experimental and observational studies to demonstrate the benefit of NA immunity is key to developing NA-based vaccines. The benefits of NA vaccination are easily observed in animal models where all components can be controlled. Clinical studies will need to use a variety of end points to evaluate the benefits of NA immunity. For example, peak virus titer, duration of infection, symptom severity, transmission to uninfected subjects, and induction of HA-specific antibodies may be primary measures. Initial human challenge studies would be very useful because they could provide important information regarding signs of disease to better design observational studies. While design and execution of observational studies to demonstrate effectiveness of NA-based vaccines is a daunting task, they are essential to draw generalizable conclusions.

Given the difficulties associated with demonstrating efficacy of an NA-only vaccine, the benefit of NA immunity is more likely to be realized by including consistent, immunogenic amounts of NA in HA-containing seasonal or pandemic influenza vaccines, with the goal of increasing vaccine efficacy or the breadth of immunity. NA-inhibiting antibodies in the upper and lower respiratory tracts are likely to be the most effective in limiting virus infection and spread. Therefore the ideal NA-containing vaccines may be those administered intranasally. However, parenteral administration clearly induces antibodies and such approaches may be more practical. Studies that carefully measure NA-inhibiting antibodies at the mucosa and in serum are urgently needed to identify titers indicative of protection. Establishing the mucosal and serum NA inhibition titers that are surrogates of vaccine efficacy is an essential step towards interpretation of increases in NA-inhibition antibody titers.

FORMATS OF NA-CONTAINING VACCINES

Depending on the goals, NA-based vaccines could be implemented in several forms as shown in Table 1. One goal may be to use NA in pandemic vaccines that aim to protect against severe disease. A universal-pandemic vaccine comprised of NA alone would contain a cocktail of N1 (prior seasonal, pdm09, zoonotic N1), N2 (current and zoonotic), N6, N7, N8, N9, and any other NA subtype associated with emerging zoonotic strains. The goal of such a vaccine would be to induce high NI antibody titers, with antibodies against conserved epitopes

Table 1. Potential Vaccine Types With Defined NA Content

Vaccine Type	Antigens	Dose/Goal	Clinical Outcome
Universal NA only	NA of various influenza types and subtypes	Enough NA to induce NI titers sufficient for heterologous protection	Permissive immunity; protection against severe disease
Universal multiple antigens	NA of various influenza types and subtypes and HA (head and/or stem), NA, NP, M2e, M	As described for universal NA only vaccines	As described for universal NA only vaccines
Seasonal high dose	All antigens in split or subunit vaccines	Vaccine dose based on amount of NA needed for homologous protection	Infection prevented by HA-specific antibodies; protection against clinical disease mediated by NA-specific antibodies in years of HA drift
Seasonal NA supplemented	Purified or rNA added at defined dose to established vaccine	Standard vaccine with NA added at amount needed for homologous protection	As described for seasonal high dose vaccines

Abbreviations: HA, hemagglutinin; NA, neuraminidase; NI, neuraminidase inhibition; rNA, recombinant, neuraminidase.

restricting release of virions from infected cells and causing virions to aggregate. This is likely to reduce virus titers and aerosol transmission, thereby limiting virus spread during an unexpected pandemic. Universal vaccine candidates that contain NA in addition to other antigens such as HA stem and/or M2e to induce antibodies against other conserved epitopes and T cells specific for M or NP epitopes, would similarly be designed to induce NI antibodies that limit virus spread and transmission. It is anticipated that these vaccines would be effective if administered either prior to or very soon after a pandemic is declared, with a vaccine that contains the homologous HA and NA administered as soon as it is available.

Alternatively, an NA-based vaccine may be designed to prevent symptomatic disease or even transmission of seasonal influenza. The breadth of protection against seasonal viruses could be improved by including NA that is antigenically matched to viruses in circulation at a dose that is shown to induce a protective response—this could be achieved by setting homologous NA content as the threshold, thereby increasing the overall HA content, or by supplementing a vaccine with purified or recombinant NA. Alternatively, vectors that express NA can be used as stand-alone immunogens or in combination with other antigens or established vaccines.

WHAT IS NEEDED TO FURTHER THE DEVELOPMENT OF NA-BASED VACCINES?

There is considerable interest in addressing gaps in knowledge to support development of NA-based vaccines [46] and the work already begun needs to be accelerated. Recent studies have shown the independent protection against infection of NA antibodies following natural infection [47] and vaccination [38]. These investigations used natural infection as the outcome. The human challenge design has also demonstrated the value of such a method to efficiently define the critical contribution of NA protection [48] and cohort studies have allowed the evaluation of the role of NA antibody in protection from different A subtypes in various age groups [49]. It is not clear what immune mechanisms contribute to protection by NA-specific immunity. Animal and human studies to examine the relative contribution of antibodies that inhibit enzyme activity and those that mediate protection through C or FcR-mediated mechanisms would provide important information toward development of an NA-based vaccine.

In addition to examining the benefits of NA immunity, there is continued need to understand the antigen more fully—antigenic mapping studies will provide information to support NA vaccine design, and biochemical and structural studies to support the production of stable, immunogenic forms of NA will undoubtedly be of value. Much of our current information regarding NA structure and immunogenicity is based on studies with NAs of circulating A(H1N1) and A(H3N2) viruses, and while this work should continue, data are needed to determine similarities and differences in immunogenicity of NAs, including those of influenza B viruses and zoonotic strains.

Antigenic drift of the NA needs to be followed as carefully as drift in the HA. The most effective seasonal and pandemic vaccines, whether NA-based or not, benefit from containing antigens that are antigenically similar to the circulating viruses. Support for antigenic studies of NA, especially of NA drift, should therefore be a priority, and the antigenic analysis of both HA and NA considered when viruses are recommended for seasonal and pandemic vaccine production.

There are a number of questions that need to be addressed before vaccines containing a predetermined amount of NA are possible, including that of the added value of ensuring an antibody response to the NA. As with most questions involving influenza, the answers may be different in different situations, based on drift or other changes in the vaccine HA and the history of the individual recipient in terms of past infection or immunization. While much work remains to fully understand the contribution of NA immunity to vaccine effectiveness, the tools are currently available to move forward with NA-containing vaccines. We can now make sure that the once forgotten antigen is a part of developments leading to vaccines of broadened and heightened protection.

Notes

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