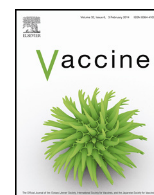
Vaccine 32 (2014) 2225–2230

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Stability of neuraminidase in inactivated influenza vaccines

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ARTICLE INFO

Article history:
Received 8 October 2013
Received in revised form 4 January 2014
Accepted 28 January 2014
Available online 6 March 2014

Keywords:
Influenza
Vaccines
Neuraminidase
Stability
Immunogenicity

ABSTRACT

Influenza vaccines are effective in protecting against illness and death caused by this seasonal pathogen. Antibodies that block the function of either hemagglutinin (HA) or neuraminidase (NA) contribute to vaccine efficacy, however vaccine potency is based only on HA content. NA protein content in vaccines varies from season to season due to differences in the relative amounts of HA and NA in influenza A, H1N1 and H3N2, and influenza B viruses that are selected for each manufacturing campaign. This, as well as potential inherent differences in NA immunogenicity, may result in varying responses from year to year. Moreover, the antigenic stability of NA is likely to dictate whether similar antibody responses will be obtained to this antigen throughout the shelf-life of the vaccine. To address this factor, we subjected NAs of influenza A (subtypes N1 and N2) and B viruses to denaturing conditions to evaluate the stability of enzyme activity. Each NA type/subtype had unique sensitivity to denaturing conditions. The N2 enzyme activity was more thermostable than that of N1 or influenza B, while the NA activity of influenza B was most resistant to detergent. N1 enzyme activity was most resistant of the three NAs to freeze–thaw cycling. In these experiments, enzyme activity was indicative of the immunogenicity of NA, but was strain-dependent, with greater neuraminidase inhibiting (NI) antibody titers elicited following immunization with the 2009 H1N1 pandemic virus A/California/7/2009, than the previously circulating seasonal H1N1 strain, A/Brisbane/59/2007. Robust NI antibody titers against both N1 and N2 components were induced following vaccination of mice with a trivalent inactivated influenza vaccine. When stored under recommended conditions, the NA of both N1 and N2 subtypes remained immunogenic well after the vaccine expiry date.

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1. Introduction

There are several types of influenza vaccines licensed in the U.S. to date – a live attenuated quadrivalent vaccine; several inactivated split or subunit vaccines produced in eggs formulated to contain either three (trivalent) or four (quadrivalent) influenza viruses; an inactivated split trivalent vaccine produced in mammalian cells (Flucelvax), and a recombinant hemagglutinin (HA) influenza vaccine produced in insect cells (Flublok). The dose of each of these vaccines is standardized by the amount of HA. Except for Flublok, all vaccines contain a number of additional influenza proteins, including neuraminidase (NA), the second most abundant glycoprotein on the surface of the influenza virion. Antibody responses to HA and NA are independent correlates of immunity [1] and therefore the amount of NA present with capacity to elicit NA inhibiting (NI) antibodies could impact vaccine efficacy.

While immunogenic amounts of NA are present in each of the inactivated influenza vaccines [2], the amounts cannot be standardized because there are different relative proportions of HA and NA for each constituent virus in a given vaccine preparation [3]. For example, since each vaccine dose aims to contain 15 μg of HA, the amount of NA per dose would be ~7.5 μg for a virus with an HA:NA ratio of 2:1 but only 1.5 μg for a virus with an HA:NA ratio of 10:1. Clinical studies have demonstrated that 2.6 μg of purified NA is immunogenic in the majority of healthy young adults [4], and high dose vaccines increase responses to NA in the elderly [5]. Enzyme activity is a practical way to estimate the relative amount of immunogenic NA protein in monovalent vaccines or vaccine intermediates, because an intact native tetrameric structure is needed for NA activity and is also optimal for immunogenicity [6]. Since this measure is not specific for any particular NA subtype, it cannot be used to estimate the amount of the immunogenic form of each separate NA subtype in a formulated trivalent or

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.vaccine.2014.01.078

quadrivalent vaccine. Moreover, the absence of enzyme activity does not necessarily infer a lack of NA immunogenicity since native protein conformation is sufficient to induce NA inhibiting antibodies [7]. An assay that measures the quantity of NA with native conformation would be extremely helpful to evaluate the relative stability of the antigenic form of N1, N2 and influenza B NA components in multivalent vaccine formulations. Since such a test is not available, we have used both enzyme activity and immunogenicity in an animal model to examine the stability of the antigenic form of NA in split inactivated influenza preparations. A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008 monovalent bulks (MVBs), as well as the formulated trivalent influenza vaccine (TIV) containing these components, were used in this study.

2. Methods

2.1. Virus and vaccine samples

The following vaccines were used in this study: vaccine intermediates (monovalent and trivalent bulks) produced for the 2011/12 influenza season consisting of the high growth variants of the following recommended viruses: H1N1: A/California/7/2009 (CA/09), H3N2: A/Victoria/210/2009 (VIC/09), and B/Brisbane/60/2008 (B/BR/08); a monovalent 2009 H1N1 pandemic vaccine; and expired inactivated, split trivalent influenza vaccines produced for the 2008/09 influenza season. The samples were stored at 4 °C. Heat-treated samples were incubated for various times at room temperature (25 °C), or in a water bath at 37 or 50 °C. Freeze-thawing was performed by rapid freezing on dry ice followed by thawing in a 37 °C water bath. H6N1 and H6N2 viruses used in this study to measure NA inhibition titers were generated as described previously [7].

2.2. Neuraminidase activity

The activity of NA was measured using 4-methylumbelliferyl-N-acetylneuraminic acid (MU NANA, Sigma, St Louis, MO) as substrate. Briefly, serial 2-fold dilutions of sample (50 μl) were mixed with PBS in triplicate wells of a black 96-well plate, and an equal volume of 20 μM MU-NANA added. The plate was incubated at 37 °C for 1 h, and then the reaction was stopped by addition of 100 μl of 0.1 M glycine, pH 10.7, 20% EtOH. The fluorescence was measured on a Victor V plate reader (Perkin Elmer, Shelton, CT), with excitation at 355 nm and emission at 460 nm. Assay variability was ≤10%. The NA of *Vibrio cholerae* (Sigma) was used as a positive control or to generate a reference standard curve in each assay. Specific activity was defined as the activity (units) per mg protein. In many assays the NA activity of sample post-treatment was compared to the activity of the original untreated sample by determining the percent relative fluorescence units at the equivalent sample dilution.

2.3. Determination of protein concentration

Total protein concentration was measured using a BCA assay kit (ThermoScientific, Rockford, IL). A published label-free MS^E method was used to quantify total amounts of HA and NA [3]. The concentration of NA in virus preparations used for immunogenicity studies was determined by isotope dilution mass spectrometry following a published method [8,9]. A cocktail of native and isotope labeled peptides used to generate a calibration curve for this analysis was kindly provided by Dr Tracie Williams (National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA).

2.4. Immunogenicity of NA in mice

BALB/c mice (6–8 week old, Jackson Laboratories, Bar Harbor, MA) were housed following federal regulations, with procedures approved by the institutional Animal Care and Use Committee. Each group ($n = 5$) was immunized intramuscularly (IM) with 50 μl of antigen. In some experiments this contained a 1:1 mixture of antigen and adjuvant (Titermax, Sigma). Mice were bled 3 weeks after vaccination from the tail vein, and the serum stored at −30 °C until serologic assays were conducted.

2.5. Neuraminidase inhibition assay

Neuraminidase inhibition (NI) titers were determined using an enzyme-linked lectin assay [10]. Briefly, H6N1 and H6N2 viruses containing the NA of the targeted antigen were titrated to determine the least amount of virus eliciting maximum signal. Serial dilutions of serum samples were mixed with an equal volume of virus and transferred to 96 well plates coated with fetuin. The plates were incubated for 16–24 h at 37 °C, and then washed with PBS–0.05% Tween 20 before incubating for 2 h at room temperature with peanut agglutinin (PNA) conjugated to peroxidase (Sigma). The plates were washed prior to addition of substrate, O-phenylenediamine dihydrochloride (Sigma). After 10 min the reaction was stopped by adding 1 N H_2SO_4 and absorbance read at 490 nm. All reagents used in this assay were purchased from Sigma. The inverse of the last dilution that resulted in ≥50% inhibition of NA activity was defined as the NI titer.

3. Results

3.1. NA content of vaccine intermediates and final product

To compare the stability of NA of different virus types and subtypes in inactivated vaccines, we measured the enzyme activity of split, inactivated CA/09 (H1N1), VIC/09 (H3N2), and B/BR/08 viruses contained in monovalent bulks (MVBs), and the overall NA activity in formulated trivalent inactivated vaccine (TIV) bulk and in syringes filled with TIV. Enzyme activity and HA and NA concentrations were measured in three lots of each MVB and in three lots of trivalent formulated bulk (Table 1). The NA activities of lots for the same strain were very similar, but were significantly different between H1N1, H3N2 and B preparations, reflecting the amounts of NA measured by label-free MS^E and small differences in NA specific activity. Comparison of the protein concentration and enzyme activity of monovalent bulk and trivalent material suggests that approximately 20-fold dilution of each MVB was used to formulate the vaccine.

3.2. The stability of NA is strain dependent

One lot of each monovalent and trivalent bulk was selected to evaluate the stability of NA when subjected to increasing temperature, freeze–thaw or detergent treatments. The enzyme activity was measured after 0.5, 2, 7 and 24 h incubation at 4, 25, 37 and 50 °C (Fig. 1A–D). It was stable at 4 and 25 °C for 24 h for all samples. At 37 °C, the NA activity was retained for at least 7 h in the monovalent bulks, but by 24 h the H1N1 monovalent bulk had only ∼50% of it original activity. A greater proportion of activity was retained in the H3N2 monovalent bulks (∼75% of initial activity). Interestingly, the trivalent formulated vaccine lost almost 50% of its activity at 37 °C after only 30 min and very low amounts of NA activity were measured after 24 h at this temperature (Fig. 1D). Since MVBs are diluted to formulate the trivalent product, the total protein concentration of TIV is less than the MVB. The greater loss of activity in the TIV suggests that the reduction in total protein

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