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Influenza virus-like particles as a new tool for vaccine immunogenicity testing: Validation of a neuraminidase neutralizing antibody assay

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ABSTRACT

Detection of neutralizing antibody to viral neuraminidase (NA) by testing for enzyme inhibition has been recognized as an important part of the immunogenicity of influenza vaccines. However, the absence of a well characterized standard source of active NA and validated assays has significantly limited clinical studies of NA immunity. Influenza virus-like particles (VLPs) containing hemagglutinin (HA), NA, and M1 proteins were produced from insect cells infected with a recombinant baculovirus and used as the NA source for the NA inhibition (NAI) assay. The NA activity of 6 different VLP strains varied from 0.43 to 1.61 ($\times10^{-3}$) enzyme units per μg of HA and was stable over 6 months of storage at 2–8 °C. The NAI assay using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid as a substrate was modified for testing the antibody titer in clinical samples and validated. The advantages of the assay include: (1) stable, reproducible, and standardized source of NA; (2) testing the antibody titer specific to each subtype of NA in serum from subjects immunized with trivalent vaccines (H1N1, H3N2, B) with no interference from antibodies specific to the HA and to heterologous subtypes of the NA; (3) suitability for conducting long-term clinical trials as a result of low intra- and inter-assay variability, and (4) a wide analytical range due to 25% inhibition cut-off value for the NAI titer estimation.

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1. Introduction

The immunogenicity of influenza vaccines is evaluated historically by measuring antibody (Ab) titer to hemagglutinin (HA), the major virus surface glycoprotein. Antibody to enzymatically active neuraminidase (NA), the other major surface glycoprotein, essentially enhances protective immunity induced by influenza vaccines (Sylte and Suarez, 2010; Sylte et al., 2007). NA removes sialic acid from both viral and host proteins and participates in the release of viruses from infected cells. Only functional antibodies which inhibit the enzyme are protective and provide so-called permissive immunity: they do not prevent viral infection by themselves but significantly reduce virus spreading throughout the body and the severity of disease (Johansson et al., 1989; Sylte and Suarez, 2010).

Although the concept of an improved influenza vaccine with both the HA and active NA proteins present together has been discussed (Sylte and Suarez, 2010), no commercial vaccine with controlled NA activity is produced today. The lack of control for NA activity (NAA) in influenza vaccines could be explained by a significant loss of enzyme activity during storage (Kendal et al., 1980). The problem with NA instability has also limited testing of anti-NA immune response during clinical studies.

The principle of testing NA inhibition (NAI) by antibody includes the comparison of NA activity in samples incubated without and with NA-specific immune serum (Cate et al., 2010). There are several well-established methods for testing the NAA in viruses and these methods are used for monitoring enzyme activity in vaccines during manufacturing (Kalbfuss et al., 2008) or screening mutants resistant to chemical NA inhibitors (Gubareva et al., 2002; Wetherall et al., 2003). For these applications the highly variable NA activity in virus samples is expected and the NAA level represents the object of analysis. In contrast, the neutralizing antibody assay requires a stable and reproducible source of active NA with a specified absolute activity (enzyme units/ml) and storage life as for any other analytical enzymatic reagent. Thus, existing publications discussing NA immunity are able to present scientific proof of concept for NA neutralizing antibody but do not provide comparable results for antibody titers and the activity of the used source of NA.

Using live influenza viruses for analytical purposes remains very complicated due to their pathogenicity (especially for pandemic

Abbreviations: NA, neuraminidase; NAA, neuraminidase activity; NAI, neuraminidase inhibition; NIT, neuraminidase inhibition titer; VLP, virus-like particle.

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virus strains), laborious and expensive preparation, and lack of standardization. Therefore, an inactivated virus vaccine (Cate et al., 2010) and a purified NA protein (Johansson et al., 1989) were proposed as a more convenient source of neuraminidase. However, the conditions required for adequate measurement of NAI using these substitutes of live virus, have not been established. For inactivated vaccine preparations which most often have a very low level of NA activity (Kendal et al., 1980), and a high contribution of unfolded (denatured) proteins (Feng et al., 2009), the requirements for the NA qualification may include the enzyme stabilization for long-term storage and setup of the range providing NAA results relevant to those in live viruses. The active H1N1 and H5N1 neuraminidases can now be purchased from Sino-Biological (Beijing, China) and RnD Systems (Minneapolis, USA). However, these preparations may contain unfolded NA molecules or reduced associated forms (dimeric or monomeric) of NA instead of a tetrameric complex present in virus (Sylte and Suarez, 2010) and their binding properties to antibodies could thus be altered compared to live virus.

Recent progress achieved in creating influenza virus-like particles (VLPs) that contain the HA and NA viral proteins with relevant functional activity and immunoreactivity makes VLP an excellent candidate to substitute for live viruses (Kang et al., 2009; Pushko et al., 2005; Lai et al., 2010). The unique combination of comparable structural and biological properties to influenza viruses, enhanced stability and ease of handling gives VLPs a big advantage over other discussed virus substitutes. This allows for expanding VLP applications outside of vaccine development into the study of different areas of virus properties (Kang et al., 2009; Lai et al., 2010). Unfortunately, NA in VLPs has not been fully characterized: only a few publications have reported detectable NA activity without proper quantification in absolute units (Lai et al., 2010). Furthermore, it has not been demonstrated that the activity and stability of NA in VLPs are sufficient for analytical applications.

To test NA activity, the fluorogenic 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (MUNANA) substrate was chosen. This is the only substrate which gives comparable results for NA activity from multiple sources as the assay is based on quantification of the enzymatic reaction product (Potier et al., 1979; Wetherall et al., 2003; Kalbfuss et al., 2008). To adjust the MUNANA-based NAA assay for testing NAI titer, we need to determine (1) the acceptable range of initial NA activity in VLP samples that would provide consistent results for a given antibody titer; (2) whether VLP could be applied for testing the antibody titer specific to only one subtype of NA in the serum from subjects immunized with a trivalent vaccine with no interference from the antibody specific to HA and heterologous subtypes of NA; and (3) how the assay performance depends on the NAI cut-off value for the antibody titer estimation and what is the optimal cut-off value. Currently, no MUNANA-based NAI assay has an established analytical range nor has been validated.

Therefore, the objective of this study was to qualify the VLP as a new source for active neuraminidase in the NAI-based antibody assay. To optimize the assay, the range for the initial NA activity and the optimal NAI cut-off value for antibody titer estimation were established. To set up an analytical range, the modified assay was validated for specificity, precision, accuracy, and linearity as per ICH guidelines (ICH, 1996).

2. Materials and methods

2.1. VLP preparation

Purified VLPs are comprised of recombinant influenza virus hemagglutinin (HA), neuraminidase (NA) and matrix 1 (M1) proteins. The appropriate genes are cloned into a baculovirus expression vector with each under the control of their own set of transcription regulatory elements. VLPs are then assembled from recombinant HA, NA, and M1 proteins expressed in recombinant virus infected *Spodoptera frugiperda* (sf9) insect cells (ATCC CRL-1711) (Invitrogen, Carlsbad, USA). Abbreviations for influenza viruses or proteins used in this study are A/Indo (A/Indonesia 05/2005 (H5N1)), A/Cal (A/California 04/2009 (H1N1)), A/NewCal (A/New Caledonia/20/99(H1N1)), H1N1 A/Br (A/Brisbane 59/2007 (H1N1)), A/NY (A/New York/55/2004 (H3N2)), H3N2 A/Br (A/Brisbane 10/2007 (H3N2)), B/Br (B/Brisbane 60/2008), B/Fl (B/Florida 04/2006) and B/Sh (B/Shanghai/ 361/2002).

The cloning of influenza HA, NA, and M1 into baculovirus (BV) expression vectors was described previously (Pushko et al., 2005; Bright et al., 2007; Mahmood et al., 2008). The sf9 insect cells were infected with a recombinant baculovirus. VLPs were purified from the supernatant harvested from the sf9 cell culture (Bright et al., 2007). After removal of the sf9 cells, the VLP preparation was concentrated and diafiltered through tangential flow filtration (TFF). The separation of VLPs from BV particles and contaminating DNA, RNA and sf9 proteins was achieved by anion exchange (IEX) followed by size-exclusion (SEC) chromatography. To ensure sterility the VLP preparation was filtered through a 0.22 μm PVDF membrane and stored refrigerated (2–8 °C).

The expression of HA, NA, and M1 proteins in VLP preparations was confirmed by SDS–PAGE and Western blot analysis and the HA concentration was quantified by a single-radial-immunodiffusion (SRID) as described (Pushko et al., 2005; Mahmood et al., 2008). The total protein (TP) concentration was tested using the BCA assay (Pierce, Rockford, USA).

2.2. Purified neuraminidase and hemagglutinin protein preparations

NA and HA proteins from H5N1 A/Indo, H3N2 A/NY and B/Fl viruses were produced by the following procedure. The corresponding native HA and secreted NA (fusion of NA with HA secretion sequence) genes were individually cloned into the BV expression vector used to produce VLPs. The sf9 cells were infected with the resulting recombinant viruses and the proteins were expressed in a similar manner as VLPs described in this paper. The HA proteins were extracted from the cell paste and purified through TMAE anion exchange, lentil lectin affinity chromatography, and hydroxyapatite (CHT) chromatography. The secreted NA proteins were harvested from the culture supernatant and purified through three column chromatography steps including Fractogel EMD TMAE, lentil lectin, and hydroxyapatite columns. The purified HA and NA proteins with >95% purity as determined by SDS–PAGE, and densitometric scanning were used for animal immunization to raise mono-specific polyclonal antiserums.

2.3. Antisera against purified neuraminidase and hemagglutinin

Sheep immunization with purified HA and NA proteins from B/Fl, H3N2 A/NY and H5N1 virus strains, followed by blood collection and antiserum preparation were performed by Covance (Princeton, USA). Sheep were immunized ID/IM with 50 μg of antigen in complete Freund's adjuvant and boosted three weeks later by the same route with 30 μg of antigen mixed with in-complete Freund's adjuvant. The antibody titers were determined by ELISA as described below.

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