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Assessment of influenza A neuraminidase (subtype N1) potency by ELISA

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Antibodies that inhibit neuraminidase (NA) activity of influenza virus provide resistance against disease and have been associated with milder epidemics. Although studies have demonstrated a correlation between NA inhibition antibody titers and vaccine efficacy, neither the quantity nor form of NA is measured in seasonal and pandemic influenza vaccines. In this report, we describe development of enzyme-linked immunosorbent assays (ELISAs) that are suitable for quantitation of the native form of NA of subtype N1. The assays use mouse monoclonal antibodies (mAbs) 1H5 and CD6 to capture NAs of viruses, and a different mAb 4E9 to detect bound antigen. The 1H5-capture ELISA detects NAs of seasonal and pandemic H1N1 viruses as well as H5N1 viruses and has a limit of quantitation (LOQ) of 5.5 ng/mL for seasonal H1N1A/Brisbane/59/2007 NA. The CD6-capture ELISA is specific for NA of the 2009 pandemic viruses with a LOQ of 67 ng/mL for A/California/07/2009 NA. The ELISA signals in both assays are proportional to NA enzymatic activity and correlate with NA immunogenicity. The ELISAs we describe may expedite the development of NA-based influenza vaccines by providing a practical assay to measure NA potency.

1. Introduction

Most inactivated influenza vaccines are split virus preparations that contain a mixture of influenza viral proteins, including hemagglutinin (HA) and neuraminidase (NA), the viral surface glycoproteins responsible for virus attachment and release from host cells, respectively (Wong and Webby, 2013). Antibodies that inhibit HA function correlate with protection against infection and therefore influenza vaccines are formulated to contain a specified amount of HA that is in its native form (Minor, 2015). Antibodies that inhibit NA activity are an independent correlate of immunity (Couch et al., 2013; Monto et al., 2015), and are associated with reduced disease symptoms (Memoli et al., 2016; Rockman et al., 2013). Although NA is contained in most influenza vaccines, its potential to contribute to vaccine efficacy throughout the vaccine's shelf-life is currently not measured. Given the predominant contribution of HA-specific antibodies to vaccine efficacy this is not of great concern, however new vaccines are being developed, some of which depend on the induction for NA-specific antibodies for their effectiveness. A NA potency assay is critical for further development of such NA-based vaccines.

The enzyme activity of NA reflects its native conformation and correlates with NA's ability to induce antibodies that inhibit its function (Sultana et al., 2014). However, enzyme activity is not suitable for measuring potency because it cannot distinguish between NA types and subtypes in a multivalent vaccine. Enzyme-linked immunosorbant assays (ELISAs) have been described for the quantitation of NA of H3N2 and B viruses (Aymard, 2002; Gerentes et al., 1998). These tests used monoclonal antibodies (mAbs) to capture NA, with subsequent detection of the captured NA by rabbit polyclonal antisera generated by immunizing animals with whole virus. The sensitivity of the reported N2 and B tests was approximately 7–10 ng/mL, a sensitivity that would be suitable for quantifying NA in vaccines. Since NA undergoes antigenic drift (Sandbulte et al., 2011; Zhong et al., 2013), mAbs are often strain specific. Therefore, it is not surprising that the previously-described N2 assay that used a mAb to A/Beijing/32/92 (H3N2), allowed quantification of NA in viruses circulating between 1992 and 1995 but not later (Gerentes et al., 1998). Similarly, the mAb selected for reactivity with the influenza B NA was not broadly-reactive (Aymard, 2002).

Our goal was to develop ELISA-based potency assays for N1 subtype that could be applied to heterologous viruses, regardless of HA

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subtype. We identified mAbs that are broadly reactive to the NA of H1N1 viruses (Wan et al., 2013; Wan et al., 2015) and used these antibodies to develop ELISAs for assessment of N1 potency. In this report, we describe the development of these assays and show that the amount of NA measured is indicative of its immunogenicity.

2. Methods and materials

2.1. Viruses and vaccine strains

The following viruses were used in this study: H1N1 viruses A/New Jersey/76 (NJ/76), A/Texas/36/1991 (TX/91), A/New Caledonia/20/1999 (NC/99), A/Solomon Islands/3/2006 (SI/06), A/Brisbane/59/2007 (BR/07), A/California/07/2009 X-179A (CA/09), A/Brisbane/70/2011 (BR/11), and A/Michigan/45/2015 (MI/15); attenuated H5N1 virus A/Vietnam/1203/2004 (VN/04), a reassortant virus containing HA (with polybasic residues in the cleavage motif deleted) and NA genes of H5N1, and the internal genes of A/Puerto Rico/8/1934 (Wan et al., 2013); H3N2 virus A/Victoria/361/2011 (VI/11); and influenza B virus B/Brisbane/60/2008 (B/BR/08). All viruses were grown in 9–11 day old embryonated chicken eggs (Sunrise Farms, Catskill, NY) for 60–72 h at 33 °C. Whole virus preparations used in ELISAs were inactivated by incubating allantoic fluid with 0.05% beta-propiolactone (Sigma-Aldrich, St. Louis, MO) overnight at 4 °C, and then purified by sucrose-gradient centrifugation. H6 reassortant viruses bearing the NA of BR/07 and CA/09 ($H6N1_{BR/07}$ and $H6N1_{CA/09}$, respectively) (Jiang et al., 2015) were used in NA inhibition (NI) assays. The total protein concentration of the virus preparations was measured by Pierce™ BCA protein assay kit (ThermoFisher Scientific, Rockford, IL).

Influenza vaccines were obtained from commercial sources. The monovalent 2009 H1N1 pandemic (H1N1pdm) vaccine was obtained through an agreement with the Biomedical Advanced Research and Development Authority (BARDA). Split inactivated monovalent bulks and trivalent formulations containing antigens recommended for the 2012/13, 2015/16 Northern Hemisphere, and 2015 Southern Hemisphere influenza seasons were provided by CSL (now Seqirus, Melbourne, Australia).

2.2. mAbs

Hybridomas secreting mAbs against the NA of seasonal influenza virus BR/07 (H1N1) were generated and characterized as previously described (Wan et al., 2013). The hybridoma secreting mAb CD6 against the NA of CA/09 (H1N1) was selected using the same methods, and the NA epitope bound by this antibody was elucidated by x-ray crystallography (Wan et al., 2015). The mAbs selected for assay development were purified using protein G columns (GE Healthcare, Uppsala, Sweden). mAbs tested as secondary antibody reagents were conjugated to horseradish peroxidase (HRP) with a peroxidase labeling kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

2.3. Determination of NA concentration by isotope-dilution mass spectrometry (IDMS)

The concentration of NA in each purified whole virus standard was measured by IDMS following published methods (Williams et al., 2008; Williams et al., 2012). Briefly, 10 μL of purified whole inactivated virus was mixed with an equal volume of 0.1% RapiGest (Waters Corp., Milford, MA) and then digested for 2 h with sequencing grade modified trypsin (Promega, Madison, WI). An aliquot (10 μL) of 500 fmol/μL C^{14}, N^{15}-labeled YNGIITDTIK, YNGIITETIK and GDVRFIR peptide cocktail was added to each sample and to various concentrations of native peptides used to establish calibration curves. These peptides were targeted as they are present in the NA of the majority of H1N1 viruses and were previously shown to have characteristics suitable for identification by mass spectrometry (Williams et al., 2012). The peptide cocktails were kindly provided by Dr. Tracie Williams (Centers for Disease Control and Prevention, Atlanta, GA). Samples were run on an H-class Acquity UPLC system (Waters Corp., Milford, MA) and fractions were analyzed on a Waters TQS triple quadrupole mass spectrometer. Results were corrected for sample dilution and the NA concentrations reported as μg/mL.

2.4. Determination of NA concentration by N1 ELISA

Samples were detected with 1H5- or CD6-capture ELISA. Immulon 2HB Microtiter® plates (ThermoScientific, Rochester, NY) were coated with 100 μL of NA-specific mAb diluted in coating buffer (KPL, Gaithersburg, MD) or 50 mM sodium bicarbonate, pH 9.6. The plates were incubated at 4 °C overnight. After washing the plates with PBS, the wells were blocked by incubating with 200 μL 15% fetal bovine serum (FBS) in PBS for 1 h at 37 °C. Samples were diluted in PBS-0.05% Tween 20 (PBST) containing 15% fetal bovine serum (FBS) and 0.1% zwittergent 3–14 (Calbiochem, Darmstadt, Germany). Serial dilutions of the standard or sample were added to duplicate wells (100 μL per well), and the covered plates incubated at 37 °C for 1 h. After washing with PBST, HRP-labeled NA-specific mAb (100 μL) was added to each well and the plates incubated at 37 °C for 1 h. A solution of o-phenylenediamine (OPD) (Sigma-Aldrich, St. Louis, MO) was prepared immediately before adding 100 μL/well to the washed plates. The colorimetric reaction was stopped after 10 min at room temperature in the dark by adding 50 μL 1N H_2SO_4. Optical density at 490 nm (OD_{490}) was read on a Victor V plate reader (PerkinElmer, Waltham, MA).

Two-fold dilutions of a virus preparation with a known NA concentration (the standard) were included in the assay. The OD_{490} readings corresponding to different NA concentrations were used to generate a standard curve that was used to calculate the NA concentration/potency of the samples. The limit of detection (LOD) and limit of quantitation (LOQ) were analyzed with GraphPad Prism software.

2.5. Measurement of NA activity

The NA activity of N1 and N2 viruses was measured by fluorescence assay using 4-methylumbelliferyl-N-acetylneuraminic acid (MU-NANA, Sigma-Aldrich, St. Louis, MO) as substrate. Briefly, each sample was mixed with an equal volume (50 μL) of 100 μM MU-NANA in duplicate wells of a 96 well plate. After 1 h incubation at 37 °C, the reaction was stopped by addition of 100 μL of 0.1 M glycine, pH 10.7, 20% ethanol. The released product was quantified by measuring fluorescence intensity with a Victor V plate reader (PerkinElmer, Waltham, MA) at excitation of 355 nm and emission of 460 nm. Bacterial NA purified from *Vibrio cholerae* (Sigma-Aldrich, St. Louis, MO) was used as a positive control and sample diluent was used as a negative control. Relative fluorescence units (RFU) values were reported.

2.6. Mouse immunogenicity studies

Immunogenicity experiments were conducted in 8–10 week old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) using procedures approved by the institutional animal care and use committee and following federal guidelines. Each group (n = 5) was immunized intramuscularly with 50 μL purified split influenza viruses (monovalent bulks) or vaccines without the addition of adjuvant. These included monovalent bulk preparations of A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2)