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An optimized enzyme-linked lectin assay to measure influenza A virus neuraminidase inhibition antibody titers in human sera

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A B S T R A C T

Antibodies to neuraminidase (NA), the second most abundant surface protein on influenza virus, contribute toward protection against influenza. The traditional thiobarbituric acid (TBA) method to quantify NA inhibiting antibodies is cumbersome and not suitable for routine serology. An enzyme-linked lectin assay (ELLA) described by Lambre et al. (1990) is a practical alternative method for measuring NA inhibition (NI) titers. This report describes optimization of the ELLA for measuring NI titers in human sera against influenza A viruses, using H6N1 and H6N2 viruses as antigens. The optimized ELLA is subtype-specific and reproducible. While the titers measured by ELLA are somewhat greater than those measured by a miniaturized TBA method, seroconversion rates are the same, suggesting similarity in assay sensitivity under these optimized conditions. The ELLA described in this report provides a practical format for routine evaluation of human antibody responses to NA.

1. Introduction

Neuraminidase (NA) inhibiting antibodies are associated with protection against influenza (Murphy et al., 1972) and correlate with reduced viral shedding and disease symptoms in a human challenge study (Clements et al., 1986). These antibodies contribute to immunity by inhibiting release and spread of newly formed virus particles from infected cells (Compans et al., 1969). Despite the established importance of NA inhibiting (NI) antibodies, these titers are rarely measured in seroepidemiologic or vaccine studies because the traditional thiobarbituric acid (TBA) assay used to quantify these antibodies is impractical for large numbers of samples and employs hazardous chemicals. Alternative assays that have been developed include a miniaturization of the TBA method (Sandbulte et al., 2009) and an enzyme-linked lectin assay (ELLA) (Lambre et al., 1990). The read-out of each method is different – while both quantify products of enzyme activity, the TBA method measures the amount of free sialic acid, the soluble product of NA activity, whereas ELLA measures the amount of penultimate galactose that becomes available after the terminal sialic acid is cleaved from substrate. Despite this difference, the assay principals are the same, usually employing whole virus as a source of antigen and using fetuin, a highly glycosylated protein, as substrate.

Since HA-specific antibodies in human sera can block access of substrate to NA, it is essential to use viruses with a HA subtype that is not in circulation when performing either of these assays for human serology. The assay described in this manuscript uses reverse genetics-derived H6 reassortant viruses that contain the targeted NA (Sandbulte et al., 2011). This strategy follows the original approach to measure NA inhibition antibody titers in which H6 reassortant viruses generated by classical reassortment were used in the traditional TBA method (Kilbourne et al., 1968, 1990).

The substrate for NA, fetuin, is coated onto the surface of 96 well plates used in the ELLA. Virus is incubated in the wells in the presence or absence of serial serum dilutions. NA cleaves terminal sialic acid moieties from glycoprotein complexes, and therefore enzyme activity can be quantified by measuring the amount of galactose that is consequently at the terminus of each carbohydrate complex. This is accomplished using peroxidase-conjugated peanut-agglutinin (PNA), a lectin with specificity for terminal

Abbreviations: ELLA, enzyme-linked lectin assay; HA, hemagglutinin; HI, HA inhibition; NA, neuraminidase; NI, NA inhibiting/inhibition; PBS, phosphate-buffered saline; PNA, peanut agglutinin; TBA, thiobarbituric acid.

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galactose. Following incubation with PNA-peroxidase, a substrate for peroxidase is added, leading to a colorimetric change proportional to NA activity. The ELLA has recently been used to measure NI antibody titers in a number of studies (Cate et al., 2010; Couch et al., 2012, 2013; Fritz et al., 2012; Fries et al., 2013), but assay optimization and details of the assay procedure to measure titers against the NA of human seasonal viruses have not been reported. This report describes steps that are important for obtaining consistent results and provide data to support the use of ELLA in human serology.

2. Materials and methods

2.1. Viruses

Reassortant influenza viruses were generated by reverse genetics as described previously (Hoffmann et al., 2000; Sandbulte et al., 2011). These viruses contain the HA (H6) gene from A/turkey/Massachusetts/3740/1965, gene segments encoding internal proteins from A/Puerto Rico/8/1934 (PR/34), and one of the following NA gene segments: N1 of 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 (CA/09); and N2 of A/Wisconsin/67/2005 (WI/05) or A/Uruguay/716/2007 (UR/07). The following wild type influenza B viruses were used: B/Florida/4/2006 (B/FL/06) as representative of the B/Yamagata lineage and B/Brisbane/60/2008 (B/BR/08) as representative of the B/Victoria lineage. Viruses were cultured in the allantoic cavity of 9–12 day old embryonated chicken eggs at 33 °C, harvested 72 h post-inoculation and stored in aliquots at −80 °C.

2.2. Serum samples

The following animal sera were used: ferret antisera against NC/99, UR/07, B/FL/06 and B/BR/08 generated by infecting ferrets with the respective wild-type influenza viruses, and cotton rat sera from naïve as well as PR/34 (H1N1)-immune animals. Ferret and cotton rat inoculations were performed following federal guidelines under a protocol approved by the institutional Animal Care and Use Committee. Pooled rabbit antisera (Capralogics, Hardwick, MA, USA) collected before and after immunization with purified NA were also used in this study. The NA was purified from WI/05 by cellulose acetate electrophoresis (Sultana et al., 2011). De-identified human sera were obtained from a clinical vaccine study in which groups of young, healthy adults were immunized with either a live or inactivated trivalent seasonal influenza vaccine. The study was approved by the Institutional Research Involving Human Subjects Committee. Antibody and cellular immune responses were measured before and 4 weeks after vaccination; these results were reported previously (Eichelberger et al., 2011). Unless otherwise noted, antisera were heat-inactivated at 56 °C for 45 min before conducting assays.

2.3. ELLA procedure

The principles of the ELLA described by Lambre et al. (1990) and Cate et al. (2010) were followed to optimize and validate the method. The standard operating procedure (SOP) for this method is included in this manuscript as supplementary information. Fetuin (Sigma, St. Louis, MO, USA) was diluted to 25 μg/ml in 0.1 M phosphate buffered saline (PBS) and 100 μl added to each well to coat high-binding 96-well plates (Nalge Nunc, Rochester, NY, USA). Plates were stored at 4 °C and used 24 h to 2 months after coating. To determine the amount of antigen (virus) to use in ELLA, serial dilutions of the targeted H6 reassortant virus were prepared in Dulbecco's PBS (pH 7.4)–0.9 mM $CaCl_2$–0.5 mM $MgCl_2$ containing 1% bovine serum albumin (BSA) and 0.5% Tween and then dispensed (50 μl/well) into fetuin-coated plates containing an equal volume of PBS. The plates were incubated for 16–18 h at 37 °C, then washed 6 times with PBS–0.05% Tween 20 (PBST) before adding 100 μl peanut agglutinin (PNA) conjugated to horse-radish peroxidase (HRPO, Sigma). PNA–HRPO was used at the highest dilution that gave the maximum signal when titrated on fully digested fetuin. Plates were incubated at room temperature for 2 h and washed 3 times with PBST before adding o-phenylenediamine dihydrochloride (OPD, Sigma) to the plate. The color reaction was stopped after 10 min by the addition of 1 N H_2SO_4. The plates were read at 490 nm for 0.1 s using a Victor V 96-well plate reader (PerkinElmer, Waltham, MA, USA). The dilution of virus (antigen) that resulted in 90–95% maximum signal was elected for use in serology.

To measure the NI titers, each serum sample was heat treated (56 °C for 45 min) and then diluted serially in PBS–BSA. Fifty microliters of each dilution was added to duplicate wells of a fetuin-coated plate. An equal volume (50 μl) of the selected virus dilution was added to all serum-containing wells in addition to at least 4 wells containing diluent without serum that served as a positive (virus only) control. At least 4 wells were retained as a background control (PBS only). The plates were incubated for 16–18 h at 37 °C. As described for the virus titration, the plates were washed and PNA–HRPO was added to all wells. After a 2 h incubation period, the plates were washed and peroxidase substrate (OPD) was added. The color reaction was stopped after 10 min and absorbance read. The mean absorbance of the background (A_{bkg}) was subtracted from the test wells and positive control (A_{pos}) wells. The percent NA activity was calculated by dividing the mean absorbance of duplicate test wells (A_{test}) by the mean absorbance of virus only wells and multiplied by 100, i.e. $(A_{test} - A_{bkg})/(A_{pos} - A_{bkg}) \times 100$. To determine percent NA inhibition, the percent activity was subtracted from 100. The NI titers were defined as the reciprocal of the last dilution that resulted in at least 50% inhibition. An alternative way to report results is to calculate the titers of replicate wells independently and then report the geometric mean of the duplicates as the 50% end-point NI titer. In some instances the exact 50% inhibition (IC_{50}) was determined by 4 parameter logistics regression analysis (GraphPad Prism software). An assay was considered valid if the background absorbance was less than 10% of the virus only control, control sera had a similar NI titer to the median established in previous assays ($\leq$2-fold difference), and the raw A_{490} values of the duplicates did not vary more than 20%.

2.4. Miniaturized TBA method

The miniaturized TBA method was followed as described previously (Sandbulte et al., 2009).

2.5. Statistical analysis

Microsoft Office Excel was used to calculate standard deviations (SDs) of NI titers, percent coefficient of variation (%CV) for repeat assays and Pearson's correlation coefficient. Bland–Altman analysis (Bland and Altman, 1999) was performed using GraphPad Prism to assess the agreement between ELLA and TBA results.

3. Results

3.1. Assay optimization

The published ELLA method (Lambre et al., 1990) was optimized for routine analysis of human sera. This assay uses reassortant viruses with a mismatched HA as antigen (source of NA enzyme) to avoid non-specific inhibition by H1 and H3-specific antibodies in human sera. The H6 reassortant viruses containing the targeted