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Conformationally selective biophysical assay for influenza vaccine potency determination

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ABSTRACT

Influenza vaccines are the primary intervention for reducing the substantial health burden from pandemic and seasonal influenza. Hemagglutinin (HA) is the most important influenza vaccine antigen. Subunit and split influenza vaccines are formulated, released for clinical use, and tested for stability based on an *in vitro* potency assay, single-radial immunodiffusion (SRID), which selectively detects HA that is immunologically active (capable of eliciting neutralizing or hemagglutination inhibiting antibodies in an immunized subject). The time consuming generation of strain-specific sheep antisera and calibrated antigen standards for SRID can delay vaccine release. The limitation in generating SRID reagents was evident during the early days of the 2009 pandemic, prompting efforts to develop more practical, alternative, quantitative assays for immunologically active HA. Here we demonstrate that, under native conditions, trypsin selectively digests HA produced from egg or mammalian cell in monovalent vaccines that is altered by stress conditions such as reduced pH, elevated temperature, or deamidation, leaving native, pre-fusion HA, intact. Subsequent reverse-phase high pressure liquid chromatography (RP-HPLC) can separate trypsin-resistant HA from the digested HA. Integration of the resulting RP-HPLC peak yields HA quantities that match well the values obtained by SRID. Therefore, trypsin digestion, to pre-select immunologically active HA, followed by quantification by RP-HPLC is a promising alternative *in vitro* potency assay for influenza vaccines.

1. Introduction

Vaccination is considered the most effective means to reduce the substantial morbidity and mortality caused by influenza infection [1,2]. Influenza hemagglutinin (HA) binds host cell surface receptors and mediates viral entry by mediating membrane fusion [3,4]. HA is the major target for virus-neutralizing antibodies and the most important antigen in subunit and split influenza vaccines (inactivated influenza vaccine – IIV) [5]. The potency of IIV is determined primarily by the quantity of immunologically active (able to elicit neutralizing or hemagglutination inhibiting antibody responses) HA that a dose contains. For IIV formulation, release, and stability testing, a surrogate *in vitro* potency test is used, single-radial immunodiffusion (SRID) [6,7]. This modified Ouchterlony test quantifies immunologically active HA based on the diameter of the immunoprecipitin ring that forms when Zwittergent-dispersed vaccine antigen (or an antigen standard) diffuses from a circular well into an agarose gel that has been cast with a homogeneous concentration of a strain-specific sheep antiserum. SRID has been used for influenza vaccine manufacture for almost four decades. It is accepted by regulatory agencies, and correlation has been shown between SRID-measured vaccine potency and vaccine immunogenicity in clinical trials [8–11].

Despite its selectivity for immunologically active HA, SRID has significant shortcomings, the greatest of which is the need to generate large quantities of strain-specific reference reagents for each strain change using time consuming processes that can delay vaccine release. The strain-specific HA reference antigens are produced by growing, inactivating, and purifying whole influenza viruses. The corresponding strain-specific antisera are generated

Abbreviations: AUC, area-under-curve; HA, hemagglutinin; IA, immunologically active; IDMS, isotope dilution mass spectrometry; IIA, immunologically inactive; IIV, inactivated influenza vaccine; SRID, single-radial immunodiffusion; RP-HPLC, reversed-phase high pressure liquid chromatography.

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by immunizing sheep multiple times with HA cleaved from whole virus by bromelain [6,12]. Reference antigens and antisera are calibrated and distributed by the World Health Organization (WHO) Essential Regulatory Laboratories (ERL). Generation and calibration of reagents require the shipment of materials between geographically dispersed organizations and is a particularly significant barrier for timely pandemic responses [13]. An alternative influenza vaccine potency assay for immunologically active HA that does not require the generation of strain-specific immunological reagents could accelerate influenza vaccine production and release.

HA is a homotrimeric membrane bound glycoprotein. Each monomeric HA subunit consists of two disulfide-linked protease fragments, HA1 and HA2. Each monomeric subunit also has two domains exposed outside the virus envelope (or cell membrane), a globular "head" composed entirely of HA1 residues, and an elongated "stem" composed of residues from HA1 and HA2 [4]. HA generally maintains a "metastable", pre-fusion conformation at neutral pH. That is, once an energy barrier is overcome, HA refolds irreversibly to a more stable, post-fusion conformation [14]. During cell entry, moderate reduction of pH in endosomes drop the energy barrier, and HA rearranges in a series of conformational changes that mediate viral and endosomal membrane fusion [15]. The head domains separate from the stem (but remain tethered by disulfide bonds) and the stem jack-knifes, but remains trimeric [16]. Heat can also trigger similar HA rearrangement [17,18].

Low-pH treatment abolishes HA detection by SRID and greatly reduces its ability to elicit binding and neutralizing antibodies in mice [19], in an example of the correlation between the HA immunological activity and detection by SRID. The transition to the post-fusion conformation also increases HA susceptibility to proteolysis [17,20]. HA1 of prefusion HA is trypsin-resistant; HA1 of post-fusion HA is trypsin-sensitive. Often, compact, well-folded, protein domains are protease resistant, and denatured or misfolded proteins are trypsin sensitive. These observations suggest that trypsin digestion, under native conditions, might selectively cleave HA1 not only in post-fusion HA, but also in HA damaged by chemical or physical stressors.

Alternative influenza potency assays are in development [21]. Many of the most quantitative, reproducible, easily calibrated, and practical assays (such as RP-HPLC [22–24], isotope dilution mass spectroscopy [IDMS] [25] and SDS-PAGE) are run with denatured protein samples. Although such assays might represent a major improvement over SRID in many regards, the experimental denaturation of HA during sample preparation for the assays or during the assays themselves makes the assays incapable of providing sufficient information on HA conformational integrity. Monoclonal antibody based assays (such as enzyme-linked immunosorbent assay [ELISA] [26,27] and antibody-dependent surface plasmon resonance [SPR] [28]) can potentially measure conformational integrity. However these assays rely on a strain-specific monoclonal antibody panel that would need to be updated and calibrated along with a reference antigen that likely would be quantified with a conformationally insensitive biophysical assay.

Here, we report an alternative influenza potency assay that includes a selective trypsin pre-treatment of HA under native conditions, designed to leave immunological active HA intact followed by a quantification step (RP-HPLC) of the intact HA. To determine assay robustness, we analyzed HA from several subtypes or types (H1N1, H3N2, B), two manufacturing platforms (chicken egg-produced and mammalian cell-produced) and multiple stress conditions (moderately reduced pH, moderate elevated temperature and deamidation) with results that parallel those of SRID. These studies indicate that the combination of approaches has generated a stability indicating assay independent of strain specific reagents.

2. Methods

2.1. Influenza standards and vaccines

SRID polyclonal sheep reference antisera and calibrated reference antigens for A/Brisbane/59/2007 (H1N1), A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), A/Texas/50/2012 (H3N2), B/Massachusetts/02/2012, B/Wisconsin/1/2010 and B/Brisbane/60/2008 were provided by the US Food and Drug Administration's Center for Biologics Evaluation and Research (FDA CBER, Silver Spring, MD, USA) and National Institute for Biological Standards and Control (NIBSC, London, UK).

A/Brisbane/59/2007 (H1N1), A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), A/Texas/50/2012 (H3N2), B/Massachusetts/02/2012, B/Wisconsin/1/2010 and B/Brisbane/60/2008 monobulks (unblended lots of subunit vaccine antigen) were produced by Novartis Vaccines. The chicken egg-produced monobulks were produced from embryonated chicken eggs by the Agrippal® subunit influenza vaccine process from pilot or engineering batches. The mammalian cell-produced monobulks were produced from Madin-Darby canine kidney (MDCK) cells by the Flucevax® subunit influenza vaccine process from pilot or engineering batches. The production process includes whole virus isolation and inactivation, virus splitting and HA antigen purification.

Trivalent bulks were formulated by mixing the three monobulks at a ratio of 1:1:1 with a 30 μg/ml final HA concentration for each strain.

2.1.1. SDS-PAGE

Samples mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer and reducing agent (Invitrogen, Grand Island, NY, USA) were heated at 90 °C for 5 min, and then separated by NuPAGE 4–12% Bis-Tris gels with 2-(N-morpholino)ethanesulfonic acid (MES) running buffer (Invitrogen). Protein bands were visualized using Coomassie blue staining and sliver staining.

2.1.2. RP-HPLC

Samples pretreated with Zwittergent 3-14 (Calbiochem-Novabiochem, Billerica, MA, USA), 1% volume/volume, at room temperature for 30 min followed by treatment with 25 mM dithiothreitol (DTT) at 90 °C for 10 min were injected onto a POROS® R1/10 column (Applied Biosystems, Grand Island, NY, USA) and eluted with a gradient of 30–35% 0.1% trifluoroacetic acid (TFA) in 100% acetonitrile (Waters Alliance, Milford, MA, USA). Sample HA was quantified by comparing the area-under-curve (AUC) of HA1 peaks to those from SRID reference antigens.

2.1.3. SRID

Agarose solution in phosphate buffered saline (PBS), 1% weight/volume, was prepared by mixing 3.0 g SeaKem Agarose (Lonza, Basel, CH) with 300 ml PBS. The mixture was boiled to dissolve the agarose, cooled to 60 °C and mixed with strain-specific SRID reference antiserum at the dilution recommended by FDA CBER and NIBSC. The agarose was poured into plastic casts to a uniform 2 mm thickness and cooled at room temperature for 20 min. Then, 4 mm diameter round wells were cut 10 mm apart in the solidified gel.

SRID reference antigen was diluted with PBS to 40 μg HA/ml. Reference and unknown samples were treated with Zwittergent 3–14, 1% volume/volume, for 30 min at room temperature. After treatment, reference and samples were diluted 3:4, 2:4 and 1:4 in PBS and dispensed into the agarose wells at 20 μl sample/well in duplicate.

SRID plates were incubated 16–24 h at 25 °C to allow complete diffusion of the antigen. Proteins that had not formed immune