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Inactivated influenza vaccine stress can affect *in vitro* potency assay relationship to immunogenicity

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ABSTRACT

Influenza vaccines are the most effective intervention to prevent the substantial public health burden of seasonal and pandemic influenza. The capability of hemagglutinin (HA), the main antigen in inactivated influenza vaccines (IIVs), to elicit functional neutralizing antibodies determines IIV effectiveness. When HA is subjected to environmental stress during manufacturing or while stored prior to administration, such as low pH and temperature excursions, the HA immunological activity can be affected. Single-radial immunodiffusion (SRID), the standard *in vitro* potency assay for IIVs, is believed to specifically detect immunologically active HA and has been applied to evaluate HA stability against stress. Here we report that transient low pH treatment and freeze/thaw cycles with HA in PBS abolish SRID-quantified *in vitro* potency for all HAs of multiple influenza strains. Raised temperature substantially decreases *in vitro* potency with more extensive HA structural changes. Chemical stress and mechanical stress moderately change SRID *in vitro* potency values in a strain-dependent manner. Trypsin digestion, which selectively degrades stressed HA, followed by RP-HPLC quantification as a candidate alternative *in vitro* potency assay yields results comparable to SRID. Mouse immunogenicity studies confirm that HA stressed by transient low pH treatment does not elicit functional antibodies *in vivo,* nor does it have a measureable SRID value. However, HA stressed by raised temperature elicits high titers of functional antibodies *in vivo* despite substantial loss of SRID *in vitro* potency. This discrepancy between SRID *in vitro* potency and vaccine immunogenicity suggests that SRID may not reliably indicate IIV potency under all conditions. Further efforts to develop alternate potency assays that can better predict *in vivo* immunogenicity should continue along with additional studies exploring HA conformation, SRID values and consequent immunogenicity.

1. Introduction

Influenza is a highly infectious virus that can be transmitted via the aerosol route and is responsible for seasonal epidemics and periodic, unpredictable pandemics. Vaccination is the most cost-effective strategy to combat the substantial morbidity and mortality caused by influenza infection [1,2]. Hemagglutinin (HA), the dominant influenza viral surface protein, plays a key role during infection, by attaching to host cell surface receptors and facilitating membrane fusion to mediate viral entry into the cell [3,4]. HA is the major viral neutralizing antibody target and is the most important antigen in inactivated influenza vaccines (IIVs) [5].

HA forms a membrane-bound homologous trimer of disulfide linked HA1/HA2 heterodimers. HA1 primarily forms the membrane-distal globular domain and it contains the receptor binding domain (RBD). Upon binding the cellular receptor, sialic acid, the virus is endocytosed. HA2 forms a central stem structure with HA1 and mediates viral and endosomal membrane fusion through a low pH-induced conformational change [6]. HA original folds in a "metastable", pre-fusion conformation at neutral pH. Once an energy barrier is overcome by endosomal low pH drop, HA refolds extensively and irreversibly to a more stable, post-fusion conformation. The refolding involves dramatic HA2 structural rearrangement and dissociation of the trimeric HA1 globular domain which remains tethered to HA2 [7–9]. Besides low pH, other energy input, such as raised temperature and chemical

Abbreviations: AUC, area-under-curve; Bv, B Victoria lineage; By, B Yamagata lineage; HA, hemagglutinin; HI, hemagglutination inhibition; IDMS, isotope dilution mass spectrometry; IIV, inactivated influenza vaccine; MDCK, Madin-Darby Canine Kidney; MN, microneutralization; RBD, receptor binding domain; SRID, single-radial immunodiffusion; RP-HPLC, reversed-phase high pressure liquid chromatography.

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denaturant stress can also trigger a HA conformational change that is biochemically similar to the low pH-triggered state [10,11].

The HA1 head region, including the RBD, contains the dominant set of potent neutralizing antibody epitopes. The RBD region appears to be largely structurally similar between the pre-fusion structure and the low pH-dissociated post-fusion state [12]. However in mouse immunogenicity studies, only native pre-fusion HA, not low pH-triggered post-fusion HA, elicits strong neutralizing or hemagglutination inhibiting (HI) antibody responses against influenza [13,14]. In addition, HA modifications that improve HA pre-fusion conformation stability enhance vaccine immunogenicity [15], while mutations that destabilize the pre-fusion conformation impair immunogenicity [16].

Single-radial immunodiffusion (SRID) is an *in vitro* potency test for IIV HA that was developed and validated in the 1970s to predict immunogenicity [17–19]. This modified Ouchterlony test quantifies HA based on the diameter of immunoprecipitin ring that forms when vaccine antigen diffuses radially from a circular well cut into an agarose gel that has been cast with strain-specific sheep antiserum generated by immunization with HA from bromelain treated whole virus [18,20]. Although the sheep antiserum binds to pre-fusion and post-fusion HA equally [21], through the SRID format [14], a readable SRID immunoprecipitin ring is produced with a native pre-fusion HA but not with low pH-triggered post-fusion HA [22]. Similarly the low response of low pH-stressed vaccine correlates with low immunogenicity in mouse studies [14]. The time consuming generation of strain-specific sheep antisera and calibrated antigen standards is the limitation for SRID assay and was evident during the early days of the 2009 pandemic, prompting efforts to develop more practical, alternative potency assays.

HA conformation is also correlated with HA susceptibility to proteolysis [10,23]. HA1 from pre-fusion HA, the more immunogenic form, is trypsin resistant whereas HA1 from the less immunogenic, post-fusion HA form is trypsin sensitive. This is consistent with the observation that native, well-folded protein domains are often protease resistant and stressed, denatured proteins are trypsin sensitive. Trypsin digestion has been shown to distinguish HA conformation [21] and confer functional specificity on biophysical techniques (e.g. reversed-phase high pressure liquid chromatography (RP-HPLC), isotope dilution mass spectrometry (IDMS) and SDS-PAGE) that are generally insensitive to conformation, without the requirement for strain-specific antibodies.

HA in IIVs can potentially be subjected to other environmental stresses, such as transient temperature changes, low pH excursions and oxidation during manufacturing, distribution, storage and administration [24]. These stresses, even at mild level under controlled condition, have various impacts on HA conformation and potentially affect vaccine immunogenicity. Virus strain, host culture, vaccine production processes and formulation further complicate the impact of these stresses [25]. The essential role for an *in vitro* potency assay is to accurately measure dose of potent vaccine, assess vaccine potency changes and predict vaccine immunogenicity with correlation to protective efficacy.

Here we report the analysis of mass and SRID potency changes caused by multiple stress techniques: low pH, raised temperature, oxidation, deamidation, freeze/thaw cycles and mechanical stress by vortexing. By examining subunit monovalent bulks (monobulks) from four virus strains, it was determined that low pH and freeze/thaw cycles with HA in PBS buffer consistently abolish vaccine potency as measured *in vitro*. However, raised temperature treatment results in varying outcomes for different viral strains. Mouse studies further show that *in vitro* potency loss due to transient low pH treatment correlates with decreased immunogenicity, but *in vitro* potency loss due to raised temperature stress does not.

2. Results

2.1. Reduction of mass and potency of A/H3N2 HA caused by low pH, raised temperature, oxidation, deamidation, freeze/thaw cycles and mechanical stress

Egg-derived A/Texas/50/2012 (H3N2) HA was stressed by multiple treatments as described in the Materials and Methods section, e.g. low pH, raised temperature, oxidation, deamidation, freeze/thaw cycles or vortexing. Total HA mass was quantified by RP-HPLC by comparing the area-under-curve (AUC) of the HA1 peak using the SRID reference antigen as the standard. Relative HA mass in stressed monobulks was calculated against HA mass from non-stressed monobulk (Fig. 1A). RP-HPLC results showed that the mass for HA stressed by either low pH, oxidation, deamidation, freeze/thaw cycles in either PBS or Tris buffer, or vortexing, was not significantly changed (<+/−20%). Raised temperature reduced HA mass more substantially (>40%).

The same set of stressed A/Texas/50/2012 (H3N2) HAs were tested for *in vitro* potency by SRID and RP-HPLC with trypsin digestion as a preparative step [21]. SRID results (Fig. 1A) showed low pH, raised temperature and freeze/thaw cycles in PBS eliminated SRID signal. Oxidation, freeze/thaw cycles in Tris buffer and vortexing did not substantially change relative SRID values (< +/−20% versus non-stressed HA potency), but deamidation reduced HA potency (∼30%). HA stressed by low pH, raised temperature or freeze/thaw cycle in PBS was also sensitive to trypsin. Quantification with RP-HPLC following trypsinization showed a loss of mass of typsin-resistent HA consistent with SRID potency loss (Fig. 1A). Similar to SRID results, RP-HPLC following trypsinization also showed minor loss of HA potency due to oxidation, freeze/thaw cycles in Tris buffer or vortexing (<+/−20% difference from non-stressed HA potency), and ∼40% potency reduction by deamidation.

The stressed A/Texas/50/2012 (H3N2) HA and their corresponding trypsin-digested samples were analyzed by reduced SDS-PAGE with Coomassie blue staining for HA protein detection (Fig. 1B). The band density for HA1 and HA2 indicating HA mass from the non-digested HA was not significantly changed by the different stress conditions, with the exception of raised temperature, that reduced the signal for both HA1 and HA2, consistent with RP-HPLC results showing no significant HA mass change by all the stress except raised temperature. Post trypsinization, the HA1 bands for HA stressed with low pH or freeze/thaw cycles in PBS were undetectable, while HA2 bands were unchanged, suggesting a similar degradation mechanism by either low pH or freeze/thaw cycles in PBS. Raised temperature, instead, reduced both HA1 and HA2 bands, suggesting a different degradation mechanism. The HA1 and HA2 bands in HA stressed by other stress conditions were not changed by trypsin digestion.

2.2. B Yamagata (By) HA, A/H1N1 HA and B Victoria (Bv) reduction of mass and potency

To compare the effect of these stress conditions on different strains, egg-derived B/Brisbane/9/2014 (By) HA, A/California/07/2009 (H1N1) HA and B/Brisbane/60/2008 (Bv) HA were stressed under the same conditions applied to A/Texas/50/2012 (H3N2) HA. RP-HPLC quantification of B/Brisbane/9/2014 (By) HA showed that the total HA mass was not significantly changed by any of the stress conditions (<+/−20% difference from non-stressed HA mass). Unlike A/Texas/50/2012 (H3N2) HA, B/Brisbane/9/2014 (By) HA mass stressed by raised temperature was not changed (Fig. 2A). Measurements by SRID and RP-HPLC with trypsinization were consistent in showing different levels of