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Oligomerization of Bacterially Expressed H1N1 Recombinant Hemagglutinin Contributes to Protection Against Viral Challenge

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Oligomerization of bacterially expressed H1N1 recombinant hemagglutinin contributes to protection against viral challenge

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Vaccination is the most effective intervention to prevent influenza and control the spread of the virus. Alternatives are needed to the traditional egg-based vaccine strategy for a more rapid response to new outbreaks. Two different hemagglutinin (HA) fragments ($rHA1_{1\text{-}326}$ and $rHA1_{53\text{-}269}$) derived from influenza A virus subtype H1N1 were expressed in *Escherichia coli* and characterized by immunoblot, gel filtration, hemagglutination, and competitive binding assays. $rHA1_{1\text{-}326}$ included neutralizing epitopes and the trimerization domain, whereas $rHA1_{53\text{-}269}$ included only the head of HA with the neutralizing epitopes. Mice were immunized with $rHA1_{1\text{-}326}$ or $rHA1_{53\text{-}269}$, and sera were tested for the presence of neutralizing antibodies. Mice were then challenged with H1N1 and infection severity was monitored. $rHA1_{1\text{-}326}$ trimerized, whereas $rHA1_{53\text{-}269}$ was unable to form oligomers. Both $rHA1_{1\text{-}326}$ and $rHA1_{53\text{-}269}$ elicited the production of neutralizing antibodies, but only oligomerized $rHA1_{1\text{-}326}$ protected against live virus challenges in mice. This study demonstrated that bacterially expressed HA was capable of folding properly and eliciting the production of neutralizing antibodies, and that HA oligomerization contributed to protection against viral challenge. Therefore, prokaryotic-derived vaccine platforms can provide antigenic and structural requirements for viral protection, as well as allow for the rapid and cost-effective incorporation of multiple antigens for broader protection.

Influenza seasonal infections lead to approximately 36,000 deaths in the United States alone each year with an associated annual economic burden of $87.1 billion dollars[1,2]. Vaccinations are the primary method employed to control the seasonal spread of the influenza virus, as well as aid in pandemic preparedness. Almost all current influenza vaccines utilize a lengthy egg-based vaccine manufacturing process, requiring a minimum of five months to generate the vaccine[3]. Additional drawbacks to current egg-based vaccine manufacturing include the vulnerability of chicken populations to disease and limited scale-up capacity. Recent studies have also shown that the virus mutates to adapt to growth in the egg[4], contributing to antigenicity that differs from circulating viral strains by the time the vaccine is ready for use. Therefore, a more rapid process that increases the efficiency and availability of an influenza vaccine is needed.

Seasonal vaccine strains for the northern hemisphere are selected at least six months before the flu season starts. This can result in a mismatch of the vaccine and circulating strains from antigenic drift, resulting in poor protection from the influenza virus[5]. For example, during the 2007–2008 influenza season, A/Wisconsin (H3N2) was selected as the strain to be included in the vaccine, yet A/Brisbane (H3N2) virus became the dominant circulating strain; therefore, the available vaccine provided no protection[6]. Lengthy manufacturing times also impact responsiveness to emerging strains and antigenic shift in the pandemic strains. The 2009 H1N1 pandemic vaccine was released six weeks behind schedule due to manufacturing delays[7]. If vaccines could have been distributed just one month earlier, an estimated 2,200 lives would have been saved in the United States[8].

For influenza, hemagglutinin (HA) is the primary viral protein recognized by the immune system and subsequently is the primary target for vaccine design[9]. HA is composed of two subunits, HA1 and HA2 (Fig. 1). HA1 consists of a globular head, which is responsible for receptor binding and contains neutralizing epitopes Ca1, Ca2,

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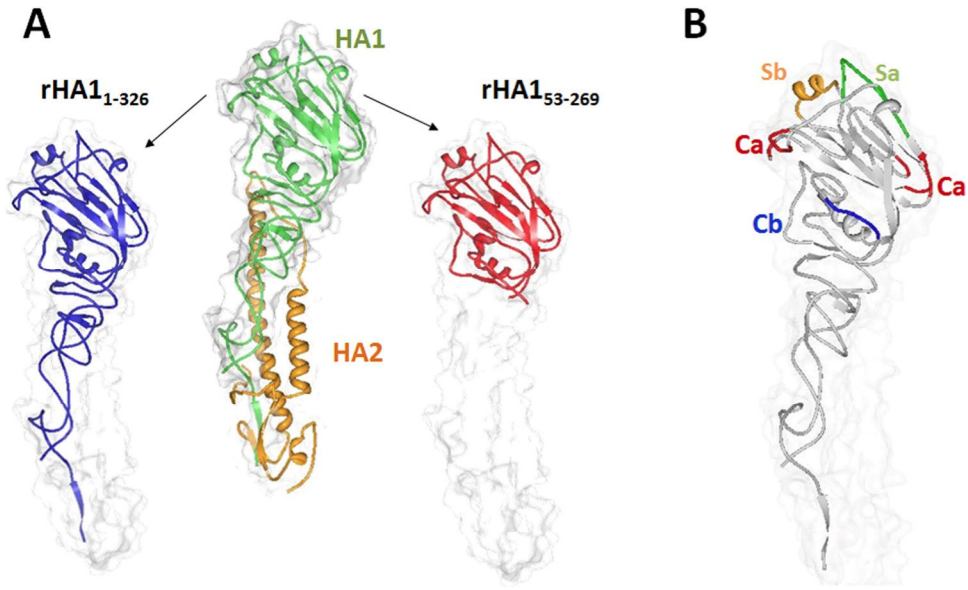


Figure 1. Structural representations of rHA1$_{1-326}$ and rHA1$_{53-269}$. (**A**) Crystal structure models of rHA1$_{1-326}$ and rHA1$_{53-269}$ containing residues 1-326 and 53-269, respectively, are shown and were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB)[21], using the Visualize 3D Viewer (www.rcsb.org) for PDB ID 3AL4. The PDB ID 3AL4 crystal structure was derived from swine-origin A (H1N1)-2009 influenza A virus hemagglutinin[22]. (**B**) The relative location of the H1N1 neutralizing epitopes (Ca, Cb, Sa, and Sb) are shown, and both rHA1$_{1-326}$ and rHA1$_{53-269}$ contained these regions.

Cb, Sa, and Sb. HA2 is composed of a stem structure that supports HA1 and mediates membrane fusion during viral entry. Trimerization of HA is required for complete antigenicity of epitopes Ca1, Ca2, Cb, and Sa[10].

Alternatives to the egg-based production of influenza vaccines include the use of mammalian cell culture or baculovirus expression systems, since appropriate glycosylation of specific epitopes is often needed in order to generate protective antibodies against the infecting pathogen. Therefore, improvements in vaccine manufacturing have typically focused on these expression platforms, due to the belief that post-translation modifications (e.g., glycosylation) were critical for producing HA antigens that elicited neutralizing antibodies[11]. Although investigations to determine the functional role of HA glycosylation are ongoing, others have shown that glycosylation of HA is not required for generating protective antibodies[12], prompting our investigation of a recombinant HA vaccine using a prokaryotic expression system.

Advantages of vaccine generation utilizing a prokaryotic expression platform include rapid production times, readily amendable genetics, and economic savings over cell culture expression systems. Several studies have demonstrated that bacterial expression and subsequent immunization with recombinant HA from H5N1 conferred protection in animal models[13–15]. These findings suggested that bacterially expressed HA immunogens may provide an alternative to egg-based vaccines, especially when folded properly and combined with the appropriate adjuvant. The fast production of large amounts of HA-based vaccines that were derived from a bacterial expression platform may provide timely protection against seasonal influenza and newly emerging pandemic influenza outbreaks. However, essential elements or critical domains of H1N1 have yet to be defined for viral protection, especially when using a prokaryotic expression platform. The herein study reports several critical factors that are required to confer protection when using bacterially expressed HA1 from H1N1.

Results

Cloning and expression of the recombinant HA proteins. Trimerization of HA is hypothesized to be essential for the complete antigenicity of epitopes Ca1, Ca2, Cb, and Sa and the effective generation of protective antibodies. Residues 63–286 have been shown to elicit the production of neutralizing antibodies due to a functional binding site; however, they do not form functional trimers *in vitro*[16]. Structural analyses identified the location of conserved cysteines outside of this region (amino acids 4, 42, 275, 279, and 303), which were incorporated into a recombinant HA protein that was evaluated in this study.

To investigate whether oligomerization of HA monomers would contribute to the overall effectiveness of a recombinant vaccine, two constructs were generated from the influenza A virus subtype H1N1 strain A/California/04/2009 (Fig. 1A). The first recombinant HA construct, rHA1$_{1-326}$, contained residues 1–326, and the second recombinant HA construct, rHA1$_{53-269}$, was comprised of only residues 53–269. Both recombinant proteins were expressed in the soluble fraction with a typical recovery of 2–3 g/L of purified protein. To determine the contribution of oligomerization for enhancing protection, both constructs contained all previously characterized Sa, Sb, Ca1, Ca2, and Cb H1N1 neutralizing epitopes (Fig. 1B).

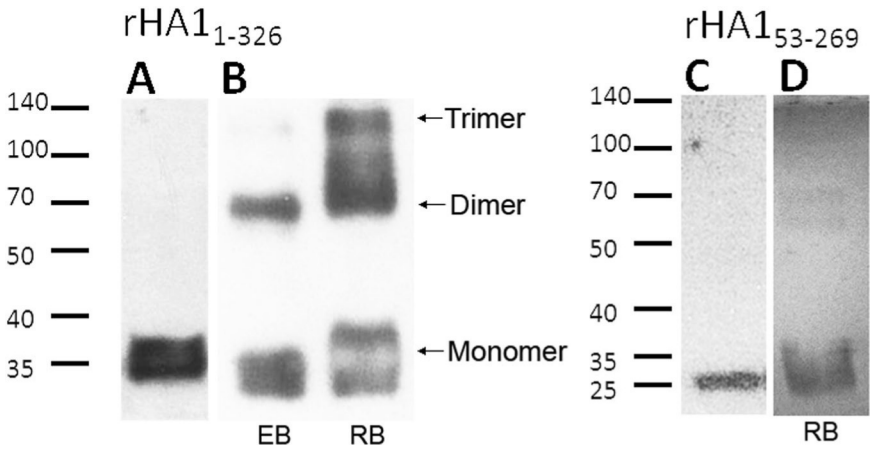
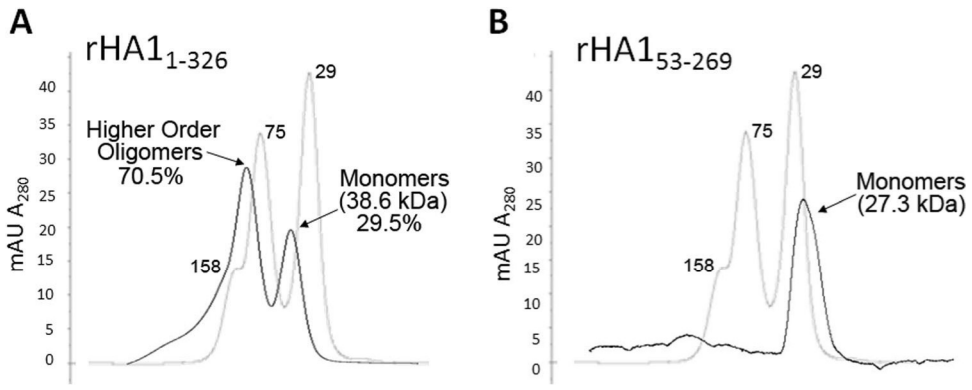


Figure 2. Detection of H1N1 recombinant HA1 oligomers. Immunoblot analyses of the $rHA1_{1-326}$ and $rHA1_{53-269}$ purified proteins were performed under denaturing and non-denaturing conditions with subsequent detection using an anti-His antibody. (**A**) Electrophoretic mobility of $rHA1_{1-326}$ (100 ng) with a predicted MW of 38.6 kDa under denaturing conditions is shown. (**B**) Analysis of $rHA1_{1-326}$ (100 ng) under non-denaturing conditions in elution buffer (EB), and also after buffer exchange in refolding buffer (RB) are shown. (**C**) Analysis of $rHA1_{53-269}$ (40 ng) with a predicted MW of 27.3 kDa under denaturing conditions, and (**D**) under non-denaturing conditions, after buffer exchange in refolding buffer are shown. Molecular weight size markers in kDa are indicated to the left of panels A and C for the denaturing gels. The recombinant HA1 proteins were ran in separate gels under denaturing and non-denaturing conditions, and the uncropped immunoblots of these purified proteins are shown in Supplementary Fig. S1. EB = Elution Buffer, RB = Refolding Buffer.



Characterization of rHA1 proteins by immunoblot and gel filtration chromatography. Both $rHA1_{53-269}$ and $rHA1_{1-326}$ were purified by affinity chromatography and evaluated for their ability to form higher order oligomers, including dimers and trimers. For immunoblot analysis, these recombinant HA1 proteins were probed with an anti-His antibody under denaturing and non-denaturing conditions (Fig. 2A–D). A panel of commercially available buffers designed to assist in protein folding was used to evaluate protein oligomerization under non-denaturing conditions. The refolding buffer that was identified to generate optimal oligomerization of $rHA1_{1-326}$ contained 1.1 M guanidine, 440 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl, 1 mM EDTA, 1 mM glutathione, and 1 mM glutathione disulfide at a pH of 8.2. Following buffer exchange of $rHA1_{1-326}$ with the refolding buffer, there was a significant shift to dimeric and trimeric forms of the protein (Fig. 2A,B). However, no oligomerization of $rHA1_{53-269}$ occurred in this refolding buffer nor any of the other buffers tested, and was present only as a monomeric species under both denaturing (Fig. 2C) and non-denaturing conditions following refolding buffer exchange (Fig. 2D).

Gel filtration chromatography was used as a second method to characterize the composition of higher order oligomers in $rHA1_{1-326}$. Using this approach $rHA1_{1-326}$ dimers and trimers were found to comprise 70.5% of the total recombinant protein species, whereas monomers comprised 29.5% (Fig. 3A), as determined by

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