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Recombinant H7 hemagglutinin forms subviral particles that protect mice and ferrets from challenge with H7N9 influenza virus

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ABSTRACT

A novel avian-origin influenza A H7N9 virus emerged in China in 2013 and continues to cause sporadic human infections with mortality rates approaching 35%. Currently there are no approved human vaccines for H7N9 virus. Recombinant approaches including hemagglutinin (HA) and virus-like particles (VLPs) have resulted in experimental vaccines with advantageous safety and manufacturing characteristics. While high immunogenicity of VLP vaccines has been attributed to the native conformation of HA arranged in the regular repeated patterns within virus-like structures, there is limited data regarding molecular organization of HA within recombinant HA vaccine preparations. In this study, the full-length recombinant H7 protein (rH7) of A/Anhui/1/2013 (H7N9) virus was expressed in Sf9 cells. We showed that purified full-length rH7 retained functional ability to agglutinate red blood cells and formed oligomeric pleomorphic subviral particles (SVPs) of ~20 nm in diameter composed of approximately 10 HA0 molecules. No significant quantities of free monomeric HA0 were observed in rH7 preparation by size exclusion chromatography. Immunogenicity and protective efficacy of rH7 SVPs was confirmed in the mouse and ferret challenge models suggesting that SVPs can be used for vaccination against H7N9 virus.

1. Introduction

Q2 Cases of human infection caused by an avian-origin influenza A H7N9 virus emerged in eastern China in 2013 and have continued into 2015 [1,2]. As of February 23, 2015, the World Health Organization (WHO) had reported 571 infections and 212 deaths, mostly in East China [3]. Cases of H7N9 infection in travelers have also been reported [4,5]. Because humans are immunologically naïve to this subtype, the emergence of a transmissible H7N9 virus presents a significant public health concern. There are currently no approved human vaccines for H7N9 viruses. Experimental vaccines have been previously developed for H7 subtype viruses including live attenuated H7N7 and H7N3 vaccines [6–8], inactivated H7N7 vaccine [9], cell-based H7N1 split virus vaccine [10], and viral vector vaccines [11,12].

Recombinant approaches have resulted in novel influenza vaccines with advantages in safety and manufacturing, and they have been shown to be efficacious against influenza [13–20]. For example, H7N9 recombinant virus-like particle (VLP) vaccine induced protective immunity against H7N9 virus in mice and ferrets [21,22]. Recombinant hemagglutinin (HA) H7 vaccine has also been prepared [23]. HA, the major protein of influenza virus envelope, contains virus-neutralizing epitopes and is included in all currently approved human influenza vaccines, as well as in the majority of experimental vaccines. The observed high immunogenicity of influenza VLP vaccines has been attributed to the organization of HA into regular, highly repeated patterns in the virus resembling structures that favor activation of immune effector functions [14,24]. However, recombinant full-length HA alone is also capable of protecting against infection with influenza viruses [25,26]. Recombinant subunit HA FluBlok vaccine has been approved for

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human vaccination [27]. Studies have shown that immune response can depend on the molecular structure of recombinant HA protein [28]. A previous study has shown that purified rH3 HA forms rosette-shaped subviral particles (SVPs) of approximately 30–50 nm in diameter [29]. However, it is not clear if such SVPs are also formed by rH7 HA and if rH7 HA SVPs are capable of inducing protective immunity in animal models.

In this study, the influenza H7N9 virus H7 gene was cloned into a baculovirus expression vector and expressed in *Spodoptera frugiperda* (Sf9) insect cells. We demonstrate that rH7 protein from Sf9 cells forms high-molecular weight SVPs that elicit protective immunity in mouse and ferret challenge models. These results have implications for understanding immunogenicity of rH7 HA protein and vaccination against H7N9 influenza virus.

2. Materials and methods

2.1. Expression of H7 gene and preparation of rH7 protein

The sequence of influenza H7 HA gene of A/Anhui/1/2013 (H7N9) virus was obtained from the GISAID EpiFlu database (www.gisaid.org), accession number EPI439507. The HA gene was codon-optimized for high-level expression in Sf9 insect cells (Life Technologies, Carlsbad, CA) and synthesized biochemically (Genscript, Piscataway, NJ). For expression, full-length H7 HA gene was cloned into baculovirus (BV) pFastBac1 transfer vector between BamHI–HindIII sites downstream from a polyhedrin promoter, as described elsewhere [17,21]. Recombinant BV expressing H7 HA gene was generated by using a Bac-to-Bac baculovirus expression system (Life Technologies). The presence of BV during virus stock preparation was monitored by Virus Counter (ViroCyt, Boulder, CO). For infection of Sf9 cells, the titer was determined by plaque assay in Sf9 cells and expressed as plaque forming units (PFU)/ml. Recombinant baculovirus stocks were prepared by infecting cells at a low multiplicity of infection (MOI) of 0.01 PFU per cell and harvested at 68–72 h post infection.

For expression of rH7 HA, Sf9 cells were maintained as suspension cultures in SF-900 II insect serum free medium (Life Technologies) at 27 ± 2 °C. Sf9 cells (2×10^6 cells/ml) were infected at a MOI of 3 for 70–72 h with BV expressing H7 gene. Briefly, infected Sf9 cells were incubated with continuous agitation at 27 ± 2 °C and harvested by centrifugation at 4000 × g for 15 min. To confirm expression of rH7, a 0.3 ml aliquot of infected Sf9 cells was seeded into eight-well Nunc chamber slide for immunofluorescence assay (IFA). Following 72 h incubation at 27 °C, Sf9 cells were fixed with cold acetone, and IFA was carried out as described elsewhere [30] using H7-specific chicken antiserum. Antigen-expressing cells were visualized using FITC-conjugated goat IgG (H+L) (KPL, Gaithersburg, MD).

The rH7 protein was prepared from the 2 l Sf9 culture. Sf9 cells were harvested and solubilized using treatment with Triton X-100 or Tergitol-NP9 non-ionic surfactant for 1 h at 20 °C, essentially as described elsewhere [29,31]. After solubilization, the cell lysate was clarified by centrifugation and the rH7 protein was purified from the lysate by lectin affinity chromatography. The purified rH7 protein was dialyzed against buffer containing 20 mM Tris–HCl pH 7.4, 150 mM NaCl and 0.01% Polysorbate 80 (PS80) non-ionic surfactant. This procedure resulted in a highly purified rH7 protein preparation. Finally, purified rH7 protein was filter-sterilized using a 0.2 μm filter and stored at −80 °C.

2.2. Characterization of rH7 protein

Protein expression was examined by SDS-PAGE using 4–12% polyacrylamide gels followed by staining with GelCode Blue stain (Pierce, Rockford, IL). The content of rH7 protein was quantified by Qubit 2.0 fluorometer (Life Technologies). Western blot was performed using chicken antibody specific to avian H7N3 virus (a gift from Darrell Kapczynski). Cross-linking studies were done using treatment of rH7 protein with 0.025% glutaraldehyde for 5 min at 37 °C. Before cross-linking reaction, rH7 protein sample was diluted to reduce the effects of free amine in the reaction. Furthermore, due to the dilution, cross-linking is expected to occur within the trimers, rather than between different trimers. The cross-linking reaction was stopped by adding 0.1 volume of 1 M Tris–HCl pH 8.0, and the rH7 preparation was as examined by SDS-PAGE and western blot.

The hemagglutination titer was determined by serially diluting rH7 protein at twofold increments in 50 μl in phosphate buffered saline (PBS) in a 96-well plate. To each dilution, 50 μl of 1% turkey red blood cell (RBC, Lampire, Pipersville, PA) working solution was added, mixtures of rH7 and RBCs were gently agitated and the plate was incubated at 25 °C for 30–60 min before visual examination as recommended by the WHO [32]. Negative hemagglutination results appeared as dots in the center of the wells and activity was expressed in hemagglutination units (HAU). The titer was determined as the highest dilution factor that produced a positive reading.

Purified rH7 preparations were also evaluated by transmission electron microscopy (TEM) to demonstrate presence of SVPs. Purified rH7 was adsorbed at concentrations 50–200 μg/ml onto freshly discharged 400 mesh carbon parlodion-coated copper grids (Poly-Sciences, Warrington, PA). The grids were rinsed with buffer containing 20 mM Tris–HCl, pH 7.4, and 120 mM KCl, negatively stained with 1% phosphotungstic acid, and dried by aspiration. SVPs were visualized on a Hitachi H-7600 transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL) operating at 80 kV and digitally captured with a CCD camera at 1k × 1k resolution (Advanced Microscopy Techniques Corp., Danvers, MA).

2.3. Analytical chromatography

A size exclusion chromatography (SEC) column XK16/40 containing S300 resin (GE Healthcare Bio-Sciences, Pittsburgh, PA) was calibrated essentially as described previously [33] using molecular weight standards (Bio-Rad Laboratories, Hercules, CA), which included thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa). Then, rH7 preparation was subjected to chromatography. Because of the presence of PS80 surfactant in the rH7 preparation, optical density readings were unreliable, and the chromatography profile was determined by western blot. Fractions (2 ml each) from the S300 column were collected and chromatography peaks were determined by SDS-PAGE and western blot for the presence of rH7 antigen.

2.4. Vaccinations and challenge of mice

All animal experiments were performed under the guidance of the Institutional Animal Care and Use Committees and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facilities. For vaccination studies, 8–9 week old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) (five per group) received two vaccinations 3 weeks apart with 5 μg of rH7 SVP in PBS in 50 μl volume intranasally (i.n) with, or without, 5 μg of Monophosphoryl Lipid A (MPL) adjuvant (Invivogen, San Diego, CA). Three weeks following boost, mice were challenged i.n. with 10 mouse 50% lethal dose ($10 \times MLD_{50}$) of A/Anhui/1/2013 (H7N9) virus and morbidity (weight loss) and mortality were determined. Survival and body

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