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Development of influenza H7N9 virus like particle (VLP) vaccine: Homologous A/Anhui/1/2013 (H7N9) protection and heterologous A/chicken/Jalisco/CPA1/2012 (H7N3) cross-protection in vaccinated mice challenged with H7N9 virus



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ABSTRACT

The recent emergence of severe human illness caused by avian-origin influenza A(H7N9) viruses in China has precipitated a global effort to rapidly develop and test vaccine candidates. To date, non-A(H7N9) H7 subtype influenza vaccine candidates have been poorly immunogenic and difficulties in production of A(H7N9) virus seed strains have been encountered. A candidate recombinant A(H7N9) vaccine consisting of full length, unmodified hemagglutinin (HA) and neuraminidase (NA) from the A/Anhui/1/2013 and the matrix 1 (M1) protein from the A/Indonesia/05/2005 (H5N1) were cloned into a baculovirus vector. Baculovirus infected *Spodoptera frugiperda* (Sf9) insect cells secreted virus like particles (VLP) composed of HA, NA, and M1 that resemble mature influenza virions. Genetic construction of vaccine from acquisition of an H7N9 genomic sequence to production of A(H7N9) VLP occurred in 26 days. The immunogenicity and efficacy of A/Anhui/1/2013 (H7N9) VLP vaccine administered on days 0 and 14 were evaluated in a lethal wild-type challenge Balb/c mouse model. Control groups included a non-homologous H7 vaccine (A/chicken/Jalisco/CPA1/2012 (H7N3)-VLP), and A/Indonesia/05/2005 (H5N1)-VLP, or placebo. All vaccines were administered with or without ISCOMATRIX. A(H7N9) VLP elicited hemagglutination-inhibition (HAI) antibody titers of $\geq 1:64$ against the homologous virus, cross-reactive HAI against the heterologous A(H7N3), and 3- to 4-fold higher HAI responses in corresponding ISCOMATRIX subgroups. Similarly, all doses of H7N9 VLP elicited anti-neuraminidase (NA) antibody, with 3- to 4-fold higher responses measured in the corresponding ISCOMATRIX subgroups. The non-homologous H7 vaccine induced both H7N3 and H7N9 HAI but no N9 anti-NA antibodies. A lethal murine wild-type A/Anhui/1/2013 (H7N9) challenge demonstrated 100% survival of all animals receiving A(H7N9) and A(H7N3) vaccine, versus 0% survival in A(H5N1) vaccine and placebo groups. Together, the data demonstrate that recombinant H7N9 vaccine can be rapidly developed that was immunogenic and efficacious supporting testing in man as a pandemic influenza H7N9 vaccine candidate.

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1. Introduction

Following recognition of the first human infections with avian-origin influenza A(H7N9) and their attendant severity in March 2013, public health officials from around the world have called for immediate and preemptive development of surveillance,

diagnostic and clinical intervention tools in the event that these viruses become readily transmissible among humans [1–3]. The Arg292Lys mutation in the virus neuraminidase (NA) gene known to confer resistance to both zanamivir and oseltamivir has already been identified in two patients [4], which places further pressure on the development and clinical testing of candidate vaccines.

Nearly all influenza vaccines commercially distributed in the world today are conventional inactivated “split” or subunit vaccines that require a vaccine virus that grows well enough in eggs or cultured mammalian cells to produce sufficient amounts of the essential vaccine antigen, hemagglutinin (HA). This process is iterative and often time-consuming, and occasionally requires selection of an HA that is less than optimally matched to the HA of

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predominantly circulating strains [5]. The development of recombinant HA-based vaccines offer the potential for overcoming these limitations [6], but some observers remain concerned that HA-based vaccines could be inadequately effective, especially in vulnerable populations such as the elderly, who appear to be a critical target population for A(H7N9)-mediated severe disease [7].

An A(H7N9) vaccine candidate was constructed from full length, unmodified influenza hemagglutinin (HA) and neuraminidase (NA) from the A/Anhui/1/2013 strain and the matrix 1 (M1) protein from the A/Indonesia/05/2005 (H5N1) strain. The corresponding genes were cloned into a baculovirus expression vector to produce A(H7N9) VLP in *Spodoptera frugiperda* (Sf9) insect cells. The expressed HA, NA, and M1 self-assembled into virus-like particles (VLP) and were secreted as enveloped, pleomorphic particles that resemble live, mature influenza virions. Previous VLP vaccine candidates developed for H1N1, H3N2, B strains and H5N1 have been shown to display conformational-dependent antigenic epitopes associated with HA oligomers and have induced robust anti-HA and anti-NA antibody responses in clinical studies in over 6000 subjects [8,9].

In this report, we describe the rapid development of an A/Anhui/1/2013 (H7N9) vaccine candidate and accompanying potency reagents. The immunogenicity, efficacy, and induction of cross-protective antibodies in immunized mice challenged with a lethal dose of A/Anhui/1/2013 influenza virus following immunization with VLPs representing homologous A(H7N9), heterologous A(H7N3), and a control A(H5N1) with and without the saponin-based ISCOMATRIX are described.

2. Materials and methods

2.1. Recombinant baculovirus and insect cells

The HA and NA protein sequences of influenza A/Anhui/1/2013 (H7N9) were obtained from GISAID Epiflu database (www.gisaid.org) with accession number EPI439507 and EPI439509, respectively. A/Shanghai/1/2013 (H7N9) HA and NA were obtained from GISAID Epiflu database with accession number EPI439486 and EPI439487, respectively. The HA and NA protein sequences of A/chicken/Jalisco/CPA1/2012(H7N3) were obtained from NCBI GenBank with accession number AFN85519 and AFN70735, respectively. The HA, NA and M1 protein sequences of A/Indonesia/05/2005 (H5N1) were from NCBI GenBank with accession number ABP51969, ABW06107 and ABI36004, respectively. The genes were codon optimized for optimal expression in insect cells and biochemically synthesized for A(H5N1) HA, NA, M1 (Geneart AG, Regensburg, Germany); and for A(H7N9) and A(H7N3) HA and NA genes (Genscript, Piscataway, NJ). Influenza VLP were produced using full length, HA and NA genes specific for each strain combined with common influenza A/Indonesia/05/2005 M1 as described [10,11]. Full length HA, NA, and M1 genes were cloned between BamHI–HindIII sites in pFastBac1 baculovirus transfer vector plasmid (Invitrogen, Carlsbad, CA) such that each gene was under the transcriptional control of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin promoter. Recombinant baculovirus construct was plaque purified and master seed stocks prepared, characterized for identity, and used to prepare working virus stocks. The titers of baculovirus master and working stocks were determined by using rapid titration kit (BacPak Baculovirus Rapid Titer Kit; Clontech, Mountain View, CA).

S. frugiperda Sf9 insect cells (ATCC CRL-1711) were maintained as suspension cultures in HyQ-SFX insect serum free medium (HyClone, Logan, UT) at $27 \pm 2^\circ\text{C}$. Recombinant baculovirus stocks were prepared by infecting cells at a low multiplicity of infection (MOI) of ≤ 0.01 plaque forming units (pfu) per cell and harvested at 68–72 h post infection (hpi).

2.2. Recombinant influenza VLP, HA, and NA

A(H7N9), A(H7N3), and A(H5N1) VLP and influenza A(H7N9) HA and NA antigens were produced in Sf9 cells at $2\text{--}3 \times 10^6$ cells/ml infected with specific recombinant baculovirus. Infected Sf9 cells were incubated with continuous agitation at $27 \pm 2^\circ\text{C}$ and harvested at 68–72 hpi by centrifugation at $4000 \times g$ for 15 min. VLP were purified from the infected cell media. Briefly, media containing VLP and baculovirus particles was concentrated and dialyzed against buffer using hollow fiber tangential flow filtration. Separation of VLP from baculovirus and other host contaminants was done using anion exchange followed by gel filtration chromatography. Purified VLP in phosphate buffered saline were 0.2 micron filtered and stored at $2\text{--}8^\circ\text{C}$.

Full length A(H7N9) HA and NA glycoproteins were produced in Sf9 cells as described above. Recombinant HA and NA were extracted from cellular membranes with 25 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.5% Tergitol NP9 and 2 $\mu\text{g}/\text{ml}$ Leupeptin and insoluble material removed by centrifugation at $10,000 \times g$ for 30 min. HA and NA oligomers were purified using a combination of anion exchange, lectin affinity and cation exchange chromatography. During purification the detergent is removed allowing HA trimers and NA tetramers to form higher ordered protein–protein micellar nanoparticles. Purified, detergent-free NA and HA nanoparticles were 0.2 μm filtered and stored at -80°C .

2.3. Analytical methods

H7N9 HA, NA and VLP were analyzed by SDS-PAGE using 4–12% gradient polyacrylamide gels (Invitrogen), stained with GelCode Blue stain reagent (Pierce, Rockford, IL) and quantified by scanning densitometry using OneDscan system (BD Biosciences, Rockville, MD). Purified A(H7N9), A(H7N3), and A(H5N1) VLP were tested for total protein concentration (BCA bicinchoninic acid protein assay, Pierce Biochemicals) and particle size by dynamic light scattering using ZETASizer Nano (Malvern Instruments, PA) using standard manufacturer recommended methods.

To identify the major proteins in purified A(H7N9) NA and HA, samples were tested by peptide mapping and liquid chromatography–mass spectrometry (LC–MS). 50 μL of NA or HA were mixed with 100 μL 25 mM ammonium bicarbonate containing 0.08% RapiGest and 4 μL of 250 mM DDT solution and heated at 60°C for 30 min. After cooling to room temperature (RT), 8 μL of 250 mM iodoacetamide was added and incubated at room temperature in the dark for 30 min. 10 μL of 1 $\mu\text{g}/\mu\text{L}$ trypsin solution was added to digest the sample for 5 h at 37°C . The digested samples were incubated for 20 min, followed by centrifugation at $20,000 \times g$ for 20 min, and LC–MS analysis performed on an Agilent 1200 system (Agilent, Santa Clara, California). Protome Discover 1.3 software (Thermo Scientific, Logan, UT) was used for database searching of a custom protein database including sequences for baculovirus, insect cells, and influenza genes.

Capillary gel electrophoresis (CE) was carried out using a Beckman PA 800 plus Pharmaceutical Analysis System equipped with an untreated fused-silica capillary of 60 cm, 375 μm I.D. detector at a UV absorbance of 220 nm. Prior to each run, the capillary is regenerated by base and acid wash then rinsed with water and refilled with buffer before each sample injection. Electrophoretic separations were carried out at a constant voltage of 30 K volts for 30 min. 32 Karat software from the manufacturer was used to process data. Sample buffer was 1% SDS, 10% β -mercaptoethanol, 100 mM Tris and 1 μL of P10KD marker per 50 μL of sample. A(H7N9) VLP sample was mixed 1:1 with sample buffer and incubated at 70°C for 10 min to denature and reduce VLP proteins. Denatured samples were incubated with Triton X-100 and PNGase F for >2 h at 37°C to remove N-linked glycans prior to analysis. A P10KD marker was

used as internal standard to align peaks from different electropherograms.

2.4. Influenza viruses

The A/Anhui/1/2013 (H7N9) virus was kindly obtained from Todd Davis, Ph.D., Team Lead, Zoonotic Virus Team, Influenza Division, CDC, Atlanta, GA. The wild-type A/Anhui/1/2013 (H7N9) virus stock was produced (BIOQUAL, Inc., Rockville, MD) by inoculating 10-day old embryonated hens eggs with a A/Anhui/1/2013 (H7N9) seed virus CDC #2013759189 (Centers for Disease Control, Atlanta, GA). A/Anhui/1/2013 (H7N9) virus had an infectious titer of $10^{7.95}$ tissue culture infectious doses/ml (TCID₅₀) in Madin Darby canine kidney cells (MDCK) and a 50% lethal dose (LD₅₀) in adult female BALB/c mice of 1200 TCID₅₀/ml.

2.5. Electron microscopy

A(H7N9) purified VLP were adsorbed by flotation for 2 min onto a freshly discharged 400 mesh carbon parlodion-coated copper grid (Poly-Sciences, Warrington, PA). The grids were rinsed with buffer containing 20 mM Tris, pH 7.4, and 120 mM KCl and negatively stained with 1% phosphotungstic acid, then dried by aspiration. Virus-like particles 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).

2.6. Immunological assays

Individual sera were tested for HAI titers against A/Anhui/1/2013 (H7N9) influenza virus, A/Indonesia/05/2005 (H5N1), A/chicken/Jalisco/CPA1/2012 (H7N3) and A/Shanghai/1/2013 (H7N9) VLP. Sera were treated with receptor destroying enzyme (RDE) at 37 °C overnight. The following day, RDE was inactivated by incubation at 56 °C for 1 h. Individual mouse sera (25 μL) were serially diluted (starting at an initial sample dilution of 1:10) and incubated with 4-HA units of influenza virus or VLP in 25 μL. Fresh horse red blood cells (0.75%) (Lampire, Pipersville, PA) were added to the virus for one hour. Red cell agglutination status was determined at the end of the incubation period. The HAI titer was defined as the reciprocal titer at which no agglutination was observed. The lower limit of quantitation for the assay was 10; titers lower than 10 were considered to be negative and assigned a value of 5.

Neuraminidase inhibiting (NI) antibody titers were determined in an enzyme-linked lectin assay using peroxidase-labeled peanut agglutinin (PNA-PO; Sigma, St Louis, MO) to bind to desialylated fetuin. NA activity was determined by incubating serial dilutions of purified, full length A(H7N9) NA on fetuin coated microtiter plates. The use of purified NA eliminated HA which can interfere in a fetuin-based NI assay. After 30 min incubation at RT, plates were washed and PNA-PO added. After 1 h incubation at RT, plates were again washed and the peroxidase substrate 3,3',5,5'-tetramethylbenzidine added and color development allowed to proceed for 10 min. Color development was stopped and the plates the OD450 measured using a SpectraMax plus plate reader (Molecular Devices, Sunnyvale, CA). Data were plotted as a 4-parameter-fit plot and the dilution corresponding to 95% NA activity determined.

NI titers against A(H7N9), A(H7N3), and A(H5N1) were measured beginning at a 1:20 dilution of mouse sera followed by 2-fold serial dilutions in 96-well U-bottomed tissue culture plates. NAs corresponding to 95% maximum activity were added to diluted sera and incubated for 30 min at RT after which sera/NA samples were transferred to fetuin coated microtiter plates. Plates were

incubated for 2 h at 37 °C, washed and PNA-PO added. The plates incubated at RT an additional hour, washed and peroxidase substrate TMB added. Color development was stopped after 10 min and the OD450 of the plates measured using a SpectraMax Plus plate reader (Molecular Devices, LLC, and Sunnyvale, CA). The data were analyzed using the manufacture's SoftMax software and NI titers defined as the reciprocal dilution at which 50% NA activity was inhibited. The lower limit of quantitation for the assay was 20; titers lower than 20 were considered to be negative and assigned a value of 10.

2.7. Mouse immunization and challenge

Balb/c mice (6–8 weeks old) were purchased from Harlan Laboratories, Inc. (Fredrick, MD). Mice (8 per group) were immunized intramuscularly days 0 and 14 in the quadriceps muscles with A/Anhui/1/2013 (H7N9), A/chicken/Jalisco/CPA1/2012 (H7N3), or A/Indonesia/05/2005 (H5N1) VLP (0.6–6 μg based on HA content) with and without 1.9 ISCO™ units (IU) of ISCOMATRIX™ adjuvant (ISCOMATRIX; CSL Biotherapies Inc., King of Prussia, Pennsylvania, USA; ISCOMATRIX is a registered trademark of ISCOTEC Ab a CSL company; ISCO is a registered trademark of CSL). Immunizations were done on day 1 and 14 and serum sera collected on day 0 and 21 for serological analysis. Blood was collected via the retro-orbital route and serum was obtained by centrifugation of whole blood.

Mice were transferred to a biological safety level 3 (BSL3) facility for influenza challenge. Influenza A/Anhui/1/2013 virus challenge with 4.4×10^3 TCID₅₀ pfu (equal to $3.6 \times$ LD₅₀) was performed on day 22. Prior to virus instillation, mice were anaesthetized with intra-peritoneal (IP) injection of a ketamine (125 mg/kg)/xylazine (2.5 mg/kg) HCl solution. The mice were then positioned on their back and 25 μL virus solution delivered drop-wise into each nostril. After challenge, the animals were observed for signs of illness and weighed daily for 14 days to assess morbidity. Mice identified as moribund or having lost >25% of body weight were euthanized.

On day 3 post challenge three animals per group were euthanized and lung tissue collected for virus isolation. Lung tissue was weighed and frozen. Cryopreserved lungs were quickly thawed and homogenized in 0.5 mL cold medium (DMEM/10% FBS/gentamicin) for approximately 20 s using a hand-held tissue homogenizer (Omni International, Kennesaw, GA), and spun down to remove debris. The resultant supernatant was added in 10-fold graded dilutions to MDCK (Madin-Darby canine kidney) cells (ATCC, Manassas, VA) monolayers in 96-well tissue culture plates. Specifically, the supernatant was added to the first row of wells (10 μL/well) of a 96-well tissue culture plate and then diluted 10-fold (6 wells per dilution) for a total of 6 dilutions. The first well represented a 1:10 dilution of the sample. The plates were incubated at 37 °C for a 2-day period. Fifty (50) μL from each well was transferred to a 96-well V-bottomed plate and 50 μL 0.5% Turkey RBC (Lampire, Pipersville, PA) in PBS added to each well. The hemagglutination pattern was read following 30 min at RT. The Reed–Muench formula was applied to calculate virus titer and was expressed as log₁₀ TCID₅₀/g of tissue.

All animal procedures were in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

2.8. Statistical analysis

Statistical analyses were performed using SAS® version 9.1.3 in a Windows environment (SAS Institute Inc., Cary, NC, USA). Antigen-specific antibody response as measured by neutralizing antibody to HA and NA. Results were summarized using the geometric mean titer (GMT) and corresponding 95% CI.

Differences in GMTs among vaccine groups were analyzed using an ANOVA model with fixed effects for vaccine group. Modeling was performed using PROC MIXED in SAS with data from Day 21. The data at Day 21 were log₁₀-transformed prior to statistical analysis. The overall treatment difference was assessed using global *F*-test. Pairwise comparisons of vaccine groups was analyzed and estimated by least squares mean (LSMEANS) at Day 21 adjusted by Tukey. A *p*-value <0.05 was considered significant for vaccine group comparisons.

3. Results

3.1. Influenza A(H7N9) HA, NA, and VLP

Baculovirus vectors were constructed to express A/Anhui/1/2013 (H7N9), A/chicken/Jalisco/CPA1/2012 (H7N3), and A/Indonesia/05/2005 (H5N1) VLP with HA, NA, and M1 genes in tandem such that each were flanked with a 5'-AcMNPV polyhedrin promoter (PolH), 3'-polyadenylation signal (pA), with a 5' to 3' order of NA, M1, and HA (Fig. 1A). A/Anhui/1/2013 (H7N9) HA and NA were also expressed in separate baculovirus vectors.

Purified A(H7N9) NA and HA; and A(H7N9), A(H7N3), and A(H5N1) VLP were analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 1B; left panel). At the time of the analysis, no specific A(H7N9) antibodies were available for identification. However, a mouse monoclonal antibody against another H7 subtype, A/Netherlands/219/2003 (H7N7) (SinoBiological, Beijing, China), did recognize A(H7N9) and A(H7N3) HAs in a Western blot (Fig. 2B; second panel Western blot). To identify NAs, a rabbit antibody against a conserved NA peptide ILRTQESEC reacted strongly with A(H7N9) NA and VLPs as well as NA in both A(H7N3) and A(H5N1) VLP (Fig. 1B, third panel Western blot). Sheep anti A(H5N1) HA recognized only the homologous H5 hemagglutinin (Fig. 1B, forth panel Western blot). The major proteins in purified A(H7N9), A(H7N3), and A(H5N1) VLP preparations were HA and M1 with less but detectable NA (Fig. 1B). Baculoviruses were not completely removed but major structural nucleocapsid proteins vp39 and p6.9 were not detectable by SDS-PAGE (Fig. 1B). The major baculovirus envelop glycoprotein gp64 was separated from HA by CE electrophoresis and estimated to be about 5% of the total protein in purified VLPs (data not shown).

To confirm the identity of A/Anhui/1/2013 (H7N9) HA and NA, LC-MS analysis was performed with purified antigens. Over 70% of the A/Anhui/1/2013 HA and NA were identified as having the expected amino acid sequences, confirming their identity.

HA and NA antigens in A(H7N9) and A(H7N3) VLP essentially comigrate by SDS-PAGE limiting the use of scanning densitometry to estimate the HA concentration and thus the dose for immunization. However, capillary gel electrophoresis of deglycosylated A(H7N9) VLP resolved HA and NA with a single sharp deglycosylated HA peak and a leading smaller deglycosylated NA peak (Fig. 1C). The identity of M1 and PNGase F related peaks were also identified (Fig. 1C). The percentage of each peak was calculated based on each peak area divided by total peak area with PNGase F and P10KD marker related peaks excluded. Peak area was corrected by velocity by the software. The average of triplicate measurements showed 28.5% HA and 5.1% NA of total protein in purified A(H7N9) VLP.

Transmission electron microscopy of purified A/Anhui/1/2013 (H7N9) VLP was consistent with a size of about 150 nm measured using dynamic light scattering (Fig. 1D). The A(H7N9) VLP were largely spherical, pleomorphic, enveloped and with a high density of protein spikes presumed to be HA and NA oligomers projecting from the surface of each particle.

3.2. Immunogenicity of influenza A(H7N9), A(H7N3), and A(H5N1) VLP

BALB/c mice were immunized with 0.7, 2 and 6 µg of influenza A(H7N9) VLP, 0.7 and 2 µg of A(H7N3) VLP or 1 and 3 µg of A(H5N1) VLP vaccines (based on HA content and corrected for %HA based on capillary electrophoresis) on days 0 (prime) and 14 (boost) and serum samples were obtained on day 0 and 21 for the measurement of antibody responses. Anti-HA responses were evaluated in HAI assays using A/Anhui/1/2013 (H7N9) virus (Fig. 2A), A(H7N9) VLP derived from A/Shanghai/1/2013 virus (Fig. 2B), and A(H7N3) VLP derived from A/chicken/Jalisco/CPA1/2012 virus (Fig. 2C). VLP were used in H7N9 A/Shanghai/1/2013 and H7N3 A/chicken/Jalisco/CPA1/20 HAI assays as virus reagents were not available.

Day 21 sera from unadjuvanted A(H7N9) VLP immunized mice showed HAI antibody titers to all be >1:64 against the homologous A/Anhui/1/2013 H7N9 virus (Fig. 2A). High anti HA responses at all doses tested were likely due to being at the maximum end of the response curve in mice. Immunization with VLP adjuvanted with 1.9IU of ISCOMATRIX enhanced HAI antibody responses by 3- to 4-fold. As expected A/Anhui/1/2013 (H7N9) VLP induced HAI responses against the closely related with A/Shanghai/1/2013 (H7N9) (Fig. 2B) but also the heterologous H7N3 influenza A/chicken/Jalisco/CPA1/2012 (Fig. 2C). Levels of cross-reactive HAI antibody were also enhanced 3- to 4-fold in VLP formulated with ISCOMATRIX adjuvant. A quantitative comparison of HAI titers observed with homologous and heterologous H7 is difficult as homologous H7 was presented on virus and heterologous H7 was presented on VLP. However, HAI titers were similar between the two heterologous H7 VLP even though the H7 expressed by the A/chicken/Jalisco/CPA1/2012 (H7N3) VLP is more divergent from A/Anhui/1/2013 H7 than is A/Shanghai/1/2013 H7.

Sera from mice immunized with unadjuvanted A/chicken/Jalisco/CPA1/2012 (H7N3) VLP showed HAI antibody titers of 1:32 when evaluated against the homologous A(H7N3) VLP and when formulated with ISCOMATRIX HAI titers against the A(H7N3) VLP were increased 2- to 4-fold (Fig. 2C). Sera were also evaluated against virus or VLP expressing the heterologous H7. For the 0.7 and 2 µg dose of unadjuvanted A(H7N3) VLP, HAI titers of 24 (*p* < 0.001) and 26 (*p* < 0.001) for the A/Anhui/1/2013 (H7N9) and 18 (*p* < 0.006) and 14 (*p* = 0.071) for A/Shanghai (H7N9) VLP were observed. While these titers are low, three of the four groups demonstrated significant titers when compared to pre-immunization sera. Just as the use of the ISCOMATRIX enhanced the titers against the homologous H7, titers were also significantly enhanced against the heterologous H7 (Fig. 2A and C). GMTs of 142, and 103 against A/Anhui/1/2013 (H7N9) and 56 and 56 for A/Shanghai (H7N9) VLP were observed for the 0.7 and 2 µg dose. The data again indicated the generation of cross-reactive H7 antibody and the enhancement of these responses by ISCOMATRIX adjuvant.

While cross-reactivity was observed on heterologous H7 following immunization with either A(H7N9) or A(H7N3) VLPs, no HAI responses were observed when sera was tested on A/Indonesia/05/2005 (H5N1) VLP (Fig. 2D). Similarly, no anti-H7 reactivity was observed following immunization A/Indonesia (H5N1) VLP indicating the expected strain specificity for the HAI response (Fig. 2A, B and C). A(H5N1) VLP were immunogenic and immunization with both 1 and 3 µg induced HAI titers >1:64 and responses were enhanced 4-fold in the presence of ISCOMATRIX (Fig. 2D).

The induction of anti-NA antibodies were evaluated in a NI assay using purified N9 neuraminidase. Sera from A/Anhui/1/2013 (H7N9) VLP immunized mice demonstrated good levels of NI activity and immunization of A(H7N9) VLP with the ISCOMATRIX

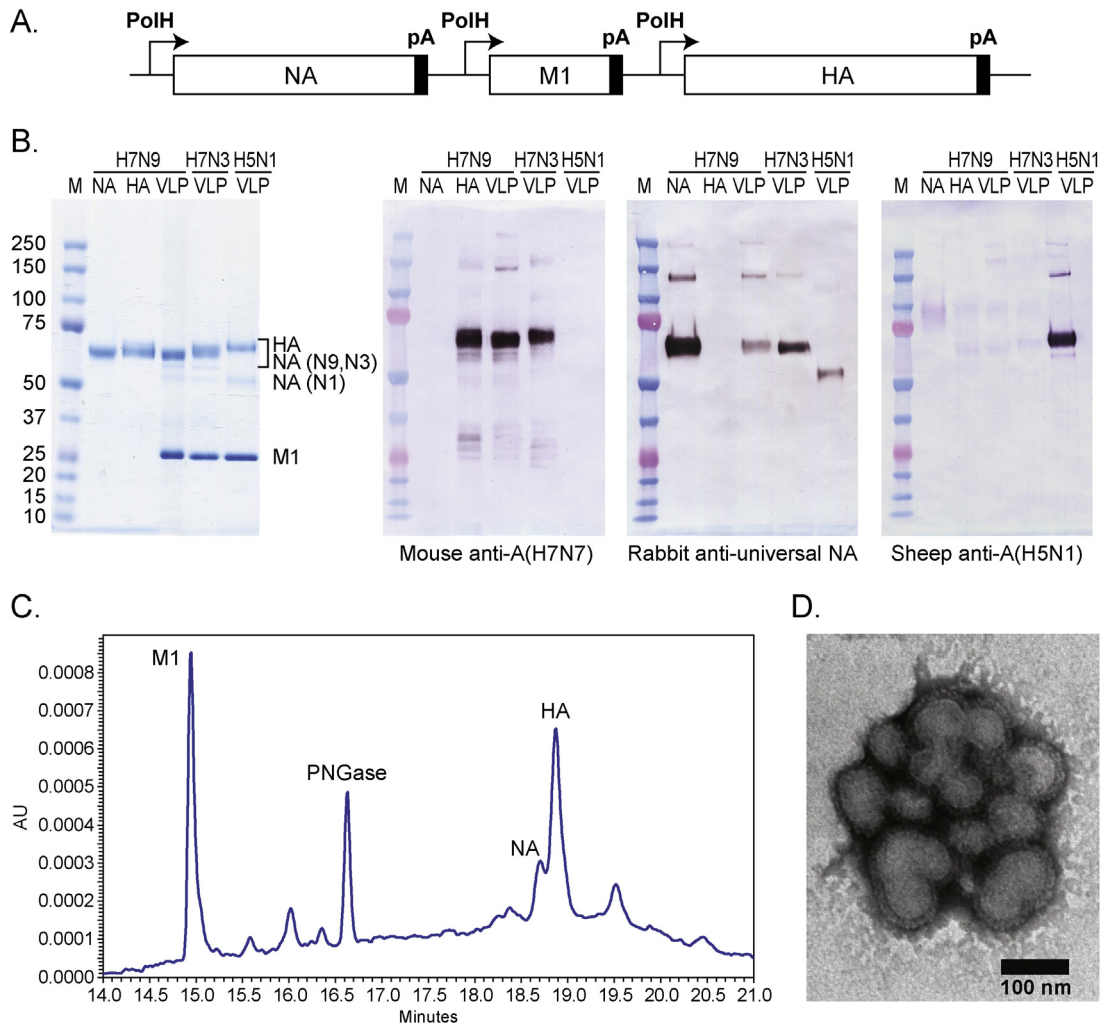


Fig. 1. (A) Diagram of influenza A/Anhui/1/2013 (H7N9) NA and HA and A/Indonesia/05/2005 (H5N1) M1 genes each under the transcriptional control at the 5' of the AcMNPV polyhedrin promoter (PoIH) and 3' polyadenylation (pA) signals. (B) SDS-PAGE and Coomassie blue stained gel and Western blot analysis of A/Anhui/1/2013 (H7N9) NA, HA, and VLP; A/Chicken/Jalisco/CPA1/2012 (H7N3) VLP and A/Indonesia/05/2005 VLP against mouse monoclonal anti A(H7N7), rabbit anti-universal conserved NA peptide, and sheep anti A(H5N1) HA; respectively. (C) Capillary electrophoresis of A/Anhui/1/2013 (H7N9) VLP. (D) Electron micrograph of negatively stained A/Anhui/1/2013 (H7N9) VLP.

resulted in a significant enhancement in NI titers (Fig. 3). Antisera from mice immunized with the A/chicken/Jalisco/CPA1/2012 (H7N3) or A/Indonesia/05/2005 (H5N1) VLP did induce detectable NI activity supporting the specificity of the NI assay, and confirming the expected lack of cross-reactivity between N9 and N1.

3.3. Protection of immunized mice against a lethal challenge with A/Anhui/1/2013 virus

Mice immunized with A/influenza VLP on day 0 and 14 were challenged with 4.4×10^3 TCID₅₀ pfu ($3.6 \times$ LD₅₀) of infectious A/Anhui/1/2013 (H7N9) virus on day 22. Following challenge, mice were monitored for weight loss and survival. A subset of mice from each group was euthanized on day 3 post challenge, and lung virus titers determined (Table 1). Viral load in the lungs of the placebo group was $10^{6.4}$ pfu. Immunization with 2 and 6 μ g A/H7N9 VLP either with or without ISCOMATRIX resulted in an absence of detectable lung virus as did immunization with 2 μ g A(H7N3) VLP with ISCOMATRIX. A single animal had a low titer of virus at the 0.7 A/H7N9 antigen dose and the H7N3 induced an overall 1 log₁₀ reduction in viral titer. Immunization of mice with A(H5N1) VLP did not result in reduction in lung virus as expected

from the lack of cross reactive antibody observed in the HAI assay (Fig. 2).

Following virus challenge, mice were monitored daily for weight loss. Analysis of average percent weight loss on day 7 post challenge indicated animals immunized with A(H7N9) and A(H7N3) VLP vaccines, irrespective of dose or adjuvant, had no significant weight loss (Table 2). However, mice in the control and A(H5N1) VLP vaccinated groups had reached or near the 25% weight loss where they were euthanized (Table 2). As immunogenicity and lung viral titers of mice within each vaccine formulation gave similar results irrespective of VLP dose, the weight loss and survival data combined of mice immunized with either 0.7 and 2 for A(H7N9) and 0.7 and 2 μ g for A(H7N3) VLP or 1 and 3 μ g for A/H5N1 groups were compared in Fig. 4. Following virus challenge, mice were monitored daily for weight loss (Fig. 4A). Weight loss was observed by day 3 for mice immunized with unadjuvanted A(H7N9) and A(H7N3) VLP as well as both adjuvanted and unadjuvanted A(H5N1) VLP as well as the placebo groups. Weight loss continued for the placebo group as well as both A(H5N1) groups until they were euthanized on day 7 and 8 after becoming moribund, with a weight loss in excess of 25% of their initial weight. Weight loss for the unadjuvanted A(H7N9) and A(H7N3) groups stabilized on days 4–5 and weight gain was observed on subsequent days. For mice immunized with either

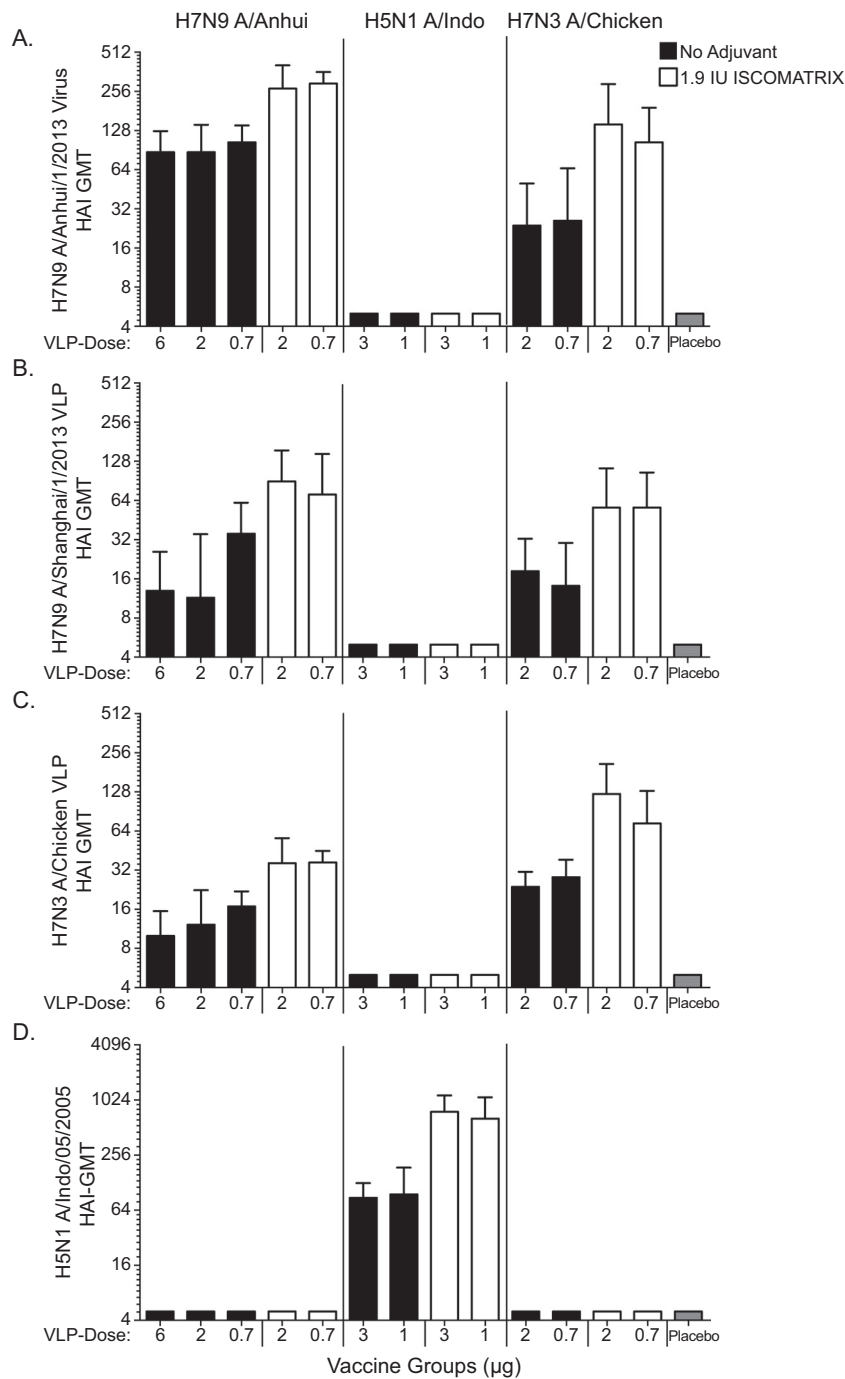


Fig. 2. Day 21 HAI responses in mice immunized with A/Anhui/1/2013 (H7N9), A/Indonesia/05/2005 (H5N1), or A/Chicken/Jalisco/CPA1/2012 (H7N3). Sera were analyzed against (A) A/Anhui/1/2013 (H7N9), (B) A/Shanghai/1/2013 (H7N9), (C) A/Chicken/Jalisco/CPA1/2012 (H7N3), and (D) A/Indonesia/5/2005 (H5N1) antigens.

A(H7N9) or A(H7N3) VLP formulated with ISCOMATRIX, minimal weight loss was observed on days 4–5 and they quickly returned to normal. The resistance to severe weight loss following challenge by mice immunized with A(H7N9) and A(H7N3) VLP formulated with ISCOMATRIX and the recovery observed for the unadjuvanted A(H7N9) and A(H7N3) groups corresponds to the anti-HA titers observed in the HAI assays.

As indicated in Fig. 4B, 100% of mice immunized with A(H7N9) and A(H7N3) VLP survived the lethal virus challenge indicating the protective efficacy of homologous A(H7N9) and heterologous A(H7N3) VLP. The survival of mice immunized with the A(H7N3) VLP is attributed to the activity of HA antibody cross-reactive on

heterologous H7. While anti-NA antibodies are known to contribute to the protective antibody response, the A(H7N3) VLP immunized group did not exhibit detectable cross-reactive NA antibody. All animals in the A(H5N1) groups as well as the placebo group exhibited severe disease characterized by huddling, ruffled fur, lethargy, anorexia leading to weight loss, and death (euthanasia at a humane endpoint). Animals in these groups had to be euthanized as early as 5 days post challenge and 100% of the animals were euthanized by day 8. Mice in the unadjuvanted A(H7N9) and A(H7N3) groups exhibited mild disease with some weight loss while those immunized with VLP ± ISCOMATRIX exhibited no disease symptoms. Immunization with either A(H7N9) or A(H7N3) VLP conferred

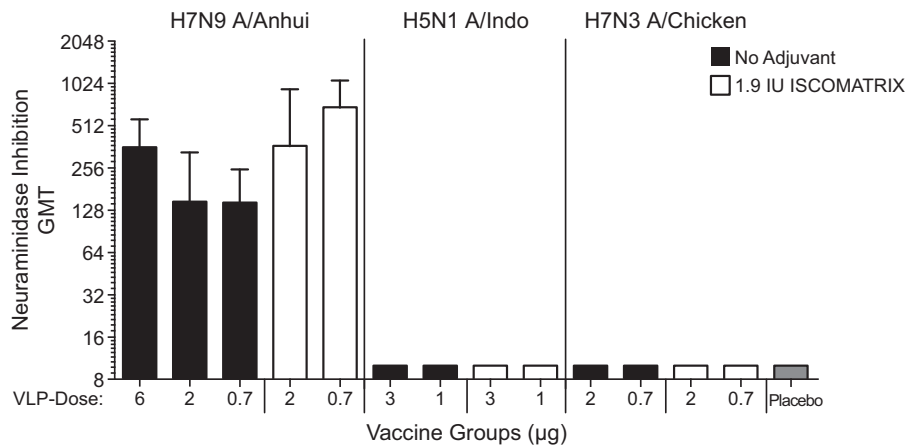


Fig. 3. Day 21 NI responses from mice immunized with A/Anhui/1/2013 (H7N9), A/Indonesia/05/2005 (H5N1) or A/chicken/Jalisco/CPA1/2012(H7N3) VLP on day 0 (prime) and day14 (boost). Graph shows NI titers of day 21 pre-challenge sera in a fetuin inhibition assay using purified A/Anhui/1/2013 (H7N9) NA as a source of neuraminidase.

Table 1

Viral recovery from lung tissue of mice challenged with A/Anhui/1/2013 (H7N9) influenza.

Immunogen ^a	Dose (μg)	ISCOMATRIX	Lung virus titer ^b Log ₁₀ TCID ₅₀ /gm tissue
None	0	+	6.4
A/H7N9	6	–	<3.1
A/H7N9	2	–	<3.1
A/H7N9	0.7	–	3.7
A/H7N9	2	+	<3.1
A/H7N9	0.7	+	<3.1
A/H7N3	2	–	5.0
A/H7N3	0.7	–	N/A ^c
A/H7N3	2	+	<3.1
A/H7N3	0.7	+	N/A ^c
A/H5N1	3	–	6.1
A/H5N1	1	–	6.4
A/H5N1	3	+	6.3
A/H5N1	1	+	6.2

^a Groups of 5 mice were immunized by intramuscular injection with A/influenza VLP as described in Section 2.7.

^b Mice were challenged intranasally with 4.4×10^3 TCID₅₀ pfu ($3.6 \times LD_{50}$) A/Anhui/1/2013 virus eight days following the last immunization. Lung tissue was harvested 3 days post challenge and viral titers in lung homogenates determined as measured in Section 2.7 and represent the average of 3 animals.

^c N/A – not available. Mice in these groups died <24 h of transfer to the challenge facility and thus were not added to either the lung virus or challenge data. Deaths were not attributed to either immunization or virus challenge.

Table 2

Percent change in body weight of mice challenged with A/Anhui/1/2013 (H7N9) influenza.

Immunogen ^a	Dose (μg)	ISCOMATRIX	N at day 1 ^b	wt. ^c (gm) day 1	N at day 7 ^d	wt. at day 7 (% of day 1)
None	0	+	5	19.3 ± 0.7	3	74 ± 1
A/H7N9	6	–	3	18.2 ± 0.8	3	99 ± 5
A/H7N9	2	–	5	17.1 ± 1.1	5	98 ± 3
A/H7N9	0.7	–	5	18.0 ± 0.8	5	98 ± 2
A/H7N9	2	+	5	18.7 ± 1.3	5	101 ± 3
A/H7N9	0.7	+	5	18.5 ± 0.9	5	101 ± 2
A/H7N3	2	–	5	17.8 ± 0.9	5	98 ± 4
A/H7N3	0.7	–	5	16.8 ± 1.2	5	98 ± 4
A/H7N3	2	+	5	17.9 ± 1.3	5	104 ± 4
A/H7N3	0.7	+	4	19.0 ± 1.1	5	104 ± 5
A/H5N1	3	–	5	18.9 ± 1.1	4	74 ± 1
A/H5N1	1	–	5	17.6 ± 0.9	3	78 ± 3
A/H5N1	3	+	5	19.1 ± 0.8	1	75
A/H5N1	1	+	5	18.1 ± 0.7	2	74 ± 1

^a Mice were immunized by intramuscular injection with A/influenza VLP as described in Section 2.7 and challenged intranasally with 4.4×10^3 TCID₅₀ pfu ($3.6 \times LD_{50}$) A/Anhui/1/2013 virus eight days following the last immunization.

^b In the two groups where N < 5 on day 1, mice died <24 h of transfer to the challenge facility and were not included in further analysis.

^c wt. = weight in gm ± standard deviation.

^d Number of mice surviving challenge on day 7.

significant protection ($p < 0.001$) in pairwise comparisons of vaccine groups analyzed and estimated by least squares mean when compared to placebo and immunization with A(H5N1) VLP vaccine.

4. Discussion

Following the announcement 31 March 2013 by the Government of China notifying the World Health Organization (WHO) of human infections with the avian influenza A(H7N9) virus [12] the first A(H7N9) sequences were reported [13]. In a previous human study of an inactivated influenza avian influenza A(H7N7) vaccine in man [14], the immunogenicity of the H7 subtype vaccine was poor suggesting that an A(H7N9) vaccine may require a high dose and likely an adjuvant or both to provide protection [2]. In addition, studies with A(H5N1) vaccines have generally required $2 \times 90 \mu\text{g}$ unadjuvanted or lower doses of adjuvanted vaccine to achieve adequate levels of HAI antibodies in a majority of subjects. However, mouse immunogenicity studies of recombinant influenza A(H5N1) VLP vaccine were predictive of immunogenicity in man [9,11]. Bright et al. [11] reported cross-clade protective immune response of an A(H5N1) VLP vaccine in mice. That same A(H5N1) VLP vaccine was subsequently shown to induce in humans cross-reactive neutralizing antibodies that preferentially bind to an oligomeric form of influenza HA [11]. The data here indicated that the A(H7N9) VLP vaccine candidate was immunogenic and protective against

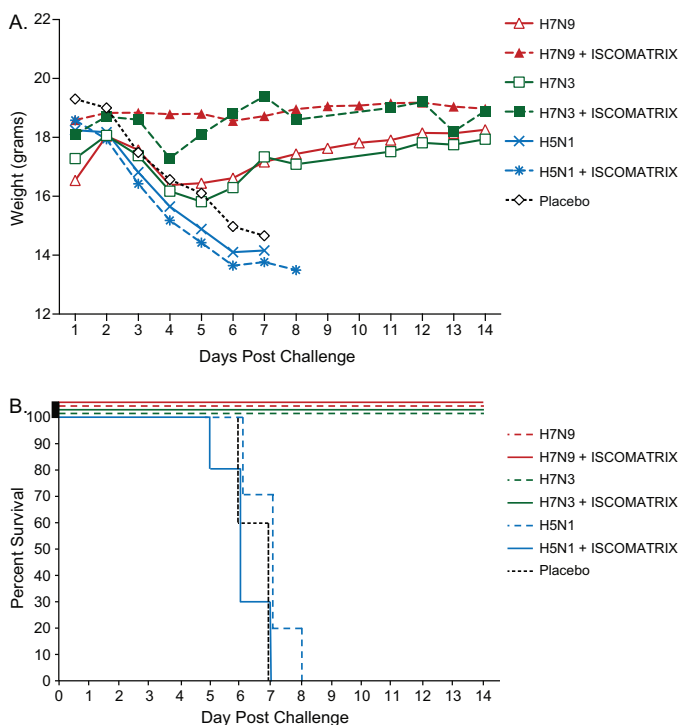


Fig. 4. Body weight (A) and percent survival (B) of mice immunized influenza A (H7N9), A(H7N3), and A(H5N1) VLPs with and without ISCOMATRIX adjuvant and challenged with a lethal dose A/Anhui/1/2013 (H7N9) virus.

wild type lethal viral challenge, even with less than a microgram of unadjuvanted vaccine. Additionally, the A(H7N3) VLP vaccine was cross-protective against a lethal challenge with A/Anhui/1/2013 and produced cross reacting HAI to non-homologous A(H7N9). A(H5N1) VLPs were not protective against A(H7N9) virus argues that the protection in A(H7N3) immunized mice was specific to the HA and not due to baculovirus or other minor contaminants as all VLPs in the study were purified in a similar manner and had low levels of baculovirus antigen contamination. However, it was not determined if VLP-induced protective immune responses were due to IgG or IgM or both, especially for A(H7N3) given that mice were challenged 8 days following a boost. Further studies including challenge of vaccinated mice at a later time point, antibody subclass analysis, duration of immunity, and identification of protective epitopes are important. However, we consider this mouse model as a reasonable and rapid *in vivo* screening method as a preliminary assessment of vaccine efficacy, and thus useful in responding to a potential pandemic by providing animal protection data prior to vaccinating humans.

The addition of ISCOMATRIX to the A(H7N9) VLP further enhanced the immunogenicity and efficacy to both the homologous and non-homologous strains and may provide dose sparing. The current A(H7N3) highly pathogenic avian influenza outbreaks in Jalisco, Mexico, with high numbers of infected animals in high-density human populations pose a risk not just to the poultry industry but of reassortants that could cause disease in humans. An effective A(H7N9) vaccine that was cross-protective with other H7 subtypes would add an additional margin of safety to the unpredictable nature of influenza pandemics.

Since 1997, avian influenza A viruses of various subtypes, including H5N1, H9N2, H7N7, H5N2, and now H7N9, have been documented to infect and cause illness in humans. Of these, Morens et al. [3] have noted the remarkable clinical-epidemiological similarity between H7N9 and H5N1 has been the most striking, with a possibility that many humans appearing to have been exposed to

both, yet without clinically apparent or immunologically detectable evidence of infection. At the same time, disease in sporadic human cases has been far more severe than in cases caused by any human-adapted influenza A virus ever encountered (59% and 28% case fatality rates, respectively), characterized by bilateral pneumonia progressing to acute respiratory distress syndrome and multi-organ failure. While there has fortunately been little or no evidence of person-to-person transmission of either virus, the potential for pandemic spread for a virus in which all 8 segments encode proteins similar to the avian influenza A consensus sequence – such as the 1918 pandemic virus – cannot be ignored [3].

In view of the potential for pandemic spread of avian viruses, government, academia and industry have devoted considerable effort over the past decade toward the development and testing of both traditional and novel manufacturing platforms for the generation of pandemic vaccine candidates for either stockpiling purposes (H5N1) or for actual deployment (H1N1 pdm09). The latter vaccines were based on the traditional inactivated split-virus or subunit platforms, but were introduced too late and appeared to have suboptimal clinical effectiveness (56%; 95% CI, 23%–75%) [2]. Adjuvanted forms of these vaccines were approved in Europe, and were shown to substantially reduce the amount of antigen required to achieve the currently accepted HAI antibody immune correlates [15–17] and improve the vaccine effectiveness [18]. However, none of these vaccines have taken an approach toward broadening the immune response, whether by attempting to elicit anti-NA antibody or to enhance T cell responses to reduce the severity of influenza disease (symptomology and virus shedding) in the absence of a sufficiently robust neutralizing antibody response.

Previous studies of our VLP candidates in animals and humans indicate that an influenza vaccine platform designed to present both full-length HA and NA antigens in VLP form may better elicit native presentation of these antigens to the immune system, yet at the same time avoid the safety concerns posed by a live, replicating agent. Because of their particulate nature, uptake of VLP by antigen-presenting cells may not only result in presentation of peptides with major histocompatibility complex II (MHC-II) molecules and stimulation of humoral immunity, but may also result in cross-presentation of peptides with MHC-I molecules for activation of cytotoxic T-lymphocytes (CTLs) [19]. In addition, the repetitive nature of the HA and NA on the VLP surface may stimulate humoral immune responses directly in the lymph nodes and also have the potential for limiting spread of virus distally to the lower airways. Finally, it is also possible that conserved T-cell epitopes on the HA and M1 proteins may induce cell-mediated immune responses that reduce the severity of influenza disease.

Of the first three published sequences of A(H7N9) that were reported A/Anhui/1/2013 and A/Shanghai/1/2013 were phylogenetically distinct with 8 amino acid difference in their hemagglutinin gene sequences and A/Anhui/1/2013 was recommended as a vaccine strain due to high titers when grown in embryonated hens eggs [13]. We choose to clone and produce both A/Anhui/1/2013 and A/Shanghai/1/2013 strain A(H7N9) VLP but to focus vaccine development on the former as recommended. The two unique HA and A(H7N9) NA genes were codon optimized, synthesized, baculovirus expression vectors constructed and confirmed to contain the genes with the correct sequences. A/Anhui/1/2013 and A/Shanghai/1/2013 VLP as well as individual A/Anhui/1/2013 HA and NA antigens were produced and purified. At this stage there were two significant challenges; first was positive identification of A(H7N9) antigens, and second and more difficult, quantification of the HA content of the VLP given that weeks were needed before specific HA antibody could be made in sheep for use in a conventional single radial immunodiffusion (SRID) assay for influenza vaccine potency.

Identification of A/Anhui/1/2013 HA was performed using Western blot and a mouse monoclonal antibody against another H7 subtype influenza A(H7N7). A/Anhui/1/2013 NA was identified with antibody against a conserved peptide [20] which reacted with all of the A strain NAs in A(H7N9), A(H7N3) and A(H5N1) VLP. The amino acid peptide sequences of A/Anhui/1/2013 HA and NA were also analyzed using LC–MS, confirming their identity.

However, this uncovered another challenge in that A(H7H9) NA and A(H7N3) NA essentially co-migrated with respective H7 HAs. Scanning densitometry of SDS–PAGE gels initially overestimated the HA content, thus the dose, of A(H7N9) and A(H7N3) VLP used in the mouse challenge study. Capillary gel electrophoresis is a widely used method for the determination of protein purity and offers greater resolution and more reproducible quantitation compared with that of SDS–PAGE. After deglycosylation, A(H7N9) HA and NA were separated and a more accurate measure of HA could be determined, thus adjustments were made to doses of A(H7N9) and A(H7N3) VLP used in the mouse challenge study.

The current study provides further support for the potential generation of desirable attributes [21] that could result in a safe and effective H7N9 vaccine candidate. First, the immunogenicity and efficacy of 2 doses of A/Anhui/1/2013 (H7N9)–VLP vaccine candidate administered on study days 0 and 14 were clearly established in the lethal challenge Balb/c mouse model, the most widely used animal model for influenza virus research. In experiments evaluating highly pathogenic strains such as H5 and H7, a lethal dose typically results in severe disease characterized by huddling, ruffled fur, lethargy, anorexia leading to weight loss, and death (euthanasia at a humane endpoint) and therefore provides an unambiguous test of a vaccine candidate. In our study, all 25 mice in the H5N1 VLP and placebo groups required euthanasia within 8 days after challenge, compared with none in the H7N9 VLP vaccine groups. Protection from death was correlated with both robust HAI and antigen-specific anti-NA antibody responses following vaccination, neither of which were elicited in the two control groups, along with evidence of cross-protective HAI responses following vaccination with both H7-based candidates.

It remains to be seen whether an A/Anhui/1/2013-like (H7N9) or a similar pathogen will emerge into the broader population but prospective development of an immunogenic and cross-protective H7N9 vaccine is a prudent public health measure and the insect cell-derived A(H7N9) VLP vaccine with or without a saponin-based adjuvant for dose sparing to be considered as candidate pandemic vaccine. This study represents a promising approach to provide a vaccine for an emerging lethal pandemic influenza virus. Based on these positive and unambiguous results, further studies in humans and other animal models were warranted and an H7N9 VLP vaccine candidate described in the report is in clinical testing [22].

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findings and conclusion in the report are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services or its components.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.07.043>.

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