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A Highly Immunogenic Vaccine against A(H7N9) Influenza Virus

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Abstract

Since the first case of human infection in March 2013, continued reports of H7N9 cases highlight a potential pandemic threat. Highly immunogenic vaccines to this virus are urgently needed to protect vulnerable populations who lack protective immunity. In this study, an egg- and adjuvant-independent adenoviral vector-based, hemagglutinin H7 subtype influenza vaccine (HAd-H7HA) demonstrated enhanced cell-mediated immunity as well as serum antibody responses in a mouse model. Most importantly, this vaccine provided complete protection against homologous A/ (H7N9) viral challenge suggesting its potential utility as a pandemic vaccine.

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Author Contributions

All authors have contributed to this work and approve the submission.

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Keywords

A/(H7N9) influenza vaccine; adenoviral vector vaccine; H7HA; cell-mediated immunity; antibody responses; protective immunity; mouse model

1. Introduction

Until the first case reported in China on March 2013, H7N9 influenza infections were confined primarily to avian species [1]. As of March 2015, however, there have been a total of 631 human infections. Many patients have had severe respiratory illness with a 35% case fatality rate [2]. Most of these infections are believed to result from the exposure to infected poultry or contaminated environment. Although direct human-to-human transmission is very limited, the continued circulation of the H7N9 virus poses a potential pandemic threat to the human population. Although prior infection or vaccination with A(H3N2) influenza A strains induce H7 cross-reactive antibodies [3], they are less frequent as only 6 monoclonal antibodies (mAb) of a total of 83 mAb isolated from 28 individuals showed reactivity against H7N9 virus. Furthermore, only 3 of these 6 mAb demonstrated virus neutralizing activity against A(H7N9) virus both *in vitro* and *in vivo* in mice. Hence, the majority of the human population still lacks protective immunity against H7N9 virus. Although vaccination is the most cost-effective intervention strategy, several independent studies have shown that H7N9 vaccines are poorly immunogenic [4, 5]. Furthermore, the circulation of H5N2 and H5N8 viruses in the United States is threatening the supply of embryonated chicken eggs, the substrate for current egg-derived inactivated and live attenuated influenza vaccines production[6]. Hence, an egg-independent vaccine production technology is beneficial for pandemic preparedness. In our earlier studies, we developed a replication-incompetent human adenoviral (HAd) vector-based, adjuvant-, and egg-independent pandemic influenza vaccine strategy and demonstrated that an HAd vaccine expressing the gene encoding hemagglutinin (HA) from A/Hong Kong/156/97 H5N1 viruses conferred long-lasting immunity and cross-protection in mice against challenge with more-recent strains of highly pathogenic H5N1 viruses [7, 8]. Therefore, in this study, we explored the potential utility of an Adenoviral vector-based delivery system expressing H7HA from A/Anhui/1/2013 influenza virus and assessed its immunogenicity and efficacy to confer protection in BALB/c mice against a homologous challenge compared to a recombinant H7HA vaccine.

2. Materials and Methods

2.1 Cell culture and vector purification

293, 293Cre and bovine-human hybrid (BHH2C) cell lines were grown in minimum essential medium (MEM) containing 10% FetalClone III (Thermo Fisher Scientific Inc., Waltham, MA) and gentamicin (50 µg/ml). HAd vector purification was done by cesium chloride density gradient centrifugation and virus titration was done in BHH2C by plaque assay.

2.2 Generation and characterization of replication deficient HAd-H7HA vector

A Cre-recombinase-mediated site-specific recombination technique [9] was used to insert the full-length coding region of the HA gene of the A/Anhui/1/2013 (AH1) A(H7N9) influenza virus under the control of the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal (polyA). An HAd gene with deletions of the early regions E1 and E3 (HAd- E1E3) served as a negative control [10]. The recombinant virus was plaque purified, and its genome was analyzed to confirm the presence of the HA gene cassette without any other major deletion or insertion. 293 cells were mock-infected or infected with an empty vector (HAd- E1E3) or HAd-H7HA at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. Thirty-six hours (h) post-infection, cells were harvested, and cell lysates were examined for the expression of H7HA protein using the ferret anti-A/Netherland/219/03 (H7N7)-specific antibody by Western blot as described [11]

2.3 Animal immunization, immunogenicity and viral challenges

Six to eight week old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with Avertin (2,2,2-tribromoethano; Sigma) by intraperitoneal (i.p.) injection and immunized (5 animals/group) with HAd-H7HA or HAd- E1E3 intranasally (i.n.). As controls, mice were immunized by the intramuscular (i.m.) route with 3 µg of the recombinant H7HA (rH7HA) from A/Shanghai/2/2013 (SH2) which has an identical HA amino acid sequence to AH1 or PBS using 50 µl in each thigh. Four weeks later, mice were boosted with the same immunization regimen. Sera were obtained three weeks post-primary and again three weeks post-boost to determine antibody responses. Mice were challenged with 50 × lethal dose 50% (LD₅₀) of wild type H7N9 virus (AH1) and monitored for weight loss and mortality. Animal research was conducted under the guidance of the CDC's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) International-accredited animal facility. Mice that lost >20% of their pre-infection body weight were euthanatized.

2.4 Cell-mediated immune responses

Single cell suspensions were prepared from the lung, spleen, lymph node and bone marrow tissues one week post-boost immunization. To detect intracellular cytokine production by cells from the lung, spleen and lymph node, 1×10^6 cells were stimulated *in vitro* with HA peptide (5 µg/ml) or A/Shanghai/2/2013(H7N9)-PR8 reverse genetic virus (SH2/PR8) virus (MOI=1) overnight with GolgiPlug™ (BD Bioscience, San Jose, CA) added during the last 6 h of incubation. Cells were surface stained with anti-CD44 antibody and with either anti-CD4 or anti-CD8 antibody (BD Bioscience), followed by intracellular staining with anti-IFN-γ, anti-IL-2 or anti-TNF-α antibodies (BD Bioscience). Samples were analyzed using LSRII Flow cytometer (BD Biosciences), and the cytometric data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

The percentage of H7N9 virus or HA-specific Antibody-Producing Cells (ASCs) in the spleen or bone marrow was detected by ELISPOT assay. Briefly, 1×10^6 cells were added onto antigen-coated plates and incubated overnight at 37°C in a humidified atmosphere with 5% CO₂. The plates were incubated with biotinylated anti-mouse IgG (Southern Biotech,

Birmingham, AL) followed by alkaline phosphatase-conjugated streptavidin and developed with Vector Blue alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA). Spot forming units were counted using ImmunoSpot® (Cellular Technology Ltd., Shaker Heights, OH) and expressed as the number of antigen-specific IgG or IgA secreting B cells/ 10^6 cells.

2.5 Serum HAI assay

Sera from all mice were subjected to overnight treatment with receptor-destroying enzyme from *Vibrio Cholerae* (Denka Seiken, Tokyo, Japan) at 37°C to destroy non-specific serum inhibitor activity. Serial dilutions of RDE-treated sera were mixed with 4 hemagglutination units of SH2/PR8 virus for 60 min, followed by addition of 50 μ l 1% horse RBC. The highest serum dilution inhibiting hemagglutination was taken as the HAI titer [12].

2.6 Serum microneutralization assay

RDE-treated sera were serially diluted in 96-well plates and incubated with SH2/PR8 viruses at a dose of 2×10^3 TCID₅₀/ml for 2 h at 37°C. MDCK cells were added and incubated overnight. Cells were then fixed with 80% acetone and incubated with biotinylated anti-nucleoprotein Ab (EMD Millipore, Billerica, MA), followed by streptavidin-HRP (Southern Biotech, Birmingham, AL). Bound HRP was visualized using 1 $\times$ TMB substrate solution (eBioscience, San Diego, CA) and the developed color was assessed using a microplate reader. The highest serum dilution that generated >50% specific signal was considered to be the neutralization titer; 50% specific signal = (OD₄₅₀ virus control – OD₄₅₀ cell control)/2 + OD₄₅₀ cell control.

2.7 ELISA

ELISA was performed to detect H7HA- or A(H7N9) virus-specific IgG antibody levels in the sera. Briefly, Immunol plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight with 1 μ g/ml H7HA or 50 HAU SH2/PR8 virus at 4°C and then blocked for 1 h with PBS/0.05% Tween-20 (PBST) containing 4% BSA at room temperature. Sera were serially titrated 4 fold in PBST and incubated with antigen-coated plates for 2 h at room temperature. After washing with PBST, wells were probed with HRP-anti-mouse IgG for 1 h at room temperature. Signal was developed using 1 $\times$ TMB substrate solution (eBioscience) and the developed color was assessed using the microplate reader.

2.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Groups were compared by one-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean $\pm$ SEM. All differences were considered statistically significant when the *p*-value was ≤ 0.05 .

3. Results

3.1 Generation and characterization of HAd vector expressing HA of H7N9 virus (HAd-H7HA)

To construct the HAd-H7HA vaccine, the full coding region of HA from the A(H7N9) virus under the control of the CMV immediate early promoter and BGH polyA were inserted into E1 of the HAd genome using the Cre-recombinase-mediated site-specific recombination system (Fig. 1A). Western blot was performed in 293 T cells to examine the expression of H7HA. A single polypeptide band of approximate molecular weights 75 KDa, representing the HA precursor (HA0), was observed in the HAd-H7HA infected 293 cell lysate (Fig. 1B).

3.2 HAd-H7HA vaccine induces cell-mediated immune responses

To first evaluate the vaccine-induced cell-mediated immune responses, 6–8 week old BALB/c mice were immunized either i.n. with 2.5×10^8 PFU HAd- E1E3 or HAd-H7HA or i.m. with 3 μ g rH7HA or PBS. Four weeks later, mice were boosted with the same immunization regimen. Spleens were harvested one week post-boost, and functional CD4 and CD8 T cells were analyzed by surface CD44 expression and intracellular cytokine production. The percentage of CD44⁺ CD4 or CD44⁺CD8 T cells was similar among all groups (data not shown). However, in response to *in vitro* A(H7N9) virus re-stimulation, the percentage of (IFN- γ , IL-2 or TNF- α producing CD4 or CD8 T cells in the spleen was significantly increased in mice that received HAd-H7HA vaccine (Fig. 1C). Similarly, HAd-H7HA vaccine also substantially increased the percentage of IFN- γ , IL-2 or TNF- α producing CD8 T cells following restimulation with HA-peptide containing CD8 T cell epitope (Fig. 1C). Similar results were observed in the lungs and draining lymph nodes (data not shown). Furthermore, the percentage of triple-cytokine (IFN- γ , IL-2 and TNF- α) producing CD4 or CD8 T in the spleen was significantly higher in HAd-H7HA vaccine group (supplementary Fig. 1). HAd-H7HA immunization also increased the frequency of antigen specific IFN- γ and IL-2 or IFN- γ and TNF- α double cytokine producing CD8 T cells and IFN- γ and IL-2 double cytokine producing CD4 T cells, as compared to H7HA vaccine (supplementary Fig. 1) indicating that HAd-H7HA vaccine increased the frequency of functional CD4 and CD8 T cells in lungs, lymph nodes and spleens.

3.3 HAd-H7HA induces antigen-specific B cell responses

The antigen-specific B cell responses in the spleen and bone marrow were examined by ELISPOT assay. As shown in Fig. 1D, the number of HA protein-specific as well as A(H7N9) virus-specific IgG antibody-secreting cells (ASC) in the spleen was significantly increased in the HAd-H7HA group compared to the HAd- E1E3 or the H7HA protein groups. However, the HAd-H7HA vaccine-induced increase in virus-specific IgG ASCs in the bone marrow was very minor and not statistically significant. The frequency of virus-specific IgA ASCs in both spleen and bone marrow was substantially increased in mice immunized with the HAd-H7HA (Fig. 1D). These data suggest that the HAd-H7HA enhanced memory B cell responses in both primary and secondary lymphoid tissues.

3.4 HAd-H7HA vaccine induces antibody responses

The serum antibody responses, especially neutralizing antibodies, are critical for protective immunity against virus infection. To assess whether HAd vaccine induced serum antibody responses, mice were immunized as described above, and serum samples were collected at three weeks post-primary and again at three weeks post-boost to detect antibody responses by ELISA, HI and microneutralization assays. Firstly, ELISA was used to detect A(H7N9) virus- and HA-specific IgG in sera. As shown in Fig. 2A, the primary as well as booster immunizations with HAd-H7HA vaccine significantly increased the A(H7N9) virus- and HA-specific IgG in sera, while no or very little IgG was detected in the rH7HA and other control vaccine immunized groups. Secondly, the HI and microneutralization assays showed that the HAd-H7HA vaccine induced significantly higher antibody titers in the sera, even after single primary immunization (Fig. 2B, 2C). In summary, the HAd-H7HA vaccine demonstrated significant enhancement of H7HA immunogenicity compared to recombinant H7HA protein.

3.5 HAd-H7HA vaccine induces protection against homologous virus challenge

To investigate the protective immunity, mice were immunized either i.n. with the HAd-H7HA vaccine or the control HAd- E1E3 or i.m. with 3 µg recombinant H7HA protein. The animals were boosted 4 weeks later with the same antigen as the primary immunization. At four weeks post-boost, mice were challenged with a homologous A(H7N9) virus [AH1 (50LD₅₀)] to assess the protective efficacy of the vaccines. All mice immunized with PBS or the HAd- E1E3 lost significant body weight after challenge and had to be euthanized at days 6–7 post-challenge (Figure 2D). Mice immunized i.m. with recombinant H7HA had substantial weight loss and only 20% of the mice survived the A(H7N9) virus challenge. However, the HAd-H7HA fully protected mice against homologous H7N9 virus challenge with no obvious body weight loss.

Discussion

The pandemic potential of H7N9 virus cannot be underestimated due to its rapid increase of case numbers in short period of time and disease severity [13]. However, H7N9 vaccines have been shown to be poorly immunogenic [4, 5] and the manufacture of vaccines by traditional egg-based methods are labor-intensive and time-consuming [14]. New production platforms with the capacity to produce large quantities of vaccines in a short period is in urgent need. The first H7N9 viral vector vaccine based on modified vaccinia virus Ankara (MVA) was proved to be immunogenic, even after a single immunization [15]. In this study, we demonstrate a feasible vaccine strategy that overcomes the poor immunogenicity of egg-derived or recombinant protein antigens. This Adenoviral vector-based H7HA vaccine is highly immunogenic, induces enhanced humoral and cellular immune responses and effectively protects mice from homologous A(H7N9) viral challenge. The advantage of an Adenoviral vectored vaccine approach is that it is an egg-independent strategy. Most of the currently marketed influenza vaccines, both split as well as live attenuated, are produced in embryonated chicken eggs. Current H5N2 and H5N8 infections in commercial poultry are threatening the supply of these embryonated chicken eggs and thus, the production of vaccines. Adenoviral vectors are known for their potent adjuvanticity by directly activating

immune responses. The concern of pre-existing immunity against Adenoviruses can be overcome by increasing the vaccine dose, using alternate routes of vaccination or using non-human Adenoviral vectors as vaccine vectors [16, 17]. The effective protection induced by HAd-H7HA vaccine is consistent with its induction of significantly higher antibody responses in sera as detected by ELISA, HI, and microneutralization assays. In addition, an Adenoviral vectored vaccine substantially increases both CD4 and CD8 T cell functional responses. CD4⁺ T cells are shown to influence effector and memory CD8⁺ T cell responses, humoral immunity, and the antimicrobial activity of macrophages by recruiting cells to sites of infection [18]. Those individuals who have high levels of virus-specific CD8⁺ T cells develop less severe illness [19]. The HAd-H7HA vaccine induces strong CD4 and CD8 T cell responses which are crucial in conferring protective immunity [14, 15]. Therefore, the HAd-H7HA vaccine developed in this study could potentially serve as a pre-pandemic or pandemic vaccine against A(H7N9) influenza virus infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. HAd-H7HA vaccine induced significant cell-mediated immune responses

(A) Diagrammatic representation of replication-deficient HAd vectors, HAd- E1E3 (HAD5 with deleted E1 and E3 regions) and HAd-H7HA (HAd- E1E3 with hemagglutinin (HA) gene from AH1 influenza virus). ITR, inverted terminal repeat; HCMV, human cytomegalovirus immediate early promoter; BGH, bovine growth hormone. Expression of A(H7N9) HA in 293 cells infected with HAd-H7HA. (B) Mock (PBS-infected), HAd- E1E3, or HAd-H7HA-infected 293 cells were harvested 36 h post-infection, and cell lysates were analyzed by Western blot using the ferret A/Netherlands/219/03 (H7N7)-specific antibody. (C) BALB/c mice (5 animals/group) were immunized with either HAd- E1E3 or HAd-H7HA (2.5×10^8 PFU/mouse) intranasally (i.n.) or rH7HA protein vaccine (3 μ g) or PBS intramuscularly (i.m.). Mice were boosted one month later with the same vaccine formulation as they received previously. Spleens were collected at 1 week after booster immunization and single cell suspensions were prepared. Cells were *in vitro*

stimulated with SH2/PR8 (MOI=1) or HA peptide (5 µg/ml) overnight with GolgiPlug™ added in the last 6 hours of incubation. The percentage of IFN γ , TNF α or IL-2-producing CD4 $^{+}$ or CD8 $^{+}$ T cells were then measured by intracellular cytokine staining. **(D)** BALB/c mice were immunized as described above. One week after booster immunization, the spleen and bone marrow were collected and the frequency of rH7HA protein-specific IgG $^{+}$ ASCs and virus-specific IgG $^{+}$ ASCs or IgA $^{+}$ ASCs was measured using the ELISPOT assay. The number of HA or virus-specific IgG $^{+}$ (or IgA $^{+}$) ASCs per million total cells are presented. The error bars represent standard error of the means (SEM).

Figure 2. HAd-H7HA vaccine enhanced serum antibody responses and protected mice from homologous virus challenge

BALB/c mice (5 mice/group) were immunized with either HAd- E1E3 or HAd-H7HA (2.5×10^8 PFU/mouse) intranasally (i.n.) or rH7HA protein vaccine (3 μ g) or PBS control intramuscularly (i.m.). Mice were boosted one month later with the same vaccine formulation they received earlier. (A–C) Serum samples were collected three weeks following primary immunization and three weeks following booster immunization. (A) A(H7N9) virus-specific or H7HA-specific IgG in sera were measured by ELISA; (B) HI titers were measured against SH2/PR8 (H7N9) virus; (C) Microneutralization titers against SH2/PR8 virus were measured. (D) BALB/c mice (5 mice/group) were immunized with either HAd- E1E3 or HAd-H7HA (2.5×10^7 PFU/mouse) intranasally (i.n.) or rH7HA protein vaccine (3 μ g) or PBS controls intramuscularly (i.m.). Mice were boosted with the same vaccine formulation one month later. Immunized mice were challenged with 50LD₅₀ of wild type A(H7N9) virus, AH1 and weight loss and survival were monitored for 15 days post-infection. The error bars represent standard error of the means (SEM).