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Humoral and Cellular Immunogenicity Induced by Avian Influenza A (H7N9) DNA Vaccine in Mice

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Background: In March 2013, human infection with avian influenza A (H7N9) virus emerged in China, causing serious public health concerns and raising the possibility of avian-source pandemic influenza. Thus, the development of an effective vaccine for preventing and rapidly controlling avian influenza A (H7N9) virus is needed. In this study, we evaluated the immunogenicity of a synthetic DNA vaccine against H7 HA antigens in mice.

Materials and Methods: The synthetic consensus H7 HA DNA vaccine (25 or 50 μg) was administered to BALB/c mice at 0, 14, and 28 days by intramuscular injection followed by electroporation. Humoral and cellular immune responses were analyzed in a hemagglutination inhibition test and interferon-gamma enzyme-linked immunospot (ELISpot) assay, respectively.

Results: H7 HA-vaccinated mice showed 100% seroprotection and seroconversion rate against H7N9 reassortant influenza virus after both second and third immunizations. The geometric mean titer by the hemagglutination inhibition test increased with an increasing number of immunizations. However, there was no significant difference in geometric titer between the two groups injected with 25 and 50 μg of H7 HA DNA vaccine after two (79.98 *vs.* 107.65, $P = 0.39$) and three (159.96 *vs.* 215.28, $P = 0.18$) doses. In addition, the ELISpot assay revealed that administration of H7 HA DNA vaccine induced potent interferon-gamma production from mouse splenocytes.

Conclusion: This study demonstrated the humoral and cellular immunogenicity of synthetic consensus H7 HA DNA vaccine in mice. This work demonstrates the potential of the H7 HA DNA vaccine as an efficient tool for the rapid control of emerging influenza A (H7N9) virus.

Key Words: Influenza A(H7N9) virus; DNA vaccine; Cellular immunity

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Introduction

Influenza virus is a member of the family Orthomyxoviridae and consists of eight genetic segments [1]. Antigenic variation is a unique characteristic of influenza virus. Antigenic drift generates seasonal influenza and antigenic shift results in the generation of pandemic influenza. Human influenza A and B viruses cause seasonal epidemics every year. In contrast to annual epidemics, worldwide pandemics of influenza occur infrequently in association with the unpredictable emergence of novel influenza A virus through antigenic shift [2].

In March 2013, the novel reassortant avian-origin influenza A (H7N9) virus was detected in patients with rapidly progressing respiratory diseases in Shanghai and Anhui Province, China [3]. Influenza A (H7N9) virus is thought to emerge by the reassortment of gene segments from at least four different influenza A viruses: H7 from duck, N9 from wild birds or chickens, and six internal gene segments from H9N2 influenza viruses of brambling birds and chickens [4-6]. Multiple studies suggested that the origin of novel H7N9 virus in humans can be traced to live-poultry markets, where most H7N9-infected patients had direct contact [4, 7]. As of December 3, 2014, 458 confirmed human cases and 177 deaths had been reported, with a mortality rate of 38.6%. Because of the increasing numbers of H7N9 infections in humans, rapid development of an efficient H7N9 vaccine is necessary. Different approaches have been applied by several manufacturers to develop influenza A (H7N9) virus vaccines [8, 9]. Here, we evaluated the humoral and cellular immunogenicity of H7 HA DNA vaccine in a mouse model.

Materials and Methods

1. Ethics

This study was approved by Korea University Institutional Animal Care and Use Committee (KUIACUC-2014-69).

2. Immunization of mice

Vector control and the synthetic H7 HA DNA vaccine were obtained from GeneOne Life Science, Inc. (Seoul, Korea) [10]. Female 6-week-old BALB/c mice were purchased from Orient Bio, Inc. (Seongnam, Gyeonggi province, Korea). Mice were divided into a naïve (n = 7), vector control (n = 9) or DNA vaccine group (n = 21 for 25 or 50 μg, respectively) for a total of three vaccinations and immunized as described previously [10]. Briefly, mice were immunized with the vector control (50 μg) or H7 HA DNA vaccine (25 or 50 μg) by intramuscular injection followed by electroporation using a CELLECTRA® adaptive constant current electroporation device (Inovio Pharmaceuticals Inc., Blue Bell, PA, USA). Two 0.1-A constant current square-wave pulses were delivered through a triangular 3-electrode array consisting of 26-gauge solid stainless steel electrodes. Each pulse was 52 ms in length, with a 1-s delay between pulses. Mice were immunized at one to three doses, two weeks apart. Naïve mice without immunizations were used as a negative control. Blood and spleen samples were collected two weeks after the last immunization (Fig. 1).

3. Hemagglutination inhibition (HI) test and analysis

Sera samples were treated with receptor-destroying enzyme (Denka Seiken Co., Tokyo, Japan) (1:3 ratio) at 37°C for 18–20 h and then heated at 56°C for 30 min to inactivate the receptor-destroying enzyme. Sera were diluted with phosphate-buffered saline to bring the final dilution to 1:10. Two-fold serial dilution was performed on 96-well V-bottom microplates. Four hemagglutinin units of H7N9 reassortant influenza virus (HA and NA from A/Anhui/01/2013; other 6 segments from A/Puerto Rico/8/1934) were added to each well and the serum-virus mixture was incubated at 25°C for 1 h. Following incubation, 50 μL of 1% horse red blood cells (10% red blood cell diluted with saline solution 1:9, Innovative Research, Inc., Novi, MI, USA) was added to each well and incubated for 1 h 30 min at room temperature. Anti-HA antibody titers below the detection limit (<1:10) were assigned a value of 1:5. Using HI data, we calculated the seroconversion rate and seroprotection rate. Seroconversion corresponds to a negative pre-vaccination serum conversion to an HI titer >1:40 or significant increase in HI antibody titer. Seroprotection was defined as the proportion of vaccinated individuals achieving an HI titer of >1:40.

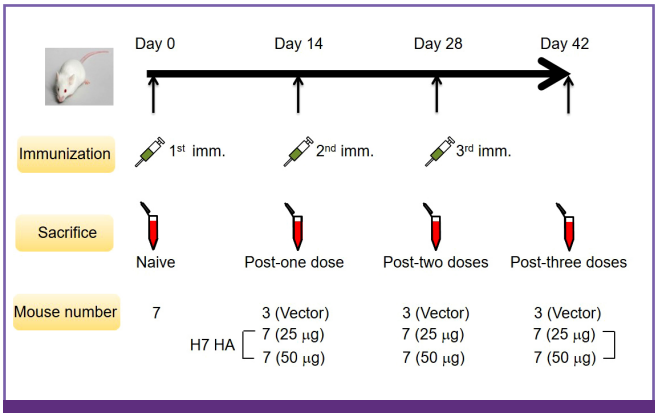


Figure 1. Schema of the study design.
imm, immunization.

4. IFN-γ enzyme-linked immunospot (ELISpot) assay

Interferon (IFN)-γ ELISpot assay was performed as described previously [10]. Briefly, mice splenocytes were isolated from spleens using a 70-mm nylon cell strainer (BD Pharmingen, San Diego, CA, USA) and incubated in media containing 10 ng/mL interleukin-2 for 48 h. Cells were plated in 96-well multiscreen filter plates (Millipore, Billerica, MA, USA) coated with purified rat anti-mouse IFN-γ capture antibody (BD Pharmingen, San Diego, CA, USA) in triplicate at 1×10^6 cells/well and stimulated by H7 HA peptides (GeneOne Life Science, Inc., Seoul, Korea) for 16 h. Biotin rat anti-mouse IFN-γ (BD Pharmingen, San Diego, CA, USA) was used as the detection antibody. Color development was performed according to the manufacturer's instructions (ELISPOT Blue Color Module, R&D Systems, Minneapolis, MN, USA). The number of spots on the plates was counted using an automated ELISPOT reader system (CTL Analyzer, Cellular Technology, Cleveland, OH, USA).

5. Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). Categorical variables were compared using chi-square test and Student's *t* test was applied to compare continuous variables. A *P* value of <0.05 was considered statistically significant.

Results

1. H7 HA DNA vaccine induced antibody production against H7N9 reassortant influenza virus in mice

Mice were immunized with vector control (50 μg) or H7 HA DNA vaccine (25 or 50 μg). After the first immunization with H7 HA DNA vaccine, the HI titers of all immunized mice were <40 (Fig. 2A and B). However, HI titers were greater than 40 after the second and third immunizations. Both the seroprotection and seroconversion rates were 100% in mice immunized with 25 or 50 μg DNA vaccine two and three times. After secondary immunization with H7 HA vaccine, the mean of geometric mean titer (GMT) was 79.98 (25 μg H7 HA) and 107.65 (50 μg H7 HA) (Fig. 2C and D). The GMTs after three doses of H7 HA vaccine were 159.96 in the 25 μg group and 215.28 in the 50 μg group ($P = 0.18$).

2. H7 HA DNA vaccine induced IFN-γ production in mouse splenocytes stimulated with H7 HA peptides

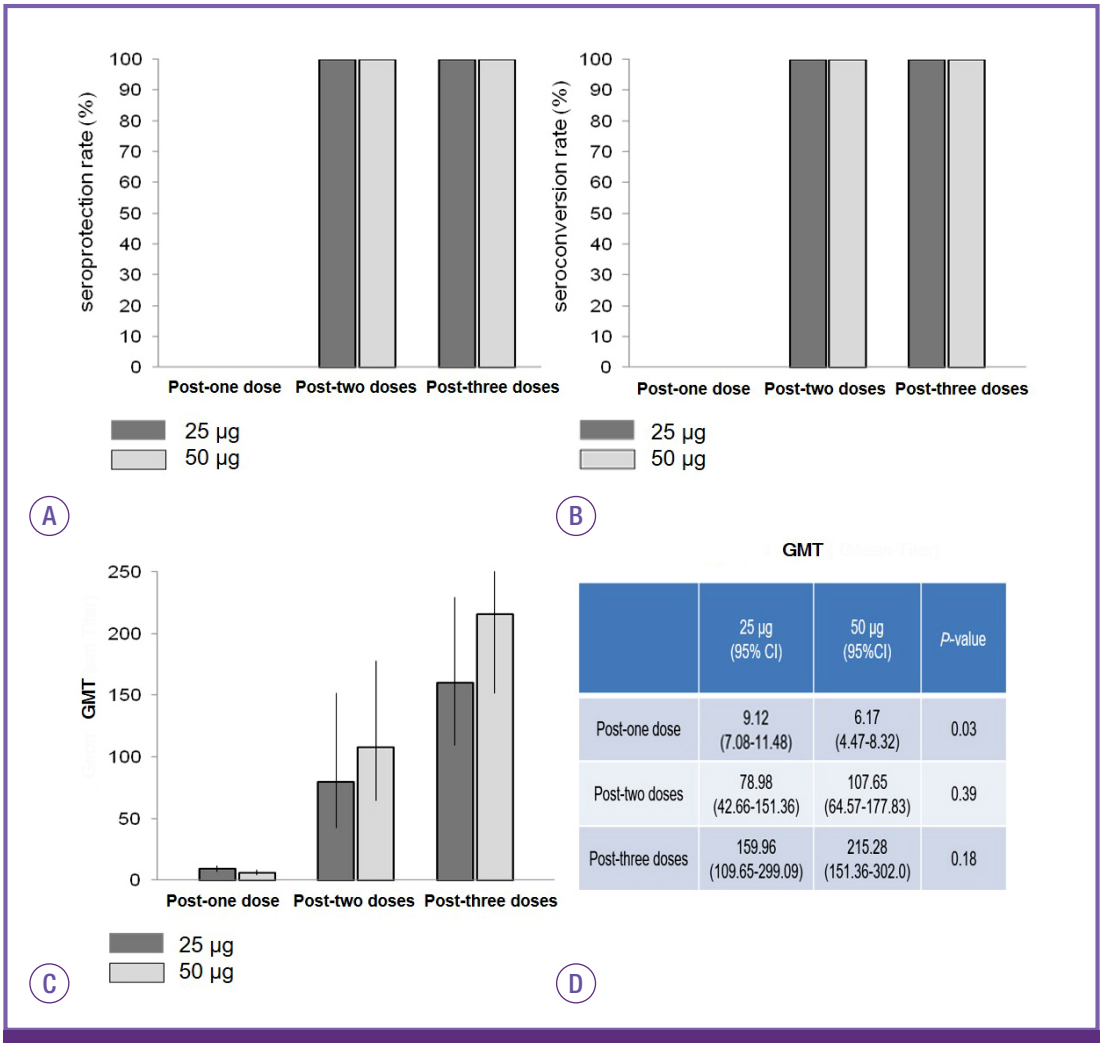
Mice immunized with H7 HA DNA vaccine showed a significant increase in IFN-γ production by splenocytes in response to stimulation with H7 HA peptides compared to the naïve and vector-immunized groups (Fig. 3). Two immunizations with H7 HA DNA vaccine induced significant increases in spot-forming units (SFUs) in both the 25 μg (234 ± 66 SFU/10^6 splenocytes) and 50 μg (255 ± 91 SFU/10^6 splenocytes) vaccination groups; this enhancement was proportional to the increased number of immunization doses. In mice with three immunizations, 50 μg H7 HA DNA vaccination (357 ± 39 SFU/10^6 splenocytes) enhanced IFN-γ production compared to 25 μg vaccination (275 ± 30 SFU/10^6 splenocytes, $P = 0.0021$, 50 μg *vs.* 25 μg vaccinations).

Discussion

DNA vaccination is considered a novel next-generation vaccine, which is achieved by the application of genetically engineered DNA to induce immunogenicity. DNA vaccines offer a variety of advantages, including the induction of broad cellular and humoral immune responses and rapid large-scale production [11, 12]. However, they also have some limitations, such as low efficiency in immunogenicity in large animals and humans [13]. To overcome the limitations of DNA vaccine, researchers have developed new delivery systems, including electroporation [14]. For example, the efficacy of hepatitis B surface antigen and human immunodeficiency virus gag DNA vaccine was shown to be greatly enhanced by an electroporation delivery method *in vivo* [15]. Moreover, when challenged with H1N1 influenza virus, mice immunized with DNA vaccine via electroporation recovered faster than mice immunized without electroporation [16].

Several reports have described DNA vaccines against veterinary and human influenza developed through animal testing and clinical trials. Immunization with the H5 HA DNA vaccine in chickens led to enhanced antibody responses and protection against avian influenza virus challenge [17]. Moreover, mice immunized with chitosan nanoparticles containing a swine influenza DNA vaccine showed humoral and cellular immunity [18], and influenza A DNA vaccine delivered via electroporation provided full protection against H1N1 challenge in a mouse model [16]. Our data demonstrate that the DNA vaccine against influenza virus induces humoral and cellular immune responses in mouse models.

In a previous study, a synthetic DNA vaccine elicited protective immunity against H7N9 influenza viruses in mouse models [10]. In this study, we investigated the immunogenicity of



	25 μg (95% CI)	50 μg (95%CI)	*P*-value
Post-one dose	9.12 (7.08-11.48)	6.17 (4.47-8.32)	0.03
Post-two doses	78.98 (42.66-151.36)	107.65 (64.57-177.83)	0.39
Post-three doses	159.96 (109.65-299.09)	215.28 (151.36-302.0)	0.18

Figure 2. Induction of HA-specific humoral immune responses in mice immunized with H7 HA DNA vaccine. BALB/c mice were immunized with vector (50 μg) or H7 HA DNA vaccine (25 or 50 μg) at one to three doses and blood was isolated 2 weeks after the last immunization. Seroprotection rate (A), seroconversion rate (B), and geometric mean titer (C and D) against H7N9 reassortant influenza virus were determined by hemagglutination inhibition (HI) test. The values are the means ± standard error of the means. Experiments were performed in duplicate and the values are the means ± standard error of the means.
GMT, geometric mean titer; CI, confidence interval.

this DNA vaccine against influenza A (H7N9) virus in detail, including two different doses of DNA vaccine and three repeated numbers of immunizations. Consistent with the previous report, DNA vaccination followed by electroporation efficiently induced antibody production and the IFN-γ response against the immunogen. However, when 25 μg DNA vaccine was injected, the induction of cellular immune responses in our study was lower than that in the previous study [10]. This difference may be related to differences in mouse strains, intervals between immunizations, and time of sacrifice of mice after immunization. Overall, our results confirmed and supported the efficacy of the H7 HA DNA vaccine in inducing immunogenicity against H7 HA peptides in mice.

Since human infections of influenza A (H7N9) virus were reported in March 2013, new cases of human infection have occurred. Various vaccines targeting influenza A (H7N9) virus have been developed in many countries. A phase 1 study showed that a recombinant virus-like particle influenza A (H7N9) vaccine administered with an adjuvant enhanced the antibody response [8]. A cell culture-derived MF59-adjuvanted pandemic A (H7N9) vaccine was shown to be immunogenic and safe in adults [9]. In addition to the development of these vaccines, our study suggests that the H7 HA DNA vaccine is also an effective tool for rapidly controlling emerging influenza viruses with pandemic potential.

In this study, we found that the H7 HA DNA vaccine confers

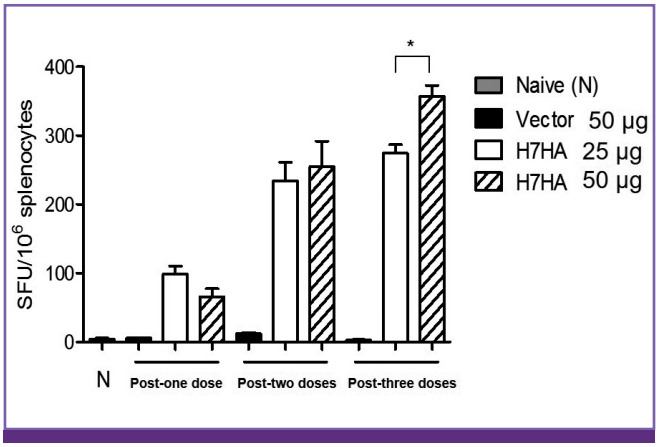


Figure 3. Induction of HA-specific cellular immune responses in mice immunized with H7 HA DNA vaccine. BALB/c mice were immunized with vector (50 μg) or H7 HA DNA vaccine (25 or 50 μg) at one to three doses and spleens were isolated 2 weeks after the last immunization. Mouse splenocytes were stimulated with H7 HA peptide pools for 16 h followed by IFN-γ ELISpot assay. IFN-γ production was determined by calculating the number of spot-forming cells per 1×10^6 splenocytes. Naïve mice (N) were used as a negative control. Experiments were performed in triplicate and the values are the means ± standard error of the means.
SFU, spot-forming unit.

significant humoral and cellular immunogenicity in mouse models. In the absence of an available vaccine against H7N9, our results suggest the potential for dealing with rapid mutations in influenza viruses including H7N9, although further studies are required, including clinical trials in humans.

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Conflicts of Interest

No conflicts of interest.

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