Protective Efficacy of a Single Dose of Baculovirus Hemagglutinin-Based Vaccine in Chickens and Ducks Against Homologous and Heterologous H5N1 Virus Infections

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Abstract

Outbreaks of the highly pathogenic H5N1 virus in poultry and humans are ongoing. Vaccination is an efficient method for prevention of H5N1 infection. Using chickens and ducks, we assessed the efficacy of a vaccine comprising H5N1 hemagglutinin (HA) protein produced in a baculovirus expression system. The immunized chickens and ducks were protected against lethal infection by H5N1 in an antigen dose-dependent manner. Complete protection against homologous challenge and partial protection against heterologous challenge were achieved in chickens immunized with 5 μ g HA protein and in ducks immunized with 10 μ g HA protein. The IgG antibody subtype was mainly detected in the sera and tissues, including the lungs. The neuraminidase (NA) inhibition assay was negative in immunized chickens and ducks. Our results indicated that the expressed HA protein by baculovirus was immunogenic to both chickens and ducks, and the immunized chickens and ducks were protected from the lethal infections of highly pathogenic H5N1 influenza virus, though ducks required more HA protein than chickens to be protected. Also, baculovirus HA-vaccinated poultry can be differentiated from infected poultry by NA inhibition assay.

Introduction

INFLUENZA VIRUSES ARE classified as types A, B, and C (3). Influenza B and C viruses mainly infect humans, and influenza A viruses can infect a variety of hosts, including humans, chickens, ducks, horses, dogs, cats, seals, and pigs (3,39). Influenza A viruses, comprising 16 hemagglutinin (HA) subtypes and nine neuraminidase (NA) subtypes, circulate in aquatic birds (9,39). Influenza A viruses have caused pandemics that have claimed thousands of human lives (10,14,16,17,19,32,33,36,37). The Spanish pandemic caused by avian-origin H1N1 influenza virus was the worst on record, resulting in the deaths of more than 50 million people from 1918 to 1919 (15).

Highly pathogenic (HP) H5N1 influenza virus was first detected in geese in Guangdong province, China, in 1996 (41). In 1997, it infected 18 humans (34), and has spread to Asia and the Middle East, including South Korea, Thailand, Vietnam, China, Egypt, Pakistan, and Turkey since 2003 (11,18,21,26,38,43,44).

While HP H5N1 influenza viruses are lethal for chickens, several duck species are quite resistant to HP H5N1 influenza viruses (12,20). The absence of diseases in some infected duck species with HP H5N1 influenza viruses led to the concept of “Trojan horses” that could act as silent spreaders of HP influenza viruses to other species (12,20). The mild disease in infected ducks was attributed to their low cytokine levels compared with infected chickens (4).

Vaccination is now considered an alternative strategy for controlling HP H5N1 infections in poultry, although slaughter strategy maintains a gold standard. A variety of approaches have been used to develop vaccines for chickens and ducks, including reverse genetic expression of H5 and N1, DNA vectors expressing H5, infectious laryngotracheitis virus expressing H5, nonpathogenic H5-subtype influenza virus, plant-expressed H5, Herpes virus of turkeys expressing H5, fowlpox virus expressing H5, Newcastle disease virus expressing H5, and baculovirus virus-like particles expressing H5 (6,8,13,25, 27,31,42).

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There are a number of disadvantages of other HP H5N1 influenza vaccines compared with the baculovirus-based vaccines. Whole inactivated vaccines are difficult to achieve because of the rapid death of inoculated eggs with HP H5N1 influenza viruses, and thus lower titers are usually obtained. The propagation of HP H5N1 influenza viruses must be done in biological safety level 3 (BSL-3) facilities, which are only available in a few countries. To generate safe vaccines from HP H5N1 influenza viruses, the cleavage sites must be modified by reverse genetic techniques.

In this study, we compared the immunogenicity of baculovirus vector-expressed HA protein in chickens and ducks. We also identified the antibody subtypes that mediate protection of immunized chickens and ducks from infection by HP H5N1.

Materials and Methods

Viruses and cells

HP H5N1 influenza viruses (A/Vietnam/1203/04 [H5N1] [clade 1] and A/Vietnam/HN31244/07 [H5N1] [clade 2]), which were isolated from the infected humans, were kindly provided by the WHO Collaborating Center for Influenza, Centers for Disease Control and Prevention. The viruses were propagated in 10-day-old specific pathogen free (SPF) chick embryo in a BSL-3 facility approved by the Korean government. SF-9 cells were maintained in Grace's insect cell culture medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 27°C.

Expression of HA protein in a baculovirus expression system

A/Vietnam/1203/04 HA protein was expressed in a BaculoDirect™ baculovirus expression system (Invitrogen). Briefly, the full-length HA gene of HP H5N1 influenza virus was polymerase chain reaction (PCR) amplified (primers: Baculo-VN-HA-F:5'-CAC CAT GGA GAA AAT AGT GCT TC-3' and Baculo-VN-HA-R:5'-AAT GCA AAT TCT GCA TTG TAA CGA-3') and recombined into BaculoDirect™ C-term linear DNA with LR Clonase™ II Enzyme Mix. Baculovirus encoding the HA protein was rescued by transfecting the recombined BaculoDirect™ C-term linear DNA containing the HA gene into SF-9 cells with Cellfectin® II reagent (Invitrogen) in 6-well plates.

Purification of HA protein

HA protein was purified from the SF-9 cells with ProBond™ nickel-chelating resin, cell lysis buffer containing 1× native purification buffer (100 mL: 80 mL of water and 20 mL of 250 mM NaH₂PO₄ and 2.5 M NaCl) and 10 mM imidazole, native wash buffer containing 1× native purification buffer and 20 mM imidazole, and native elution buffer containing 1× native purification buffer and 250 mM imidazole (Invitrogen).

Western blotting and Coomassie brilliant blue staining of HA protein

The expressed HA protein was separated by SDS-PAGE and transferred to a 0.45 µm nitrocellulose membrane (BioRad). The membrane was blocked with 5% skim milk

in phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween20 (Sigma). The blocked membrane was reacted with mouse anti-H5 antibody (polyclonal antibody prepared by immunizing inactivated H5N1 virus in mice in our laboratory) and then with HRP-labeled goat anti-mouse antibody (Invitrogen). In addition, the blocked membrane was stained with rabbit anti-histidine antibody and then with HRP-labeled goat anti-rabbit antibody (Invitrogen). The reacted membranes were added with the DAB substrate (KPL). The expressed HA protein separated by SDS-PAGE was also stained with Coomassie brilliant blue (Sigma-Aldrich).

Quantification of the purified HA protein

Purified HA protein was quantified by the Bradford method (Bio-Rad) against a standard curve of bovine serum albumin.

Immunization and challenge with HP H5N1 influenza viruses in chickens

Three-week-old leghorn chickens ($n=20$) that were serologically negative for H5N1 or H9N2 influenza viruses were purchased from local farms. Hemagglutination inhibition (HI) assay was performed to check the reactivity of sera to H5N1 and H9N2 influenza viruses using 0.5% turkey red blood cells. Immunization was administered by intramuscular (i.m.) injection of the pectoral muscle with 300 µL oil adjuvant HA protein (70% oil and 30% antigen diluted in PBS to 1, 2, 3, 4, and 5 µg). As a control, 3-week-old leghorn chickens ($n=10$) were mock immunized with 300 µL containing 70% oil and 30% PBS. The oil (Montanide IAS 70 VG) was obtained from SEPPIC Co. The immunized chickens ($n=10$ per group) were intranasally (i.n.) and intratracheally (i.t.) challenged with 0.5 mL of 100 chicken lethal dose 50/mL (100CLD50/mL) of A/Vietnam/1203/04 at 4 weeks post vaccination (p.v.) to study the homologous protection. In addition, the immunized chickens were i.n. and i.t. challenged with A/Vietnam/HN31244/2007 at 4 weeks p.v. to determine the heterologous protection. The infected chickens were monitored for survival for 14 days post challenge (p.c.) and were swabbed in the tracheas and cloacae in 1 mL of PBS for 14 days p.c.

Animal experiments were approved by the Animal Experimental Ethics Committee at Chungnam National University, and were performed at a BSL-3 facility approved by the Korean government.

Immunization and challenge with HP H5N1 influenza viruses in ducks

Three-week-old Pekin ducks ($n=20$) that were serologically negative for H5N1 or H9N2 were purchased from local farms. HI assay was performed to check the reactivity of sera to H5N1 and H9N2 influenza viruses using 0.5% turkey red blood cells. Immunization was performed by i.m. injection of the pectoral muscle with 300 µL oil adjuvant HA protein (70% oil and 30% antigen diluted in PBS to 1, 2, 3, 4, 5, 7.5, and 10 µg). As a control, 3-week-old Pekin ducks ($n=10$) were mock immunized with 300 µL containing 70% oil and 30% PBS. The immunized ducks ($n=10$ per group) were i.n. and i.t. challenged with 0.5 mL of 100 duck lethal

dose 50/mL (100DLD50/mL) of A/Vietnam/1203/04 or A/Vietnam/HN31244/2007 at 4 weeks p.v., and were swabbed in the tracheas and cloacae in 1 mL of PBS for 14 days p.c.

Antibody titers

Serum samples were collected from immunized chickens ($n=10$ per group) and ducks ($n=10$ per group) 4 weeks p.v. The samples were treated with receptor-destroying enzyme (RDE; Denka Seiken). RDE-treated sera were serially twofold diluted in PBS (pH 7.4) in V-bottom 96-well plates. Eight HA units (25 μ L) of A/Vietnam/HN31244/2007 was added, and the plates were incubated for 15 min at room temperature. Turkey red blood cells (50 μ L, 0.5%) were added and incubated at room temperature for 40 min. The HA inhibition titer was expressed as reciprocal dilutions that yielded complete HA inhibition.

Measurement of viral titers

Brain, lung, liver, kidney, and intestinal (rectal) tissue samples (1 g per organ) were obtained from immunized chickens (5 μ g; $n=3$ per group) and ducks (10 μ g; $n=3$ per group), which were different animals used for survival rate at 3 days p.c. The samples were homogenized with liquid nitrogen and a mortar and pestle. Homogenized tissues were diluted in 1 mL PBS (pH 7.4) supplemented with antibiotic-antimycotic solution (Sigma) and then serially 10-fold diluted in the same solution prior to inoculation of SPF eggs. Tissue and swabbed samples were inoculated into 10-day-old SPF chick embryo. Inoculated eggs were incubated in a humidified chamber (5% CO₂, 37°C) for 72 h. Presence of the virus in the amniotic fluid was confirmed with 0.5% turkey red blood cells, and the viral titer, log₁₀ egg infectious dose 50%/g (log₁₀EID₅₀/g) was calculated as described (30).

Histopathology and immunohistochemistry of lung tissues

We selected the lungs of chickens and ducks to determine histopathology and immunologic staining, since the lungs are the main target of influenza A viruses. Lung tissues from the same chickens and ducks used for the measurement of viral titers were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin (H&E) (1) and evaluated under an Olympus DP70 microscope (Olympus Corporation). Sections were deparaffinized, hydrated in distilled water, and fixed in 100% chilled acetone for 2 h, and were then stained with mouse anti-influenza A virus nucleoprotein (NP) antibody (Serotech). Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ for 15 min at 37°C and was followed by 5% bovine serum albumin in PBS (pH 7.4) for 1 h. The blocked tissue sections were labeled with the anti-influenza NP antibody (1:1,000 dilution) by incubating at room temperature for 1 h. Labeled tissue sections were stained with biotin-labeled goat anti-mouse immunoglobulin, VECTASTAIN ABC-AP, and Vector red alkaline phosphatase substrate (Vector). The stained tissue sections were counterstained with hematoxylin QS (Vector). The stained sections were evaluated under an Olympus DP70 microscope.

To measure histopathology scores in the lung lesions, the five sites in the lung tissues stained with H&E were randomly chosen to determine histopathology scores for inflammation. The scores were assigned according to the following criteria: 0, no inflammation; 1, mild, inflammatory cell infiltrates in the perivascular/peribronchiolar compartment; 2, moderate, inflammatory cell infiltrates in the perivascular/peribronchiolar space with modest extension into the alveolar parenchyma; and 3, severe, inflammatory cell infiltrates in the perivascular/peribronchiolar space with a greater magnitude of inflammatory foci in the alveolar parenchyma.

Detection of antibody subtypes in the sera and tissues

Sera and brain, lung, liver, kidney, and intestinal (rectal) tissues (1 g per tissue) from immunized chickens (5 μ g; $n=3$ per group), which were different animals used for survival rate, and viral titers were collected 4 weeks p.v. and homogenized in 1 mL PBS (pH 7.4) containing 5% horse serum. Homogenized samples (200 μ L) were added to a 96-well plate coated with 0.05 μ g purified H5N1 virus (A/Vietnam/1203/04) (clade 1), and the mixed plate was incubated at room temperature for 60 min. The plate was washed three times with PBS (pH 7.4) containing 0.05% Tween-20 (Sigma); horseradish peroxidase (HRP)-conjugated goat anti-chicken IgM (Serotec), IgG (KPL), IgA (Serotec), or HRP-conjugated goat anti-duck IgG (KPL; 100 μ L) was added to each well of the plate. The plate was incubated at room temperature for 30 min, and ABTS peroxidase substrate was added (KPL). Optical density was read at 405 nm in a microplate reader.

NA inhibition assay

Sera were collected 4 weeks p.v. from the immunized chickens (5 μ g; $n=5$) and ducks (10 μ g; $n=5$). Sera from PBS mock-immunized chickens ($n=5$) and ducks ($n=5$) were also collected. The sera (50 μ L) were diluted 1:10 in PBS (pH 7.4), mixed with inactivated H5N1 virus (A/Vietnam/1203/04; 50 μ L) diluted 1:10 in PBS (equivalent to 1 U NA activity), and incubated at room temperature for 60 min. Fetuin (100 μ L) was added and incubated at 37°C for 18 h, followed by addition of 1 mL arsenite and 2.5 mL thiobarbituric acid. The samples were placed in boiling water for 15 min. Warrenoff was added, and the samples were measured at an OD of 549. As a positive control, we used sera from chickens and ducks immunized with whole H5N1 antigens (7.5 μ g) containing both HA and NA proteins.

Statistical analysis

Statistical significance was determined by the Mann-Whitney *U*-test. A *p*-value of <0.05 was considered significant.

Results

Expression of HA protein from HP H5N1 influenza virus

The HA protein of HP H5N1 influenza virus was expressed in a baculovirus expression system to study vaccine efficacy in chickens and ducks. Purified HA protein was separated by SDS-PAGE, reacted with HRP-labeled anti-histidine and anti-H5 antibody, and stained with coomassie

brilliant blue (Fig. 1). The 75 kDa HA protein was stained by antihistidine antibody (Fig. 1A), anti-H5 antibody (Fig. 1B), and stained with coomassie brilliant blue (Fig. 1C).

Protective immunity in chickens

We tested the immunogenicity of expressed HA protein by immunizing chickens ($n=10$ per group) with oil adjuvant (Fig. 2). Chickens immunized with various doses of HA protein were bled 4 weeks p.v. to test for induction of antibodies against the homologous (clade 1) or heterologous (clade 2) HP H5N1 influenza virus by HI assay. Some chickens immunized with $>2 \mu\text{g}$ of HA protein were serologically converted against the homologous virus with mean HI titers from 253.3 to 612, but chickens immunized with $1 \mu\text{g}$ HA protein were not serologically converted. The mean HI titer against heterologous H5N1 influenza virus was 10 in chickens immunized with $2\text{--}5 \mu\text{g}$ of HA protein. Against the homologous H5N1 influenza virus, 6/10 chickens immunized with $2 \mu\text{g}$ of HA protein contained an HI titer >10 , 8/10 chickens immunized with 3 or $4 \mu\text{g}$ HA protein had titers >10 , and all chickens immunized with $5 \mu\text{g}$ HA protein had HI titers >10 (Fig. 2A).

The immunized chickens ($n=10$ per group) were challenged with homologous HP H5N1 influenza virus (Fig. 2B). All chickens immunized with $5 \mu\text{g}$ HA protein survived, but the survival rate of chickens immunized with 3 or $4 \mu\text{g}$ HA was 80% and that of chickens immunized with $2 \mu\text{g}$ HA protein was 60%. Chickens ($n=10$ per group) immunized with $5 \mu\text{g}$ HA protein and challenged with the heterologous HP H5N1 influenza virus exhibited a survival rate of 60% (Fig. 2C). Viral titers were measured in tissues and swabbed samples (tracheas and cloacae) of chickens immunized with $5 \mu\text{g}$ HA protein and challenged with homologous or heterologous HP H5N1 influenza virus (Fig. 2D and Table 1). No virus was detected in the brain, lung, liver, kidney, and intestine (rectum) of immunized chickens challenged with the homologous virus ($p<0.001$), but virus was detected in immunized chickens challenged with the heterologous virus ($p<0.05$). Viral titers in the lung, liver, kidney, and intestine (rectum) of immunized chickens challenged with the heterologous virus were 2.5, 1.5, 2, and $1.5 \log_{10}\text{EID}_{50}/\text{g}$ respectively ($p<0.05$), while those in the PBS mock-immunized chickens were 8, 5, 5.5, and $3.5 \log_{10}\text{EID}_{50}/\text{g}$ respectively

($p<0.05$). No virus was detected in swabbed samples from immunized chickens challenged with the homologous virus (Table 1), while 2/5 immunized chickens challenged with the heterologous shed viruses with a mean viral titer of $2.5 \log_{10}\text{EID}_{50}/\text{mL}$ in the tracheas and $3.5 \log_{10}\text{EID}_{50}/\text{mL}$ in the cloacae (Table 1). The PBS mock-immunized chickens challenged with homologous and heterologous viruses shed viruses from 2.5 to $3.5 \log_{10}\text{EID}_{50}/\text{mL}$ (Table 1).

The protective immunity conferred by HA protein in chickens was confirmed by histopathology and immunohistochemistry. Lung tissues of chickens immunized with $5 \mu\text{g}$ HA protein and challenged with the homologous or heterologous virus were stained with H&E. Samples from chickens challenged with the homologous virus exhibited no pathologic damage or infiltration of inflammatory cells in the alveolar region (Fig. 3A); samples from chickens challenged with the heterologous virus exhibited mild thickening of the alveolar septa and minor infiltration of inflammatory cells (Fig. 3B). The lung tissues of PBS mock-immunized chickens challenged with homologous or heterologous HP H5N1 influenza virus exhibited severe interstitial pneumonia with significant infiltration of inflammatory cells in the alveolar region (Fig. 3C and D). No interstitial pneumonia was observed in the lung tissue of PBS mock-immunized and mock-challenged chickens (Fig. 3E). Histopathology scores were measured in the lung tissues (Fig. 3F). Lung tissue of immunized chickens challenged with homologous virus showed a mean score of 0.4, while that of immunized chickens challenged with heterologous virus had a mean score of 1.8. Lung tissues of PBS mock-immunized chickens challenged with homologous or heterologous virus had scores of 2.4 and 2.2 respectively.

Staining of lung tissues with influenza A anti-NP antibody revealed no antigen in the lungs of immunized chickens challenged with the homologous HP H5N1 influenza virus (Fig. 4A), and few antigens were detected in the lungs of immunized chickens challenged with the heterologous HP H5N1 influenza virus (Fig. 4B). Several positive antigens were detected in the lungs of PBS mock-immunized chickens challenged with the homologous (Fig. 4C) or heterologous HP H5N1 influenza virus (Fig. 4D). No antigens were detected in the lung tissue of PBS mock-immunized and mock-challenged chickens, since no virus infected the chickens (Fig. 4E).

FIG. 1. Confirmation of the expressed highly pathogenic (HP) H5N1 HA protein by Western blotting and Coomassie brilliant blue staining. The expressed hemagglutinin (HA) protein was reacted with rabbit antihistidine antibody (A), mouse anti-H5 antibody (B), or stained with Coomassie (C). M, protein marker; 1, HA protein; Arrow, HA.

Protective immunity in ducks

Ducks ($n=10$ per group) were immunized and bled to measure HI titers (Fig. 5A). Ducks immunized with 7.5 and $10 \mu\text{g}$ HA protein were serologically converted with mean HI titers of 30 and 40 against the homologous H5N1 influenza virus. No HI titers were detected in ducks immunized with 1, 2, 3, 4, or $5 \mu\text{g}$ HA protein. The mean HI titer against the heterologous H5N1 influenza virus was 10 in ducks immunized with 7.5 or $10 \mu\text{g}$ HA protein. Six out of 10 ducks immunized with $7.5 \mu\text{g}$ HA protein exhibited HI titers >10 , and all ducks immunized with $10 \mu\text{g}$ HA protein had HI titers >10 against the homologous H5N1 influenza virus (Fig. 5A). The immunized ducks ($n=10$) were challenged with homologous HP H5N1 influenza virus (Fig. 5B). The survival rate of ducks immunized with $7.5 \mu\text{g}$ HA protein was 60%, while that of ducks immunized with $10 \mu\text{g}$

FIG. 2. Vaccine efficacy in chickens. Chickens ($n=10$ per group) were immunized via intramuscular (i.m.) injection with oil adjuvant HA protein (1, 2, 3, 4, 5 μ g) in the pectoral muscle. The immunized chickens were bled 4 weeks post vaccine (p.v.), and antibody titers were determined by hemagglutination inhibition (HI) assay using the homologous (clade 1) and heterologous (clade 2) HP H5N1 influenza virus (A). Antibody titers were a mean of 10 immunized chickens. The immunized chickens ($n=10$ per group) were challenged with homologous (clade 1) (B) or heterologous (clade 2) (C) HP H5N1 influenza virus, and mortality was observed for 14 days post challenge (p.c.). Tissues (1 g per tissue) collected from the brain, lung, liver, kidney, and intestine (rectum) of HA adjuvant immunized chickens (5 μ g; $n=3$ per group) challenged with homologous (clade 1) or heterologous (clade 2) HP H5N1 influenza virus in the homogenates with phosphate-buffered saline (PBS), and viral titers were measured in eggs by log₁₀EID₅₀/g (D). Data represent one of two experiments, since the results were similar. The statistical analysis of viral titers of immunized and challenged chickens was performed compared with PBS mock-immunized and challenged chickens. Homo virus, homologous virus; hetero virus, heterologous virus; !, number of chickens with HI titers >10/total number of immunized chickens. * $p<0.05$; ** $p<0.001$.

TABLE 1. VIRAL SHEDDING IN THE IMMUNIZED CHICKENS INFECTED WITH HIGHLY PATHOGENIC H5N1 INFLUENZA VIRUS

	Immunized chickens		Naïve chickens	
	Tracheal swab	Cloacal swab	Tracheal swab	Cloacal swab
Challenged virus	No. of positive chickens/total chickens (the mean viral titers: log ₁₀ EID ₅₀ /mL)	No. of positive chickens/total chickens (the mean viral titers: log ₁₀ EID ₅₀ /mL)	No. of positive chickens/total chickens (the mean viral titers: log ₁₀ EID ₅₀ /mL)	No. of positive chickens/total chickens (the mean viral titers: log ₁₀ EID ₅₀ /mL)
Homologous virus	0/5 (<1)	0/5 (<1)	5/5 (3.5)	5/5 (2.5)
Heterologous virus	2/5 (2.5)	2/5 (3.5)	5/5 (3)	5/5 (3)

Chickens were immunized with 5 μ g of hemagglutinin (HA) protein, and were challenged with homologous (clade 1) or heterologous (clade 2) HP H5N1 influenza virus 4 weeks after vaccination. The challenged chickens were swabbed in tracheas and cloacae in phosphate-buffered saline (PBS; pH 7.4) 36 h after challenge. Viral titers in the swabbed samples were measured by log₁₀EID₅₀/mL. The detection limit was <1 log₁₀EID₅₀/mL.

FIG. 3. Lung histopathology and histopathology scores for inflammation in lung lesions in chickens. The same lung tissues from Figure 2D were embedded in paraffin. Thin sections (5 μ m) were stained with hematoxylin and eosin (H&E). (A) Lung tissue from an immunized chicken challenged with the homologous virus. (B) Lung tissue from an immunized chicken challenged with the heterologous virus. (C) Lung tissue from a PBS mock-immunized chicken challenged with the homologous virus. (D) Lung tissue from a PBS mock-immunized chicken challenged with the heterologous virus. (E) Lung tissue from a PBS mock-immunized, mock-challenged chicken. (F) Histopathology scores for inflammation.

HA antigens was 100%. None of the ducks immunized with 1, 2, 3, 4, or 5 μ g HA survived. Ducks ($n=10$ per group) immunized with 10 μ g HA and challenged with heterologous HP H5N1 influenza virus had a 60% survival rate (Fig. 5C). Viral titers were measured in the tissues and swabbed samples (tracheas and cloacae) of ducks immunized with 10 μ g HA protein and challenged with the homologous and heterologous virus (Fig. 5D and Table 2). No viruses were detected in the brain, lung, liver, kidney, or intestine (rectum) of immunized ducks challenged with homologous HP H5N1 ($p<0.001$). Immunized ducks challenged with heterologous HP H5N1 contained viruses in the lung, liver, and kidney with mean viral titers of 2, 1.75, and 1.25 $\log_{10}$ EID₅₀/g respectively ($p<0.05$). Mean viral titers in the lung, liver, and kidney of naïve ducks challenged with heterologous HP H5N1 were 4.5, 3.5, and

4.5 $\log_{10}$ EID₅₀/g respectively ($p<0.05$; Fig. 5D). We measured viral titers in swabbed samples from the tracheas and cloacae of ducks (Table 2). No virus was detected in the tracheas and cloacae of immunized ducks challenged with homologous HP H5N1 influenza virus, but 2/5 immunized ducks challenged with the heterologous HP H5N1 influenza virus shed viruses in the tracheas and cloacae with the mean viral titer of 2.5 $\log_{10}$ EID₅₀/mL (Table 2). All PBS mock-immunized ducks challenged with the homologous or heterologous HP H5N1 virus shed viruses in the tracheas and cloacae with titers from 2.25 to 3.5 $\log_{10}$ EID₅₀/mL (Table 2).

Protective immunity in ducks immunized with 10 μ g HA protein was evaluated by histopathology and immunohistochemistry. Lung tissue staining in immunized ducks challenged with the homologous virus showed no infiltration of

FIG. 4. Lung immunohistochemistry in chickens. Paraffin sections of the same lung tissues from Figure 2D were stained with mouse anti-influenza A virus nucleoprotein antibody, biotin-labeled goat anti-mouse immunoglobulin, alkaline phosphatase substrate, and hematoxylin. (A) Lung tissue of an immunized chicken challenged with the homologous (clade 1) HP H5N1 influenza virus. (B) Lung tissue of an immunized chicken challenged with the heterologous (clade 2) virus. (C) Lung tissue of the PBS mock-immunized chicken challenged with the homologous (clade 1) virus. (D) Lung tissue of the PBS mock-immunized chicken challenged with the heterologous (clade 2) virus. (E) Lung tissue of the PBS mock-immunized, mock-challenged chicken. Arrows, positive stainings of antigens.

inflammatory cells and no thickening of the alveolar septa (Fig. 6A), but the lung tissue of immunized ducks challenged with the heterologous virus exhibited mild interstitial pneumonia with mild infiltration of inflammatory cells in the alveolar region (Fig. 6B). The lung tissues of PBS mock-immunized ducks challenged with homologous or heterologous virus exhibited severe interstitial pneumonia with severe infiltration of inflammatory cells in the alveolar region and severe thickening of the alveolar septa (Fig. 6C and D). No interstitial pneumonia was observed in the lung tissue of PBS mock-immunized and mock-challenged ducks (Fig. 6E).

Histopathology scores were measured in the lung tissues (Fig. 6F). Lung tissue of immunized ducks challenged with homologous virus showed a mean score of 0.2, while that of immunized ducks challenged with heterologous virus had a mean score of 1.8. Lung tissues of PBS mock-immunized ducks challenged with homologous or heterologous virus had scores of 2.4 and 2.6 respectively.

Lung tissue staining of ducks immunized with influenza A anti-NP antibody exhibited no antigen staining after challenge with the homologous HP H5N1 influenza virus (Fig. 7A), but minor antigen staining was detected in the lungs of immunized ducks challenged with the heterologous virus (Fig. 7B). Positive antigen staining was detected in the lung tissue of PBS mock-immunized ducks challenged with the homologous (Fig. 7C) or heterologous HP H5N1 influenza virus (Fig. 7D). No antigens were detected in lung tissue of PBS mock-immunized and mock-challenged ducks (Fig. 7E).

Antibody subtypes in the immunized chickens and ducks

Antibody subtypes were evaluated in sera and tissues (brain, lung, liver, kidney, and intestine) to determine which antibody was responsible for protecting the immunized chickens (5 µg HA protein) and ducks (10 µg HA protein)

FIG. 5. Vaccine efficacy in ducks. Ducks ($n = 10$ per group) were i.m. immunized with oil adjuvant HA protein (1, 2, 3, 4, 5, 7.5, 10 μg) in the pectoral muscle. (A) The immunized ducks were bled 4 weeks p.v., and antibody titers were determined by HI assay using the homologous (clade 1) and heterologous (clade 2) HP H5N1 influenza virus. (B) The immunized ducks ($n = 10$ per group) were challenged with the homologous (clade 1). (C) The immunized ducks (10 μg ; $n = 10$ per group) were challenged with heterologous (clade 2) H5N1 influenza virus. (D) Tissues (1 g per tissue) were collected from the brain, lung, liver, kidney, and intestine (rectum) of immunized ducks (10 μg ; $n = 3$ per group) challenged with the homologous (clade 1) or heterologous (clade 2) HP H5N1 influenza virus in PBS, and viral titers were measured in eggs by $\log_{10}\text{EID}_{50}/\text{g}$. The statistical analysis of viral titers of immunized and challenged ducks was performed compared to PBS mock-immunized and mock-challenged ducks. !, number of ducks with HI titers >10 /total number of immunized ducks. * $p < 0.05$; ** $p < 0.001$.

TABLE 2. VIRAL SHEDDING IN THE IMMUNIZED DUCKS INFECTED WITH HP H5N1 INFLUENZA VIRUS

	Immunized ducks		Naïve ducks	
	Tracheal swab	Cloacal swab	Tracheal swab	Cloacal swab
Challenged virus	No. of positive ducks/ total ducks (the mean viral titers: $\log_{10}\text{EID}_{50}/\text{mL}$)	No. of positive ducks/ total ducks (the mean viral titers: $\log_{10}\text{EID}_{50}/\text{mL}$)	No. of positive ducks/ total ducks (the mean viral titers: $\log_{10}\text{EID}_{50}/\text{mL}$)	No. of positive ducks/ total ducks (the mean viral titers: $\log_{10}\text{EID}_{50}/\text{mL}$)
Homologous virus	0/5 (<1)	0/5 (<1)	5/5 (3.5)	5/5 (2.25)
Heterologous virus	2/5 (2.5)	2/5 (2.5)	5/5 (2.5)	5/5 (2.25)

Ducks were immunized with 10 μg of HA protein, and were challenged with homologous (clade 1) or heterologous (clade 2) HP H5N1 influenza virus 4 weeks after vaccination. The challenged ducks were swabbed in tracheas and cloacae in PBS (pH 7.4) 72 h after challenge. Viral titers in the swabbed samples were measured by $\log_{10}\text{EID}_{50}/\text{mL}$. The detection limit was $<1 \log_{10}\text{EID}_{50}/\text{mL}$.

FIG. 6. Lung histopathology and histopathology scores for inflammation in lung lesions in ducks. The same lung tissues from Figure 5D were embedded in paraffin. Sections were stained with H&E. (A) Lung tissue of an immunized duck challenged with the homologous (clade 1) HP H5N1 influenza virus. (B) Lung tissue of an immunized duck challenged with heterologous (clade 2) virus. (C) Lung tissue of a PBS mock-immunized duck challenged with the homologous (clade 1) virus. (D) Lung tissue of a PBS mock-immunized duck challenged with the heterologous (clade 2) HP H5N1 influenza virus. (E) Lung tissue of the PBS mock-immunized virus-naïve duck. (F) Histopathology scores for inflammation.

from infection with HP H5N1. The IgG antibody was predominant in the sera and tissues of the immunized chickens (Fig. 8C). The O.D. values of IgG in the sera ($p < 0.001$), lung, and kidney of the immunized chickens were 1.36, 0.66, and 0.51 respectively ($p < 0.05$; Fig. 8C). We also measured IgG antibody in the serum, brain, lung, liver, kidney, and intestine (rectum) of immunized ducks (Fig. 8D). We could not measure IgM and IgA antibodies in ducks because there are no commercially available HRP labeled anti-duck IgM and IgA antibodies. The O.D. values of IgG in the sera, lung, and kidney were 0.61, 0.31, and 0.3 respectively ($p < 0.05$; Fig. 8D).

NA inhibition in the immunized chickens and ducks

On farms, immunized chickens and ducks need to be differentiated from birds carrying H5N1 infections. We

examined whether the immunized chickens and ducks could be differentiated from birds with HP H5N1 infections. We collected sera from chickens ($n=5$) immunized with $5 \mu\text{g}$ HA protein and from ducks ($n=5$) immunized with $10 \mu\text{g}$ HA protein 4 weeks p.v. for measurements of NA inhibition activity (Fig. 9A and B). NA activity in sera from chickens and ducks immunized with HA protein and sera from PBS mock control chickens and ducks was similar, but NA activity in sera from chickens and ducks immunized with whole H5N1 antigens was significantly reduced, indicating NA inhibition. The O.D. values of NA activity in sera from chickens and ducks immunized with HA protein were 0.545 and 0.585 respectively, whereas the activity in sera from chickens and ducks immunized with whole H5N1 antigens was 0.302 ($p < 0.05$). The results suggest that immunization of chickens and ducks with HA protein does not induce the production of antibody against NA protein.

FIG. 7. Lung immunohistochemistry in ducks. The same lung tissues from Figure 5D were embedded in paraffin. Sections were stained with mouse anti-influenza A virus nucleoprotein antibody, biotin-labeled goat anti-mouse immunoglobulin, alkaline phosphatase substrate, and hematoxylin. (A) The lung tissue of an immunized duck challenged with the homologous (clade 1) HP H5N1 influenza virus. (B) Lung tissue of an immunized duck challenged with the heterologous virus. (C) Lung tissue of a PBS mock-immunized duck challenged with the homologous virus. (D) Lung tissue of a PBS mock-immunized duck challenged with the heterologous virus. (E) Lung tissue of the PBS mock-immunized, mock-challenged duck.

Discussion

The HP H5N1 influenza virus is endemic to many Asian countries and is a threat to the poultry industry and humans. Most human infections appear in individuals with close contact with poultry. Efficient vaccination of chickens and ducks will reduce economic loss and human infection with HP H5N1 influenza. We expressed and purified the whole HA (H5) of HP H5N1 influenza virus in a baculovirus system. The vaccine efficacy of the purified HA protein with oil adjuvant was compared in chickens and ducks. The expressed HA protein by baculovirus was immunogenic to both chickens and ducks, and the immunized chickens and ducks were protected from the lethal infections of HP H5N1 influenza virus, though ducks required more HA protein than chickens to be protected.

Our results showed that chickens needed to be immunized with $\geq 5 \mu\text{g}$ of A/Vietnam/HN31233/2007 (H5N1) HA, and

ducks needed to be immunized with $\geq 10 \mu\text{g}$ to achieve complete protection against homologous virus, indicating different immunogenicity between the two species. We suggest that genetic differences may be responsible for the differential immunogenicity of HA protein in ducks and chickens. Our preliminary data with whole inactivated H5N1 antigens containing HA and NA proteins also indicate that ducks must be inoculated with greater amounts of H5N1 antigen than chickens to be protected from the lethality of HP H5N1 influenza virus (data not shown). Our results suggest that the different doses of vaccine antigens against HP H5N1 influenza viruses may be needed to protect chickens or ducks. The previous studies with HA protein expressed by baculovirus could provide protective immunity to ducks (5,28,29). When the expressed HA protein by baculovirus encoding HA of A/Vietnam/1203/04 (H5N1) was inoculated into ducks with water-in-oil adjuvant, the immunized ducks were protected from the lethal infections of

FIG. 8. Antibody subtypes in the tissues of immunized chickens and ducks. Tissues (1 g) from the brain, lung, liver, kidney, and intestine (rectum) or sera were collected from the immunized chickens (5 μ g; $n=3$ per group) and ducks (10 μ g; $n=3$ per group), which were different animals used for survival rate and viral titers at 4 weeks p.v. and were homogenized in PBS. Homogenized samples and sera were reacted with whole inactivated H5N1 influenza virus (clade 1). HRP-conjugated goat anti-chicken IgM, IgG, IgA, or HRP20 conjugated goat anti-duck IgG was added with peroxidase substrate. Optical density (OD) was measured at 405 nm. Statistical analysis was performed on sera from the immunized chickens and ducks compared with PBS mock-immunized chickens and ducks. * $p<0.05$; ** $p<0.001$. (A) IgM antibody in immunized chickens; (B) IgA antibody in immunized chickens; (C) IgG antibody in immunized chickens; (D) IgG antibody in immunized ducks.

FIG. 9. Neuraminidase (NA) inhibition with sera from immunized chickens and ducks. Sera were collected from chickens ($n=5$; 5 μ g) and ducks ($n=5$; 10 μ g) immunized with oil adjuvant HA or oil adjuvant inactivated whole H5N1 influenza virus (clade 1; 5 μ g) 4 weeks p.v. Sera from PBS mock-immunized chickens ($n=5$) and ducks ($n=5$) were also collected. NA inhibition activity was measured with the inactivated homologous H5N1 influenza virus (clade 1). OD was measured at 549 nm. Statistical analysis was performed on sera from the immunized chickens and ducks compared with PBS mock-immunized chickens and ducks. (A) Chicken sera; (B) duck sera. * $p<0.05$.

A/Vietnam/1203/04 (H5N1) with the reduction of duration and titer of virus shedding (5). The triple baculovirus recombinant encoding H5, N3, and M protected ducks from the infection of homologous H5N3 low pathogenicity avian influenza virus (28,29).

We showed that all chickens immunized with 5 μ g HA proteins could be protected from HP H5N1 infection. The other previous studies also showed that baculovirus-based HA vaccines could protect chickens from the lethal infections of HP H5N1 influenza viruses (2,7,23,24,35,40). Chickens immunized with HA protein expressed by baculovirus encoding HA of A/duck/China/E319-2/2003 (H5N1; 2.3.2) were 100% protected from clinical signs when they were challenged with HP H5N1 viruses belonging to clades 2.2.1 and 2.2.1.1 (2). The expressed HA protein of A/goose/Guangdong/1/96 (H5N1) by baculovirus provided protective immunity for chickens within 2 weeks after vaccination, and its protective immunity lasted for more than 6 months (24). Baculovirus pseudotyped with vesicular stomatitis virus glycoprotein expressing HA protein of A/Chicken/Hubei/327/2004 (H5N1) protected chickens from the lethal infections by A/Chicken/Hubei/327/2004 (H5N1) (40). When HA protein of A/duck/China/E319-2/03 (H5N1) was expressed by baculovirus and inoculated into chickens, they were 100% protected from the lethal infections of A/duck/China/E319-2/03 (H5N1) without shedding of viruses (23). Chickens immunized with baculovirus-expressed HA protein of A/chicken/Jalisco/14589-660/94 (H5N2) were protected against the clinical signs and death when they were challenged with A/chicken/Queretaro/14588/95 (H5N2) and A/chicken/Pennsylvania/1370/83 (H5N2) (35). A water-in-oil HA vaccine expressed by baculovirus encoding HA of A/chick/Jalisco/94 (H5N1) protected 3-week-old chickens from the lethal infections of A/chicken/Queretaro/95 (H5N2) and A/chicken/Pennsylvania/83 (H5N2) with reduced or no cloacal shedding at 3 days post infection (7).

The survival rate of immunized chickens with 5 μ g HA proteins from the infection of heterologous HP H5N1 influenza virus was about 60%, and the number of those chickens shedding viruses through tracheae and cloacae was reduced compared to infected naïve chickens, even though HI antibody titers were <10. The partial protection of immunized chickens from heterologous challenge may be mediated by antibody against HA stalk region, which is a target for universal influenza vaccine. The poor heterologous protection may be due to the antigenic difference in HA proteins between vaccine (A/Vietnam/1203/04) and challenge (A/Vietnam/HN31244/07) H5N1 viruses. The amino acid difference of HA proteins between the two viruses is 4.1%.

We measured IgM, IgA, and IgG in the tissues and sera of animals immunized with HA protein to determine which subtype mediates protection. IgG was predominant in tissues and sera. Our previous study with whole H5N1 inactivated antigens also showed that IgG was predominant in the sera and tissues of immunized chickens (13). Vaccination against HP H5N1 influenza virus in poultry can interfere with serological surveillance on farms. Our study showed that anti-HA antibody could be induced in immunized chickens and ducks, and NA inhibition antibody was not induced,

suggesting the NI assay could be used to differentiate infected from vaccinated poultry. The previous study used a reassorted virus containing an NA subtype different from that of the infecting virus to differentiate infected from vaccinated poultry (22). This study constructed recombinant H5N1 and H7N8 vaccine viruses to protect the poultry from lethal infections of H5N2 and H7N2.

In conclusion, chickens and ducks immunized with the expressed and purified HA protein from HP H5N1 were protected against lethal infection by HP H5N1 influenza.

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