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Pathogenicity and Vaccine Efficacy of Different Clades of Asian H5N1 Avian Influenza A Viruses in Domestic Ducks[▽]

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Waterfowl represent the natural reservoir of all subtypes of influenza A viruses, including H5N1. Ducks are especially considered major contributors to the spread of H5N1 influenza A viruses because they exhibit diversity in morbidity and mortality. Therefore, as a preventive strategy against endemic as well as pandemic influenza, it is important to reduce the spread of H5N1 influenza A viruses in duck populations. Here, we describe the pathogenicity of dominant clades (clades 1 and 2) of H5N1 influenza A viruses circulating in birds in Asia. Four representatives of dominant clades of the viruses cause symptomatic infection but lead to different profiles of lethality in domestic ducks. We also demonstrate the efficacy, cross-protectiveness, and immunogenicity of three different inactivated oil emulsion whole-virus H5 influenza vaccines (derived by implementing reverse genetics) to the viruses in domestic ducks. A single dose of the vaccines containing 1 μg of hemagglutinin protein provides complete protection against a lethal A/Duck/Laos/25/06 (H5N1) influenza virus challenge, with no evidence of morbidity, mortality, or shedding of the challenge virus. Moreover, two of the three vaccines achieved complete cross-clade or cross-subclade protection against the heterologous avian influenza virus challenge. Interestingly, the vaccines induce low or undetectable titers of hemagglutination inhibition (HI), cross-HI, and/or virus neutralization antibodies. The mechanism of complete protection in the absence of detectable antibody responses remains an open question.

Aquatic birds, including ducks and geese, are thought to be the natural reservoir of influenza A viruses because all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza A viruses have been isolated from these hosts. In this reservoir, influenza A viruses have been in a state of evolutionary stasis, with asymptomatic infection (24, 35, 48). Influenza A viruses can be transmitted from the natural hosts to a variety of animals, including humans, pigs, horses, sea mammals, tigers, leopards, and various avian species (11, 13, 20, 28, 32, 43, 47, 48). Once influenza A viruses are transmitted to other hosts, they may cause either mild or severe respiratory diseases. Among the 16 HA subtypes of influenza A viruses, only two HA subtypes (H5 and H7) are reported to cause respiratory and systemic diseases in the natural hosts (38).

A highly pathogenic H5N1 avian influenza A virus (A/Goose/Guangdong/1/96; Gs/GD/1/96) emerged in southern China in 1996 and subsequently spread into Hong Kong in 1997, causing serious disease outbreaks (6, 50). Now, H5N1 avian influenza A viruses are endemic in domestic poultry in many countries of Asia, including China, Japan, South Korea, Thailand, Vietnam, Indonesia, Cambodia, Malaysia, and Laos, resulting in diminution of poultry stocks and economic losses in the poultry industry. They have continued to spread across Europe and Africa (http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html/).

Moreover, the direct bird-to-human transmission of a reassortant virus containing the H5 HA gene of Gs/GD/1/96 (H5N1)-like virus and the other genes from A/Teal/Hong Kong/W312/97 (H6N1)-like virus caused 6 deaths in 18 infected people in Hong Kong in 1997 (1, 4, 36). The transmission of H5N1 avian influenza A viruses to humans has been reported in other countries of Asia, including Vietnam, Thailand, Cambodia, and Indonesia (12, 27, 30). To date, 381 laboratory-confirmed human cases have been reported by the World Health Organization, with 240 ending in death (http://www.who.int/csr/disease/avian_influenza/en/).

The H5 HA gene, traceable to Gs/GD/1/96 (H5N1), has not been replaced in a variety of highly pathogenic H5N1 influenza A virus isolates since 1996, while the other genes have undergone genetic reassortment events yielding a series of genetic groups (called genotypes) where the Z genotype is dominant (22). However, the H5 HA gene has evolved to give rise to antigenically distinguishable viruses. Therefore, the Z genotype has now been classified into 10 antigenic subgroups (called clades 0 to 9). Among the 10 clades, clade 2 is further classified into 5 subclades (2.1 to 2.5). The subclades 2.1 and 2.3 are further delineated into subclades (2.1.1 to 2.1.3 and 2.3.1 to 2.3.4, respectively). The majority of current circulating H5N1 influenza A viruses, including both avian and human

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cases, belong to clades 1 and 2 (http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/).

Ducks are thought to be the primary source of influenza A viruses, including H5N1 viruses. In particular, free-range as well as backyard domestic ducks are associated with the spread of H5N1 influenza A viruses in Southeast Asia (9, 46). The genetic variants of H5N1 influenza A viruses that emerged by rapid evolution between 2002 and 2005 in ducks have contributed not only to the continuing circulation of H5N1 influenza viruses in Southeast Asia but also to spread of them across extensive geographic areas (17, 33). Therefore, reducing the risk of H5N1 influenza A virus infection in ducks is important for controlling the continuing circulation and spread of endemic H5N1 influenza A viruses.

Currently, avian influenza A viruses, including H5N1, are mainly controlled in many countries by traditional methods, including quarantine, culling of infected animals followed by eradication, and improved sanitation (2, 45). However, because the traditional methods are not always completely effective, alternative control strategies should be considered. Vaccination would be the most effective component of alternative control strategies because influenza vaccines have been shown to reduce virus shedding and prevent symptoms of disease in chickens, turkeys, geese, and ducks (18, 37, 40, 42, 46). In 2002 to 2003, a field trial of an inactivated oil emulsion whole-virus H5 influenza vaccine successfully controlled H5N1 influenza outbreaks in Hong Kong (7).

Ideal H5N1 influenza vaccine candidates should be efficacious and induce cross-protection against H5N1 influenza A viruses from different antigenic subgroups (clades and subclades) within Z genotypes. In a previous study by our laboratory, an inactivated oil emulsion whole-virus H5N3 influenza vaccine was generated by implementing reverse genetics using the H5 HA of A/Chicken/Vietnam/C58/04 (Ck/VN/C58/04; H5N1; clade 1), the N3 NA of A/Duck/Germany/1215/73 (Dk/Ger/1215/73; H2N3), and the internal genes of A/Puerto Rico/8/34 (PR/8/34; H1N1) (46). The immunogenicity and efficacy of the vaccine were tested in ducks and chickens. The vaccine was highly efficacious and immunogenic in both ducks and chickens. However, the vaccine was only tested against homologous Ck/VN/C58/04 (clade 1) and A/Duck/Thailand/71.1/04 (clade 1) H5N1 influenza virus challenges.

In the present study, we examined the pathogenicity of the dominant clades of H5N1 influenza A viruses circulating in birds in Asia. We also investigated the efficacy, cross-protectiveness, and immunogenicity of three different inactivated oil emulsion whole-virus H5 influenza vaccines (derived by implementing reverse genetics) to the viruses against a lethal H5N1 avian influenza virus challenge in domestic ducks.

MATERIALS AND METHODS

Viruses. Five H5N1 influenza A viruses were used in this study: A/Hong Kong/213/03 (HK/213/03; clade 1), A/Chicken/Vietnam/C58/04 (Ck/VN/C58/04; clade 1), A/Whooper Swan/Mongolia/244/05 (WS/Mong/244/05; clade 2.2), A/Japanese White-Eye/Hong Kong/1038/06 (JWE/HK/1038/06; clade 2.3.4), and A/Duck/Laos/25/06 (Dk/Laos/25/06; clade 2.3.4). The viruses used in this study were obtained from collaborators in China, Vietnam, Mongolia, and Lao People's Democratic Republic. Stock viruses were grown in 10-day-old embryonated chicken eggs for 36 to 48 h at 35°C. The allantoic fluid was then harvested, and aliquots were stored at −80°C until use. Virus titers were determined by calculating the 50% egg infectious dose (EID_{50}) per ml of virus stock, using the method of Reed and Muench (31). All experiments with the H5N1 viruses were performed in biosafety level 3+ facilities at St. Jude Children's Research Hospital (St. Jude; Memphis, TN) and approved by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

Ducks. Specific-pathogen-free outbred white Pekin ducks (*Anas platyrhynchos*) were purchased from McMurray Hatchery (Webster City, IA). The ducks were leg banded and provided feed and water ad libitum in pens in biosafety level 3+ facilities. All animal experiments were approved by the Animal Care and Use Committee of St. Jude and performed in compliance with relevant institutional policies, National Institutes of Health regulations, and the Animal Welfare Act.

Pathogenicity of H5N1 viruses in domestic ducks. The pathogenicity of four H5N1 avian influenza A viruses (Ck/VN/C58/04 [clade 1], WS/Mong/244/05 [clade 2.2], JWE/HK/1038/06 [clade 2.3.4], and Dk/Laos/25/06 [clade 2.3.4]) was examined in domestic ducks. Groups of six 4-week-old ducks were inoculated via intranasal, intraocular, and intratracheal instillation of $\sim 10^{8.0}$ EID_{50} of the viruses in a total volume of 1.0 ml. As a negative control group, six 4-week-old ducks were inoculated with phosphate-buffered saline (PBS). All birds were observed daily for morbidity, mortality, and virus shedding for 2 weeks. Reduced food intake, reduced water intake, depression, possible fever, beak color change, cloudy eyes, and neurological symptoms (such as torticollis, ataxia, and seizures) were observed to follow the symptoms of disease. Tracheal and cloacal swabs were collected from all living birds for virus isolation at 3 and 5 days postinoculation.

Determination of the lethal dose of Dk/Laos/25/06 (H5N1) virus in domestic ducks. To determine the lethal dose of Dk/Laos/25/06 (H5N1; clade 2.3.4) virus in domestic ducks, groups of three 4-week-old ducks were inoculated via intranasal, intraocular, and intratracheal instillation of serial 10-fold dilutions of the virus starting with $10^{7.25}$ EID_{50} in a total volume of 1.0 ml. All birds were observed daily for symptoms of disease and mortality. Tracheal and cloacal swabs were collected from all living birds for virus isolation at 3 and 5 days postinoculation.

Generation of recombinant viruses. Three recombinant viruses (H5N3 or H5N1 subtypes) were generated to make H5 influenza vaccines using DNA transfection of eight plasmids, as previously described (15, 16, 23). One recombinant H5N3 virus was generated in a previous study by our laboratory (46), and two additional recombinant H5N1 viruses were generated in this study.

Briefly, the recombinant viruses were constructed on a genetic background containing the internal genes of PR/8/34 (H1N1), with modification of the H5 HA genes of Ck/VN/C58/04 (H5N1; clade 1), WS/Mong/244/05 (H5N1; clade 2.2), and JWE/HK/1038/06 (H5N1; clade 2.3.4) by deletion of the polybasic amino acid region at the HA1-HA2 cleavage site. The NA genes of the recombinant viruses were derived from Dk/Ger/1215/73 (H2N3), WS/Mong/244/05 (H5N1; clade 2.2), and JWE/HK/1038/06 (H5N1; clade 2.3.4). Thus, the recombinant viruses contained the H5 HA gene of Ck/VN/C58/04 (H5N1; clade 1) and the N3 NA gene of Dk/Ger/1215/73 (H2N3), generating an H5N3 subtype; the H5 HA and N1 NA genes of WS/Mong/244/05 (H5N1; clade 2.2), generating an H5N1 subtype; and the H5 HA and N1 NA genes of JWE/HK/1038/06 (H5N1; clade 2.3.4), generating a second H5N1 subtype, respectively.

Preparation of vaccines. The recombinant viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 h. Allantoic fluid was harvested and treated with formalin at a final concentration of 0.025% (vol/vol) to inactivate the viruses. After incubation at 4°C for 3 days, inactivation was confirmed by the absence of detectable infectivity after two blind passages of the formalin-treated allantoic fluid in 10-day-old embryonated chicken eggs. For the recombinant Ck/VN/C58/04 (H5N1) virus, the content of the HA protein in the allantoic fluid was standardized using a single radial immunodiffusion technique without concentration and purification (49). For the other recombinant viruses, the allantoic fluid was concentrated using a Pellicon-2 concentrator (Millipore Corporation, Bedford, MA) and purified using sucrose density gradient separation. The concentration of HA protein in the allantoic fluid was also standardized using a single radial immunodiffusion technique. The vaccines were formulated as a standard water emulsion in mineral oil. Based on the names of the H5 HA antigen donor viruses (Ck/VN/C58/04 [H5N1; clade 1], WS/Mong/244/05 [H5N1; clade 2.2], and JWE/HK/1038/06 [H5N1; clade 2.3.4]), the vaccines were denoted as rg-Ck/C58, rg-WS/244, and rg-JWE/1038 H5 influenza vaccines, respectively. A placebo vaccine contained virus-free allantoic fluid from 12-day-old embryonated chicken eggs.

Vaccination of ducks. To determine the optimal concentration of HA protein in an inactivated oil emulsion whole-virus H5 influenza vaccine, groups of seven 2-week-old ducks were immunized intramuscularly in the breast with a single dose of rg-WS/244 H5 vaccine containing 1,000, 200, 40, 8, 1.6, or 0.32 ng of HA protein in a total volume of 0.5 ml. As a negative control group, seven 2-week-old

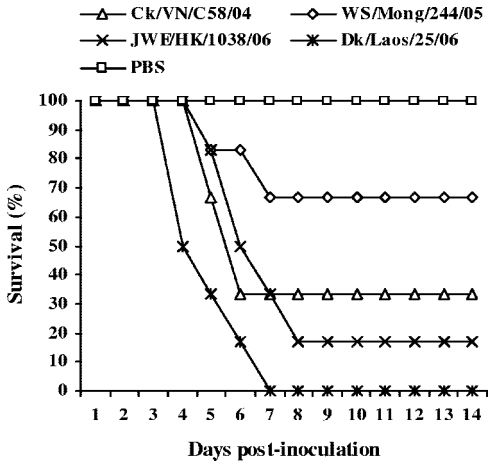

FIG. 1. Survival curves of ducks inoculated with representatives of dominant clades of H5N1 avian influenza A viruses. Groups of six white Pekin ducks were observed for 14 days after inoculation via natural routes with $\sim 10^{8.0}$ EID_{50} of the viruses in a total volume of 1.0 ml. As a negative control group, ducks were inoculated with PBS. Ducks that showed severe symptoms of disease were euthanized, and their deaths were recorded on the following day of observation.

ducks were immunized with a placebo vaccine containing virus-free allantoic fluid.

To examine the efficacy, cross-protectiveness, and immunogenicity of three different H5 influenza vaccines, groups of 10 2-week-old ducks were immunized intramuscularly in the breast with a single dose of rg-Ck/C58, rg-WS/244, or rg-JWE/1038 H5 influenza vaccine containing 1 μg of HA protein in a total volume of 0.5 ml. As a negative control group, 10 2-week-old ducks were immunized with a placebo vaccine containing virus-free allantoic fluid.

Challenge and swab sampling. The vaccinated ducks were challenged via intranasal, intraocular, and intratracheal instillation of $10^{1.25}$ EID_{50} of Dk/Laos/25/06 (H5N1; clade 2.3.4) virus in a total volume of 1.0 ml at 3 weeks postvaccination. All birds were monitored daily for 2 weeks for symptoms of disease and mortality. Tracheal and cloacal swabs were collected from all living birds for virus isolation at 3, 5, 7, and 10 days postchallenge.

Blood sampling and treatment. Blood samples were collected from all birds prior to vaccination and weekly after vaccination. Blood samples were also obtained from all living birds at 14 days postchallenge. The serum samples were treated with a receptor-destroying enzyme (Denka Seiken, Campbell, CA), following the manufacturer's instructions for the hemagglutination inhibition (HI) assay, or heat inactivated at 56°C for 30 min for the virus neutralization (VN) assay.

Serologic analyses by HI and VN assays. The HI assay was performed as described previously (25). Briefly, viruses were diluted to contain 4 agglutinating units in PBS. The diluted viruses were incubated with serial twofold dilutions of receptor-destroying enzyme-treated serum samples, starting with a 1:10 dilution at room temperature for 30 min. The antigen-antibody mixtures were tested for HA activity by the addition of 0.5% chicken red blood cells (CRBCs) or 1% horse red blood cells (HRBCs) to determine the HI titers.

The VN assay was performed as described previously (21). Briefly, Madin-Darby canine kidney cells were plated into 96-well tissue culture plates. Virus was diluted to contain 2,000 50% tissue culture infectious dose ($TCID_{50}$)/ml in 1× minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 4% (wt/vol) bovine serum albumin and L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ) at the final concentration of 1 μg/ml. The diluted virus (200 $TCID_{50}$/100 μl) was incubated with an equal volume (100 μl) of serial twofold dilutions of heat-inactivated serum samples, starting with a 1:40 dilution at 37°C for 1 h. The antigen-antibody mixtures were added into each well (four wells per mixture) of the plates containing confluent monolayers of Madin-Darby canine kidney cells. After incubation at 37°C for 72 h, 50 μl of supernatant from each well was tested for HA activity by the addition of 0.5% CRBCs or 1% HRBCs. Geometric mean titers (GMT) were calculated for each group of serum samples.

RESULTS

Pathogenicity of dominant clades of H5N1 viruses in domestic ducks. The pathogenicity of dominant clades (clades 1 and 2) of H5N1 influenza A viruses circulating in birds in Asia was examined in domestic ducks. Four representatives of dominant clades of the viruses (Ck/VN/C58/04 [clade 1], WS/Mong/244/05 [clade 2.2], JWE/HK/1038/06 [clade 2.3.4], and Dk/Laos/25/06 [clade 2.3.4]) were selected based on the year of isolation and antigenic characterization and tested in white Pekin ducks. The ducks inoculated with Ck/VN/C58/04 (H5N1; clade 1), WS/Mong/244/05 (H5N1; clade 2.2), or JWE/1038/06 (H5N1; clade 2.3.4) virus started showing mild symptoms of disease, such as depression, possible fever, and cloudy eyes, at 3 days postinoculation, while the ducks inoculated with Dk/Laos/25/06 (H5N1; clade 2.3.4) virus started showing mild symptoms of disease at 1 day postinoculation. Severe symptoms of disease, such as neurological symptoms, followed by mortality, started at 4 to 5 days postinoculation for all viral challenges (data not shown).

Figure 1 shows the proportions of lethality in the duck groups inoculated with the viruses. Four out of six birds died in the Ck/VN/C58/04-inoculated group; two out of six birds died in the WS/Mong/244/05-inoculated group; five out of six birds died in the JWE/HK/1038/06-inoculated group; and all of the birds died in the Dk/Laos/25/06-inoculated group. All surviving birds in the Ck/VN/C58/04-inoculated and the JWE/HK/1038/06-inoculated groups showed severe symptoms of disease. However, the surviving birds in the WS/Mong/244/05-inoculated group showed only mild symptoms of disease, except one bird that showed severe symptoms of disease.

As shown in Table 1, all of the viruses were predominantly shed from the respiratory tract; mean tracheal titers were ~4.9 $\log_{10}$ EID_{50}/ml at 3 days postinoculation. The viruses were also

TABLE 1. Tracheal and cloacal titers of representatives of dominant clades of H5N1 avian influenza A viruses in domestic ducks

H5N1 virus	Virus titer[a]			
	Trachea		Cloaca	
	3 dpi	5 dpi	3 dpi	5 dpi
Ck/VN/C58/04	4.6 ± 0.8 (6/6)	2.3 ± 0.8 (6/6)	2.0 ± 0.7 (6/6)	1.4 ± 1.2 (2/6)
WS/Mong/244/05	5.2 ± 0.8 (6/6)	1.9 ± 0.9 (4/6)	2.1 ± 1.1 (6/6)	1.0 ± 0.4 (2/6)
JWE/HK/1038/06	5.0 ± 1.2 (6/6)	2.8 ± 0.6 (6/6)	1.9 ± 0.7 (5/6)	1.5 ± 0.0 (1/6)
Dk/Laos/25/06	4.7 ± 1.2 (6/6)	3.4 ± 0.1 (3/3)	2.9 ± 0.6 (6/6)	1.4 ± 1.2 (2/3)

[a] Virus titers were determined in eggs and are expressed as the $\log_{10}$ EID_{50}/ml. Data are presented as means ± standard deviations of titers of positive samples (≥0.75 $\log_{10}$ EID_{50}/ml). The numbers of birds that shed virus are indicated in parentheses (number shedding/number tested). dpi, days postinfection.

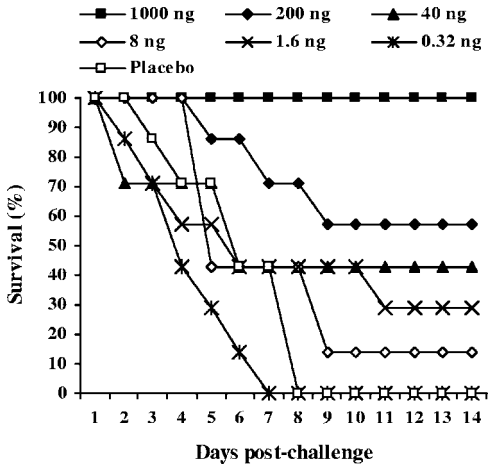

FIG. 2. Survival curves of ducks immunized with the rg-WS/244 H5 influenza vaccine containing different doses of HA protein after challenge with a lethal strain of H5N1 avian influenza A virus. White Pekin ducks were immunized with a single dose of rg-WS/244 H5 influenza vaccine containing 1,000, 200, 40, 8, 1.6, or 0.32 ng of HA protein. As a negative control group, ducks were immunized with a placebo vaccine containing virus-free allantoic fluid. The ducks were observed for 14 days after challenge with Dk/Laos/25/06 (H5N1) virus. Ducks that showed severe symptoms of disease were euthanized, and their deaths were recorded on the following day of observation.

shed through the fecal route; mean cloacal titers were ~2.2 $\log_{10}$ EID_{50}/ml at 3 days postinoculation. The tracheal and cloacal titers of the viruses were considerably reduced at 5 days postinoculation. Only traces ($\leq$2.5 $\log_{10}$ EID_{50}/ml) were detected in the cloacal swab samples at 5 days postinoculation. However, in the negative control group (to which PBS was administered), all birds survived and no symptoms of disease were observed.

These results showed that representatives of dominant clades of H5N1 influenza A viruses circulating in birds in Asia caused symptomatic infection but exhibited different lethality profiles in domestic ducks. Remarkably, Dk/Laos/25/06 (H5N1; clade 2.3.4) virus showed the highest (100%) lethality in domestic ducks. Based on these results, Dk/Laos/25/06 (H5N1; clade 2.3.4) virus was chosen as the challenge virus in the following vaccine study.

Pathogenicity of Dk/Laos/25/06 (H5N1) virus at different doses in domestic ducks. The pathogenicity of Dk/Laos/25/06 (H5N1; clade 2.3.4) virus was investigated at different doses of the virus in white Pekin ducks. Groups of three ducks were inoculated via natural routes using serial 10-fold dilutions of the virus starting with $10^{7.25}$ EID_{50}. All birds in each group receiving $10^{7.25}$ to $10^{1.25}$ EID_{50} of the virus showed severe symptoms of disease and died within 7 days (data not shown). To reconfirm the lethality of $10^{1.25}$ EID_{50} of the virus, another batch of three ducks was inoculated with $10^{1.25}$ EID_{50} of the virus. All three birds receiving the dose of the virus also showed severe symptoms of disease and died. There were no observable differences in the development of morbidity and the time to death between the groups. These results indicated that Dk/Laos/25/06 (H5N1; clade 2.3.4) virus showed extremely high pathogenicity in domestic ducks, and $10^{1.25}$ EID_{50} of the virus was sufficient to kill the domestic ducks. Therefore, we selected $10^{1.25}$ EID_{50} of Dk/Laos/25/06 (H5N1; clade 2.3.4) virus as a challenge dose in the following vaccine study.

Protective efficacy of rg-WS/244 H5 influenza vaccine at different doses of HA protein in domestic ducks. To determine the relevant dose of HA protein in an inactivated oil emulsion H5 influenza vaccine (derived by implementing reverse genetics) to provide complete protection in domestic ducks, groups of white Pekin ducks were immunized with a single dose of the rg-WS/244 H5 influenza vaccine containing 1,000, 200, 40, 8, 1.6, or 0.32 ng of HA protein. As a negative control, ducks were immunized with a placebo vaccine containing virus-free allantoic fluid. Three weeks postvaccination, all birds were challenged with Dk/Laos/25/06 (H5N1; clade 2.3.4) virus.

Figure 2 shows the proportions of survivors in the immunized duck groups after challenge with the virus. In the group of birds immunized with the rg-WS/244 H5 influenza vaccine containing 1 μg of HA protein, all birds survived with no symptoms of disease. In the group of birds immunized with the rg-WS/244 H5 influenza vaccine containing 200 ng of HA protein, four out of seven birds survived; all surviving birds in the group had no symptoms of disease. In the group of birds immunized with the rg-WS/244 H5 influenza vaccine containing 40 ng of HA protein, three out of seven birds survived; among the surviving birds, two birds had no symptoms of disease, and one bird showed mild symptoms, such as cloudy eyes. In the group of birds immunized with the rg-WS/244 H5 influenza vaccine containing 8 ng of HA protein, only one bird survived; the bird showed severe symptoms of disease, including neurological symptoms. In the group of birds immunized with the rg-WS/244 H5 influenza vaccine containing 1.6 ng of HA protein, two birds survived; among the surviving birds, one bird had no symptoms of disease, but the other bird showed severe symptoms. In the group of birds immunized with the rg-WS/244 H5 influenza vaccine containing 0.32 ng of HA protein or the placebo vaccine, all of the birds died.

Virus shedding through the respiratory tract and the fecal route was detected in all groups of birds (including the healthy birds), except for the group immunized with the vaccine containing 1 μg of HA protein (Table 2). The virus replication in the respiratory tract and the fecal route was reduced in a vaccine dose-dependent manner.

These results indicated that 1 μg of HA protein in a single

TABLE 2. Tracheal and cloacal titers of Dk/Laos/25/06 (H5N1) in domestic ducks immunized with the rg-WS/244 H5 influenza vaccine at different doses of HA protein

Dose of HA protein (ng)	Virus titer[a]			
	Trachea		Cloaca	
	3 dpi	5 dpi	3 dpi	5 dpi
1,000	<[b]	<	<	<
200	3.7 ± 1.3 (6/7)	2.6 ± 1.7 (6/7)	1.5 ± 1.1 (4/7)	1.9 ± 0.7 (2/7)
40	3.7 ± 0.7 (5/5)	3.0 ± 1.1 (4/4)	2.9 ± 0.7 (4/5)	1.7 ± 0.3 (3/4)
8	4.3 ± 1.0 (7/7)	3.9 ± 0.5 (3/3)	2.4 ± 1.1 (7/7)	2.5 ± 0.4 (2/3)
1.6	4.1 ± 1.7 (5/5)	3.8 ± 1.6 (4/4)	2.2 ± 0.8 (4/5)	1.4 ± 1.4 (2/4)
0.32	5.4 ± 0.8 (5/5)	6.6 ± 0.2 (2/2)	3.5 ± 1.0 (5/5)	3.0 ± 0.7 (2/2)
Placebo	5.6 ± 0.2 (6/6)	4.8 ± 1.2 (5/5)	2.5 ± 1.1 (5/6)	2.6 ± 1.3 (5/5)

[a] Virus titers were determined in eggs and are expressed as the $\log_{10}$ EID_{50}/ml. Data are presented as means ± standard deviations of titers of positive samples ($\geq$0.75 $\log_{10}$ EID_{50}/ml). The numbers of birds that shed virus are indicated in parentheses (number shedding/number tested). dpi, days postinfection.

[b] <, the titer was below the limit of detection (<0.75 $\log_{10}$ EID_{50}/ml).

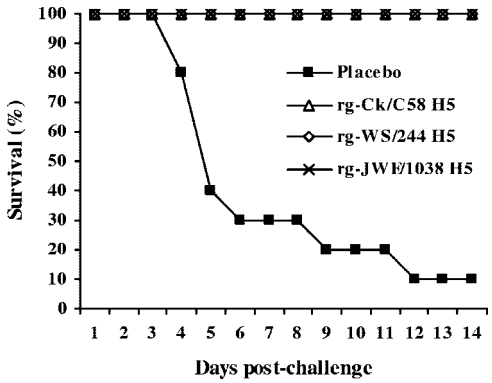

FIG. 3. Survival curves of ducks immunized with different H5 influenza vaccines after challenge with a lethal strain of H5N1 avian influenza A virus. White Pekin ducks were immunized with a single dose (1 μg of HA protein) of rg-Ck/C58, rg-WS/244, or rg-JWE/1038 H5 influenza vaccine. As a negative control group, ducks were immunized with a placebo vaccine containing virus-free allantoic fluid. The ducks were observed for 14 days after challenge with Dk/Laos/25/06 (H5N1) virus. Ducks that showed severe symptoms of disease were euthanized, and their deaths were recorded on the following day of observation.

dose of an inactivated oil emulsion whole-virus H5 influenza vaccine derived by implementing reverse genetics and prepared using concentration and sucrose density gradient purification was sufficient to provide complete protection from morbidity, mortality, and virus shedding against a lethal H5N1 avian influenza virus challenge in domestic ducks.

Protective efficacy of H5 influenza vaccines. To evaluate the protective efficacy of three different inactivated oil emulsion whole-virus H5 influenza vaccines (rg-Ck/C58, rg-WS/244, and rg-JWE/1038 H5 influenza vaccines) in domestic ducks, groups of white Pekin ducks were immunized with a single dose (1 μg of HA protein) of one of the three H5 influenza vaccines. As a negative control, other ducks were immunized with a placebo vaccine containing virus-free allantoic fluid. Three weeks postvaccination, all birds were challenged with Dk/Laos/25/06 (H5N1; clade 2.3.4) virus.

As shown in Fig. 3, all birds immunized with the H5 influenza vaccines survived, while 9 out of 10 birds of the negative control group died. None of the birds immunized with the H5 influenza vaccines showed symptoms of disease, and none of them shed detectable challenge virus at 3, 5, 7, or 10 days postchallenge (Table 3). However, all birds in the negative control group (including the surviving bird) showed severe symptoms of disease, including neurological symptoms, and virus shedding.

These results showed that all three of the H5 influenza vaccines generated and tested in this study provided complete protection by a single dose of the vaccine against a lethal H5N1 influenza virus challenge, with no evidence of morbidity, mortality, or shedding of the challenge virus. Of experimental significance, two of the three H5 influenza vaccines (rg-Ck/C58 and rg-WS/244 H5) achieved complete cross-clade and cross-subclade protection, respectively.

Serologic responses of domestic ducks to H5 vaccines. To examine the immunogenicity of the vaccinated ducks, HI antibody titers in sera from the vaccinated birds were assayed with HI tests using CRBCs or HRBCs. Prior to vaccination, all of the birds were seronegative (HI antibody titers of <10) for the influenza A viruses.

The HI antibody titers were quantified against the corresponding wild-type H5N1 viruses (Ck/VN/C58/04 [clade 1], WS/Mong/244/05 [clade 2.2], or JWE/HK/1038/06 [clade 2.3.4]) (Table 4). The HI antibody titers in sera from the rg-Ck/C58- or rg-WS/244 H5-vaccinated group, measured by the addition of CRBCs, were detectable but low (HI antibody GMT of 20 and 35 at 3 weeks postvaccination, respectively). In contrast, the HI antibody titers in sera from the rg-JWE/1038 H5-vaccinated group, measured by the addition of CRBCs, were very low or undetectable (HI GMT of 14 in 4 of 10 birds at 3 weeks postvaccination). After challenge, the HI antibody titer in the rg-Ck/C58 H5-vaccinated group was around threefold higher (HI GMT of 57) than that prior to challenge. However, the HI antibody responses in the rg-WS/244- and rg-JWE/1038 H5-vaccinated groups were not affected by challenge.

The HI antibody titers in sera from the rg-Ck/C58- or rg-WS/244 H5-vaccinated group, measured by the addition of HRBCs, were much higher than those measured by the addition of CRBCs (roughly 12-fold versus 6-fold higher at 3 weeks postvaccination). However, the HI antibody titers in sera from the rg-JWE/1038 H5-vaccinated group, measured by the addition of HRBCs, were still low or undetectable (HI GMT of 33 in 4 of 10 birds at 3 weeks postvaccination).

The HI antibody titers were also quantified using HK/213/03 (H5N1; clade 1) virus, which seems to be a better antigen for detection of HI antibody responses (14). As shown in Table 4, in the rg-Ck/C58- and rg-WS/244 H5-vaccinated groups, the HI antibody titers against HK/213/03 (H5N1; clade 1) were higher than those against corresponding viruses (HI GMTs of 171 and 61, measured by the addition of CRBCs at 3 weeks postvacci-

TABLE 3. Efficacy of H5 influenza vaccines against a lethal H5N1 influenza virus challenge[a]

Vaccine	No. of ducks shedding/no. tested							
	Tracheal shedding				Cloacal shedding			
	3 dpc	5 dpc	7 dpc	10 dpc	3 dpc	5 dpc	7 dpc	10 dpc
rg-Ck/C58 H5	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
rg-WS/244 H5	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
rg-JWE/1038 H5	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Placebo	10/10	8/8	3/3	0/1	10/10	8/8	0/3	0/1

[a] Ducks were immunized with a single dose (1 μg of HA protein) of a vaccine and then challenged with Dk/Laos/25/06 (H5N1) virus. dpc, days postchallenge.

TABLE 4. Serologic responses (HI titers) induced by H5 influenza virus vaccines

Vaccine	HI GMT[a]							
	CRBCs				HRBCs			
	Corresponding virus		HK/213/03		Corresponding virus		HK/213/03	
	3 wpv	2 wpc[b]	3 wpv	2 wpc[b]	3 wpv	2 wpc[b]	3 wpv	2 wpc[b]
rg-Ck/C58 H5	20 (10/10)	57 (10/10)	171 (10/10)	343 (10/10)	243 (10/10)	394 (10/10)	299 (10/10)	394 (10/10)
rg-WS/244 H5	35 (10/10)	37 (10/10)	61 (10/10)	106 (10/10)	197 (10/10)	172 (10/10)	80 (10/10)	101 (10/10)
rg-JWE/1038 H5	14 (4/10)	10 (3/10)	<10 (0/10)	<10 (0/10)	33 (4/10)	18 (7/10)	14 (4/10)	<10 (0/10)
Placebo	<10 (0/10)	80 (1/10)[c]	<10 (0/10)	40 (1/10)[c]	<10 (0/10)	2560 (1/10)[c]	<10 (0/10)	320 (1/10)[c]

[a] HI antibody titers were determined against corresponding H5N1 viruses (Ck/VN/C58, WS/Mong/244/05, and JWE/HK/1038/06) or HK/213/03 (H5N1) virus and are expressed as the reciprocal of the highest dilution of sera that inhibited hemagglutination by 4 HA units of virus. The results are the geometric mean titers of positive sera (≥10). The numbers of positive sera are indicated in parentheses (number of positive sera/total number of sera). wpv, weeks postvaccination; wpc, weeks postchallenge.
[b] Ducks were challenged with the lethal Dk/Laos/25/06 (H5N1) virus at 3 weeks postvaccination.
[c] HI antibody titer (against the challenge virus) of one surviving bird.

nation, respectively; HI GMTs of 299 and 394, measured by the addition of HRBCs at 3 weeks postvaccination, respectively). After challenge, the HI antibody titers, measured by the addition of CRBCs or HRBCs, rose in the rg-Ck/C58- and rg-WS/244 H5-vaccinated groups. However, HK/213/03 (H5N1; clade 1) also failed to detect a significant HI antibody response in the rg-JWE/1038 H5-vaccinated group (HI antibody titers of <10, measured by the addition of CRBCs; HI GMTs of 14, measured by the addition of HRBCs in 4 of 10 birds at 3 weeks postvaccination, respectively).

Cross-HI antibody responses against the challenge virus were also examined by the addition of CRBCs or HRBCs (Table 5). The cross-HI antibody titers measured by the addition of CRBCs in sera from the rg-Ck/C58 H5-vaccinated group were very low or undetectable (HI, 10 to 20; HI GMT, 14). In contrast, the cross-HI antibody titers measured by the addition of CRBCs in sera from the rg-WS/244- and rg-JWE/1038 H5-vaccinated groups were undetectable (cross-HI titers of <10). The cross-HI antibody titers measured by the addition of HRBCs in the vaccinated birds were higher than those measured by the addition of CRBCs. However, the titers were still low or undetectable (HI GMT of 43 in the rg-Ck/C58 H5-vaccinated group; HI GMT of 31 in 8 of 10 birds in the rg-WS/244 H5-vaccinated group; HI GMT of 14 in 4 of 10 birds in the rg-JWE/1038 H5-vaccinated group).

Additionally, the VN assay was performed to examine VN antibody responses targeting the challenge virus by the addition of CRBCs or HRBCs (Table 5). The types of RBCs did not make any differences in the VN antibody titers. The VN antibody titer in sera from the rg-Ck/C58 H5-vaccinated group was low (VN GMT of 21). In contrast, the VN antibody titers in sera from the rg-WS/244- and rg-JWE/1038 H5-vaccinated groups were very low or undetectable (VN GMT of 13 in 3 of 10 birds in the rg-WS/244 H5-vaccinated group; VN titers of <10 in the rg-JWE/1038 H5-vaccinated group).

These results suggested that the H5 influenza vaccines showed different immunogenicities in the induction of serum antibody responses. The rg-Ck/C58 and rg-WS/244 H5 influenza vaccines were immunogenic in the induction of HI antibody responses against the corresponding viruses, while the rg-JWE/1038 H5 influenza vaccine was poorly immunogenic in the induction of HI antibody responses against the corresponding virus. However, all three of the vaccines failed to elicit high cross-HI or VN antibody responses against the challenge virus.

TABLE 5. Serological responses induced by H5 influenza vaccines (cross-HI and VN antibody titers against the challenge virus)

Vaccine	Serum antibody GMT[a]			
	CRBCs		HRBCs	
	Cross-HI[b]	VN[c]	Cross-HI[b]	VN[c]
rg-Ck/C58 H5	14 (4/10)	21 (10/10)	43 (10/10)	21 (10/10)
rg-WS/244 H5	<10 (0/10)	13 (3/10)	31 (8/10)	13 (3/10)
rg-JWE/1038 H5	<10 (0/10)	<10 (0/10)	14 (4/10)	<10 (0/10)
Placebo	<10 (0/10)	<10 (0/10)	<10 (0/10)	<10 (0/10)

[a] The results are the geometric mean titers of positive sera (≥10). The numbers of positive sera are indicated in parentheses (number of positive sera/total number of sera tested).
[b] HI antibody titers of sera collected from birds at 3 weeks postvaccination were determined against the challenge virus (Dk/Laos/25/06 [H5N1]) and are expressed as the reciprocals of the highest dilutions of sera that inhibited hemagglutination by 4 HA units of virus.
[c] Virus neutralization antibody titers of sera collected from birds at 3 weeks postvaccination were determined against the challenge virus (Dk/Laos/25/06 [H5N1]) and are expressed as the reciprocals of the highest dilutions of sera that neutralized infection with virus (100 $TCID_{50}$) in MDCK cells.

DISCUSSION

Previous studies reported that ducks naturally or experimentally infected with highly pathogenic avian influenza A viruses showed resistance to disease development (3, 5, 17, 29, 44). However, the evolutionary stasis with asymptomatic infection shown between the viruses and ducks may have been disrupted with recent isolates of H5N1 avian influenza A viruses (26, 34, 39). Although recent studies have updated the data about the pathogenicity of highly pathogenic avian influenza A viruses in ducks, limited information is available.

In the present study, we examined the pathogenicity of dominant clades (clade 1 and 2) of H5N1 avian influenza A viruses in domestic ducks. Representatives of dominant clades of the viruses were selected based on the year of isolation and antigenic characterization: Ck/VN/C58/04 (clade 1), WS/Mong/244/05 (clade 2.2), JWE/HK/1038/06 (clade 2.3.4), and Dk/Laos/25/06 (clade 2.3.4).

In a previous study (19), the H5 antigenic sites were mapped by characterizing escape mutants of a recombinant virus con-

taining the HA gene of A/Vietnam/1204/04 (VN/1204/04; H5N1; clade 1). The amino acids in the H5 antigenic sites of the virus were 100% homologous with those of Ck/VN/C58/04 (H5N1; clade 1), 50% homologous with those of Dk/Laos/25/06 (H5N1; clade 2.3.4), 37.5% homologous with those of JWE/HK/1038/06 (H5N1; clade 2.3.4), and 25% homologous with those of WS/Mong/244/05 (H5N1; clade 2.2). With respect to amino acids, Ck/VN/C58/04 (H5N1; clade 1) is highly dissimilar to the other three viruses, as would be expected from a clade 1 virus, differing by 23, 25, and 24 amino acids from WS/Mong/244/05 (H5N1; clade 2.2), JWE/HK/1038/06 (H5N1; clade 2.3.4), and Dk/Laos/25/06 (H5N1; clade 2.3.4), respectively. WS/Mong/244/05 (H5N1; clade 2.2) differs from the other two clade 2 viruses by 20 amino acids each. Both representatives of clade 2.3.4 viruses are highly homologous, differing by only 5 amino acids.

The representatives of dominant clades of the viruses caused highly pathogenic infection in domestic ducks. The ducks experimentally infected with high titers of the viruses experienced lethargy, conjunctivitis, severe neurological impairment, and death. However, the viruses exhibited different proportions of lethality and extent of symptoms of disease.

Remarkably, the Dk/Laos/25/06 (H5N1; clade 2.3.4) virus showed extremely high pathogenicity in domestic ducks. The virus started causing symptoms of disease at 1 day postinoculation and killed all birds in 7 days. Moreover, this virus still showed high lethality at a very low dose ($10^{1.25}$ EID_{50}). In fact, no differences in morbidity or mortality were observed between groups of ducks inoculated with serial 10-fold dilutions of the virus starting from $10^{7.25}$ EID_{50} to $10^{1.25}$ EID_{50}. Such extremely high lethality of this virus is unusual in ducks, although some recent isolates of H5N1 influenza A viruses were reported to be highly pathogenic in ducks (26, 34, 39). Our results support the hypothesis, suggested by recent reports, that H5N1 influenza A viruses have continually evolved to cause lethal infections in ducks.

Fifteen micrograms of HA protein with no adjuvant is the typical dose of a seasonal influenza vaccine in humans. In the ferret, which represents the most optimal influenza animal model for human vaccine testing, vaccination with two injections of 7 μg of H5 HA protein with no adjuvant induced protective immunity, although it did not provide complete protection from morbidity and virus shedding against a homologous influenza virus challenge (14). However, there is no systematic information about the dose of HA protein required in agricultural influenza vaccines to provide complete protection against highly pathogenic H5N1 avian influenza A viruses in ducks.

In this study, we investigated the optimal dose of HA protein in an inactivated oil emulsion whole-virus H5 influenza vaccine (derived by implementing reverse genetics and prepared through concentration and purification) to provide complete protection against a lethal H5N1 avian influenza virus challenge in domestic ducks. Ducks were immunized with a single dose of the rg-WS/244 H5 influenza vaccine using different concentrations of HA protein and then challenged with the highly lethal Dk/Laos/25/06 (H5N1; clade 2.3.4) virus.

The vaccine containing 1 μg of HA protein provided complete protection from morbidity, mortality, and virus shedding against the lethal H5N1 avian influenza virus challenge, while the vaccine containing doses lower than 1 μg of HA protein did not provide complete protection. These findings led us to conclude that 1 μg of HA protein in agricultural inactivated oil emulsion H5 influenza vaccines is sufficient to provide complete protection against highly pathogenic H5N1 avian influenza viruses in domestic ducks. However, the dose of HA protein in the vaccine to provide complete protection may be different from those in commercial vaccines produced without concentration and purification.

In addition, these findings reinforced how important it is to use a relevant dose of antigen in vaccination for controlling H5N1 avian influenza A viruses in areas of endemicity. The partial protection provided by the vaccine containing doses lower than 1 μg of HA protein did not prevent shedding of the challenge virus. The use of inappropriate doses of antigen may cause asymptomatic infection of highly pathogenic H5N1 avian influenza A viruses to vaccinated birds, where viruses are shed and transmitted to adjacent flocks of birds, other avian species, or mammals. Such a "Trojan horse" effect in vaccinated birds may allow continuing circulation and spread of H5N1 avian influenza A viruses in areas of endemicity.

The main aim of this study was to evaluate the efficacy, cross-protectiveness, and immunogenicity of three different inactivated oil emulsion whole-virus H5 influenza vaccines against a highly lethal H5N1 avian influenza virus challenge in domestic ducks. The three H5 influenza vaccines contained the HA proteins of Ck/VN/C58/04 (H5N1; clade 1), WS/Mong/244/05 (H5N1; clade 2.2), or JWE/HK/1038/06 (H5N1; clade 2.3.4), respectively. A single dose (1 μg of HA protein) of one of the vaccines provided complete protection from morbidity, mortality, and virus shedding against a lethal Dk/Laos/25/06 (H5N1; clade 2.3.4) influenza virus challenge in domestic ducks. Remarkably, two of the three vaccines (rg-Ck/C58 and rg-WS/244 H5) achieved complete cross-clade or cross-subclade protection against the lethal H5N1 avian influenza virus challenge. Thus, the vaccines were very efficacious against the homologous or heterologous lethal H5N1 avian influenza virus challenge in domestic ducks.

It is believed that induction of an antibody response is critical for protective immunity against many pathogens, such as viruses. The protective immunity provided by influenza virus vaccines is mainly examined by HI and VN assays to measure serum antibody titers. However, in the present study, not all of the three vaccines induced substantial HI and/or VN antibody responses in the vaccinated ducks, although they provided complete protection against the lethal H5N1 avian influenza virus challenge.

Among the three vaccines, the rg-Ck/C58 and rg-WS/244 H5 vaccines induced substantial HI antibody responses against the corresponding viruses, while the rg-JWE/1038 H5 vaccine was poorly immunogenic in an HI antibody response against the corresponding virus. In cross HI antibody responses against noncorresponding viruses among the vaccine donor strains used in this study (data not shown), the sera from the rg-Ck/C58 H5-vaccinated birds cross-reacted with WS/Mong/244/05 (H5N1; clade 2.2) and JWE/HK/1038/06 (H5N1; clade 2.3.4) viruses, although the responses were much lower than that against the corresponding virus. The sera from the rg-WS/244 H5-vaccinated birds cross-reacted with JWE/HK/1038/06 (H5N1; clade 2.3.4) virus, not with Ck/VN/C58/04 (H5N1;

clade 1) virus, although the response was much lower than that against the corresponding virus. However, the sera from the rg-JWE/1038 H5-vaccinated birds did not cross-react with either the Ck/VN/C58/04 (H5N1; clade 1) or the WS/Mong/244/05 (H5N1; clade 2.2) virus. Additionally, both cross-HI antibody and VN antibody responses against the challenge virus were low (HI GMT, ≤43; VN GMT, ≤21) or undetectable (<10) in the vaccinated domestic ducks. These findings implied that the clade 1 and 2.2 H5 influenza vaccines were more immunogenic than the clade 2.3.4 H5 influenza vaccine in the induction of serum antibody responses in domestic ducks. Thus, the serum antibody responses may not be sufficient to evaluate the protective immunity provided by influenza virus vaccines in domestic ducks because the poorly immunogenic H5 influenza virus vaccine provided complete protection against the lethal H5N1 avian influenza virus challenge.

The reason for complete protection in the absence of detectable humoral immune responses shown for the rg-JWE/1038 H5 influenza vaccine is still unresolved. The unexplained phenomenon is also observed in a mammalian model (10). In ferrets immunized with an inactivated whole-virus H5 vaccine (derived by implementing reverse genetics) containing the HA and NA genes of the HK/213/03 (H5N1) virus and the internal genes of the PR/8/34 (H1N1) virus, almost no detectable HI antibody response was detected, although the vaccine provided protection against a lethal A/Vietnam/1203/04 (H5N1) virus challenge.

As previously mentioned, the unconventional observation may be the result of a compromised sensitivity of the serological assay. Recent evidence suggests that specific HA amino acid residues, such as an amino acid residue at position 223, might be related to the sensitivity of the HI assay (14). A single amino acid substitution ($S_{223}N$) in HA resulted in improved detection of HI antibodies. Sequence analyses of the HA genes of the four H5N1 viruses (Ck/VN/C58/04, WS/Mong/244/05, JWE/HK/1038/06, and Dk/Laos/25/06) revealed that they had a serine residue at position 223 (S_{223}), which was characteristically shown in recent H5N1 isolates in Asia, while HK/213/03 (H5N1; clade 1) had an asparagine residue at position 223 (N_{223}). The low or undetectable HI and cross-HI antibody responses may be a result of the serine residue at position 223 of the viruses.

Another hypothesis to explain the phenomenon is that the complete protection offered by the H5 influenza virus vaccines in the absence of detectable antibodies may be induced by antigen dose-dependent cell-mediated immune responses rather than by antibody-mediated protection. Emerging evidence indicates that influenza A virus-specific $CD8^+$ and/or $CD4^+$ T-cell responses might be correlated with protection against influenza virus challenge infection in mouse models (41). Moreover, antigen-specific T-cell-mediated immunity protected against influenza virus challenge infection in the absence of antibodies (8). The humoral immune responses mediated by antibodies are mostly effective against homologous strains of influenza A viruses because the responses target external viral coat proteins. In contrast, T-cell-mediated immune responses can be effective against both homologous and heterologous viral strains, since the responses can target more conserved internal proteins. This property may explain the cross-clade or cross-subclade protection achieved by two of the three H5 vaccines against the heterologous lethal H5N1 avian influenza virus challenge. This being said, controversy still exists, in that T-cell-mediated immune responses induced by formalin-inactivated vaccines can provide sterile resistance, as shown in this study.

In the present study, three reverse genetics-derived inactivated H5 vaccines were prepared and tested in domestic ducks. The vaccines provided complete protection against the lethal challenge of the homologous or heterologous H5N1 avian influenza A virus with no evidence of morbidity, mortality, or shedding of the challenge virus. Of specific importance, complete cross-clade or cross-subclade protection of two of the vaccines should be of value to control H5N1 avian influenza A viruses by means of vaccination. The complete protection offered by these vaccines will be useful for reducing the shedding of H5N1 avian influenza A viruses among vaccinated agricultural avian populations.

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