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Newcastle Disease Virus-Based Live Attenuated Vaccine Completely Protects Chickens and Mice from Lethal Challenge of Homologous and Heterologous H5N1 Avian Influenza Viruses▽

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H5N1 highly pathogenic avian influenza virus (HPAIV) has continued to spread and poses a significant threat to both animal and human health. Current influenza vaccine strategies have limitations that prevent their effective use for widespread inoculation of animals in the field. Vaccine strains of Newcastle disease virus (NDV), however, have been used successfully to easily vaccinate large numbers of animals. In this study, we used reverse genetics to construct a NDV that expressed an H5 subtype avian influenza virus (AIV) hemagglutinin (HA). Both a wild-type and a mutated HA open reading frame (ORF) from the HPAIV wild bird isolate, A/Bar-headed goose/Qinghai/3/2005 (H5N1), were inserted into the intergenic region between the P and M genes of the LaSota NDV vaccine strain. The recombinant viruses stably expressing the wild-type and mutant HA genes were found to be innocuous after intracerebral inoculation of 1-day-old chickens. A single dose of the recombinant viruses in chickens induced both NDV- and AIV H5-specific antibodies and completely protected chickens from challenge with a lethal dose of both velogenic NDV and homologous and heterologous H5N1 HPAIV. In addition, BALB/c mice immunized with the recombinant NDV-based vaccine produced H5 AIV-specific antibodies and were completely protected from homologous and heterologous lethal virus challenge. Our results indicate that recombinant NDV is suitable as a bivalent live attenuated vaccine against both NDV and AIV infection in poultry. The recombinant NDV vaccine may also have potential use in high-risk human individuals to control the pandemic spread of lethal avian influenza.

H5N1 avian influenza has been a considerable problem for both veterinary and public health. In 1996, the first H5N1 avian influenza virus (AIV) detected in China, A/Goose/Guangdong/1/96 (GS/GD/96), was isolated from geese in the Guangdong province (5, 41). In 1997, H5N1 AIV caused disease outbreaks in poultry in Hong Kong (31, 32) and was transmitted into humans, causing six deaths (8, 33). Starting from late 2003, H5N1 influenza viruses began to spread and caused disease outbreaks in China (39), Japan (21), South Korea (18), Thailand, Vietnam, Indonesia, Cambodia, Malaysia, and Laos (Office International des Epizooties [OIE]; http://www.oie.int), resulting in the destruction of hundreds of millions of poultry, including chickens, ducks, and geese. In May, 2005, an H5N1 highly pathogenic avian influenza virus (HPAIV) outbreak occurred in wild birds in Qinghai Lake, in western China (6, 7, 20). One of the H5N1 virus genotypes identified from the wild bird population during this outbreak, A/Bar-headed goose/Qinghai/3/2005 (H5N1) (BHG/QH/05), continued to spread to countries in Europe, Africa, the Middle East, and Middle Asia (Office International des Epizooties; http://www.oie.int) and caused disease and death in wild birds and domestic poultry. Recently, cases of human H5N1 infection have again been detected in multiple countries around the world (World Health Organization; http://www.who.int). It appears that the threat H5N1 influenza viruses pose to both domestic poultry and public health has not diminished.

Over 200 human cases of AIV infection have been confirmed around the world, and most infection cases resulted from direct contact with H5N1 influenza virus-infected poultry. The effective control of avian influenza in poultry is therefore an important issue for public health. The culling of infected poultry is the time-honored method to control or eradicate the highly pathogenic avian influenza outbreaks, and it is also the best-known way to prevent transmission to humans. However, when the viruses are spread over a wide area and have infected multiple avian species, culling and physical containment are not likely to be successful. An alternative strategy for control is the use of culling plus vaccination. Whole-virus inactivated vaccines and fowlpox virus-based recombinant vaccines have been used as control strategies for highly pathogenic avian influenza in the laboratory and in poultry farms located within a limited geographic region (4, 10, 12, 28, 29, 34, 36, 37). However, the cost of production and the laborsome administration of these vaccines are limitations for their wide application in the field.

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TABLE 1. Primers used for amplifying the cDNA of the NDV LaSota strain[a]

Fragment	Forward primer	Reverse primer
F1	5′-CTGA**AGAGCTCGTCGAC***GTAATACGACTCACT ATA*GGACCAAACAGAGAATCCGTGAG-3′	5′-AGGACTGATGCCATAC**CCATGG**
F2	5′-TACTCCTTTG**CCATGG**GTATGG	5′-CTTACTTACTCTCTGT**GATATC**G
F3	5′-GTCTATGATGGAGGC**GATATC**AC	5′-GAAGAAAGGTGCCAA**AAGCTT**AG
F4	5′-AGAG**GTGCAC**GGACTAAGCTTTTG	5′-TAGTGGCTCTCATCTGA**TCTAGA**G
F5	5′-GCTTGGGAATAATAC**TCTAGA**TC	5′-GTACTGCTTGAACTCA**CTCGAG**
F6	5′-GTCGCATTA**CTCGAG**TGAGTTC	5′-ATGTACCTGACGG**CTCGAG**TAG
F7	5′-TGTCCAGCTA**CTCGAG**CCGTCA	5′-CAAAAGCAGTAGTC**CCCGGG**TCA
F8	5′-GAAATATCGGTGA**CCCGGG**GACT	5′-GTTGTCATTGGAAAG**ATCGAT**TCG
F9	5′-GGTTTATCTCTAATCGAA**TCGAT**C	5′-GCTAGTTGATCTAGT**AAGCTT**TG
F10	5′-CAGGGTCCAATCA**AAGCTT**AC	5′-AGTC**TCTAGA**caggt**cggaccg**cgaggaggtggagatgccatg ccgacccACCAAACAAAGATTTGGTGAATG-3′[b]

[a] The restriction enzyme sites are shown in bold. T7 promoter sequences are marked in italics.
[b] The HDV ribozyme is shown in lowercase.

Newcastle disease is caused by highly pathogenic Newcastle disease viruses (NDV), which are members of the genus *Avulavirus* in the family *Paramyxoviridae*, and they have significance similar to AIV for the poultry industry. Highly pathogenic Newcastle disease virus infection could result in 100% mortality in many species of birds worldwide (2). Currently, naturally occurring avirulent NDV strains are routinely used as live vaccines throughout the world (1, 2), and the development of a reverse genetics system has provided a method to generate recombinant NDV-based, live virus-vectored vaccines (22, 23, 26). There are several advantages to the use of avirulent NDV strains as vaccine vectors. They can be administered through drinking water or spraying, making inoculation of large numbers of animals in the field feasible. In addition, the cost of production is lower and the yield of production is greater with NDV-based vaccines than with inactivated vaccines. Due to these advantages, an NDV-based live virus vaccine targeted against AIV could have success in the field in the control of infection and spread of AIV in animals, and therefore could be an effective weapon in the control of AIV transmission to humans.

In the present study, we established a reverse genetics system to generate a recombinant NDV expressing an AIV hemagglutinin (HA) gene from an H5N1 virus. The H5 HA gene of BHG/QH/05 was inserted into the LaSota NDV vaccine strain. We determined that our recombinant NDV-based vaccine was immunogenic and was efficacious as an H5 AIV-NDV bivalent vaccine in chickens. We also found that our recombinant NDV-based vaccine elicited complete protection in chickens and mice against challenge with homologous and heterologous H5N1 influenza viruses.

MATERIALS AND METHODS

Viruses and cells. HEp-2 and BHK-21 cells were grown in Eagle's minimum essential medium containing 10% fetal bovine serum. Recombinant and wild-type NDV strains were grown in 9-day-old, specific-pathogen-free (SPF) embryonated chicken eggs. The NDV vaccine strain LaSota used as vector and the highly virulent NDV strain F48E9 used for challenge studies were originally received from the China Veterinary Culture Collection. GS/GD/96, the first H5N1 HPAIV isolated in China, and A/Duck/Fujian/13/02 (DK/FJ/02), a virus isolated from healthy ducks, have been characterized as previously reported (5, 41). BHG/QH/05 virus was isolated from a bar-headed goose during the 2005 H5N1 avian influenza outbreak in wild birds in western China (6). These viruses were propagated in the allantoic cavities of 10-day-old SPF chicken embryonated eggs and kept in a −70°C freezer before RNA extraction or challenge study. The modified vaccinia virus strain Ankara (MVA) expressing the T7 RNA polymerase (a generous gift from Bernard Moss, National Institutes of Health) (40) was grown in primary chicken embryo fibroblasts.

Plasmid construction. The low-copy-number plasmid pBR322 was used as the backbone for NDV infectious clone construction. We inserted the hepatitis delta virus (HDV) ribozyme and the T7 terminator between the EcoRI and HindIII sites of pBR322 and designated the plasmid as pBRT.

The NDV strain LaSota was grown in 10-day-old embryonated SPF eggs. Viral RNA was extracted with an RNeasy mini kit (QIAGEN, Valencia, CA). The extracted RNA was subjected to reverse transcription (RT)-PCR with virus-specific primer pairs (Table 1) and high-fidelity *Pfx* DNA polymerase (Invitrogen Corp., Carlsbad, CA) to generate ten overlapping PCR fragments of the entire viral genome. The assembled cDNA containing the sequences of the T7 promoter, the full-length (15,186-nucleotide) cDNA of the NDV LaSota genome in the antigenomic orientation, and a partial HDV ribozyme sequence was inserted between the SalI and BsrII sites of pBRT, and the resultant plasmid was designated as pLa. The open reading frames (ORFs) of the NP, P, and L genes were PCR amplified from pLa for the construction of NP, P, and L expression plasmids. The amplified NP, P, and L genes were each inserted into the SmaI site of the plasmid pBluescript (Clontech, Mountain View, CA) and were designated as pT7-NP, pT7-P, and pT7-L, respectively. The assembled full-length cDNA clone and the support plasmids encoding LaSota NP, P, and L proteins were sequenced in their entirety.

The cDNA of 1,758 nucleotides representing the ORF of the HA gene of BHG/QH/05 was amplified by the primer pair of 5′GACTGTTTAAACTTAG AAAAAATACGGGTAGAACCAGTTGT*GCCACC*ATGGAGAAAAAGTG CTTCTTC3′ and 5′GCGCGTTTAAACTTAAATGCAAATTCTGCATTG3′, in which the gene end and gene start sequences (underlined) (16) and the optimal Kozak sequence (italic) were included (15). The HA gene of BHG/QH/05 was introduced into the NDV genome contained in pLa through the introduction of a PmeI site in the P-M intergenic region at nucleotide position 3165 of the NDV genome. The resultant plasmid was designated as pLa-H5w. A cDNA of 1,746 nucleotides representing a BHG/QH/05 mutant HA gene in which the multiple basic amino acids at the cleavage site were deleted (36) was also inserted into the P-M intergenic region of pLa. The resultant plasmid was designated as pLa-H5m. Both pLa-H5w and pLa-H5m maintained NDV genome lengths that were a multiple of 6 to allow for efficient replication.

Virus rescue. To generate virus from the plasmids, HEp-2 cells grown in a six-well plate were infected with MVA-T7 at a multiplicity of infection (MOI) of 1 and then transfected with 1 μg of pLa and either pLa-H5w or pLa-H5m together with the following expression plasmids: pT7-NP at 0.4 μg, pT7-P at 0.2 μg, and pT7-L at 0.2 μg. After 16 h of incubation at 37°C, the medium was replaced with 2 ml of fresh Opti-MEM (Invitrogen Corp., Carlsbad, CA) containing 0.5 μg of the protease inhibitor *N*-tosyl-phenylalanine chloromethylketone (TPCK) trypsin, and the cells were incubated for another three days at 37°C. The supernatant was then inoculated into the allantoic cavities of 10-day-old embryonated SPF eggs. After 72 h of incubation at 39°C, the allantoic fluid was harvested and the virus was identified by hemagglutination assay using 0.5% chicken red blood cells. Viral RNAs were isolated from the rescued viruses, and the presence of the inserted foreign genes was confirmed by RT-PCR and sequence analysis. The rescued viruses were designated as rLa, rLa-H5w, and rLa-H5m.

Confirmation of the expression of H5 HA in cells infected by rescued viruses. The H5 HA gene expression in the recombinant NDVs was confirmed by Western blotting and immunofluorescence assay. The Western blot analysis was per-

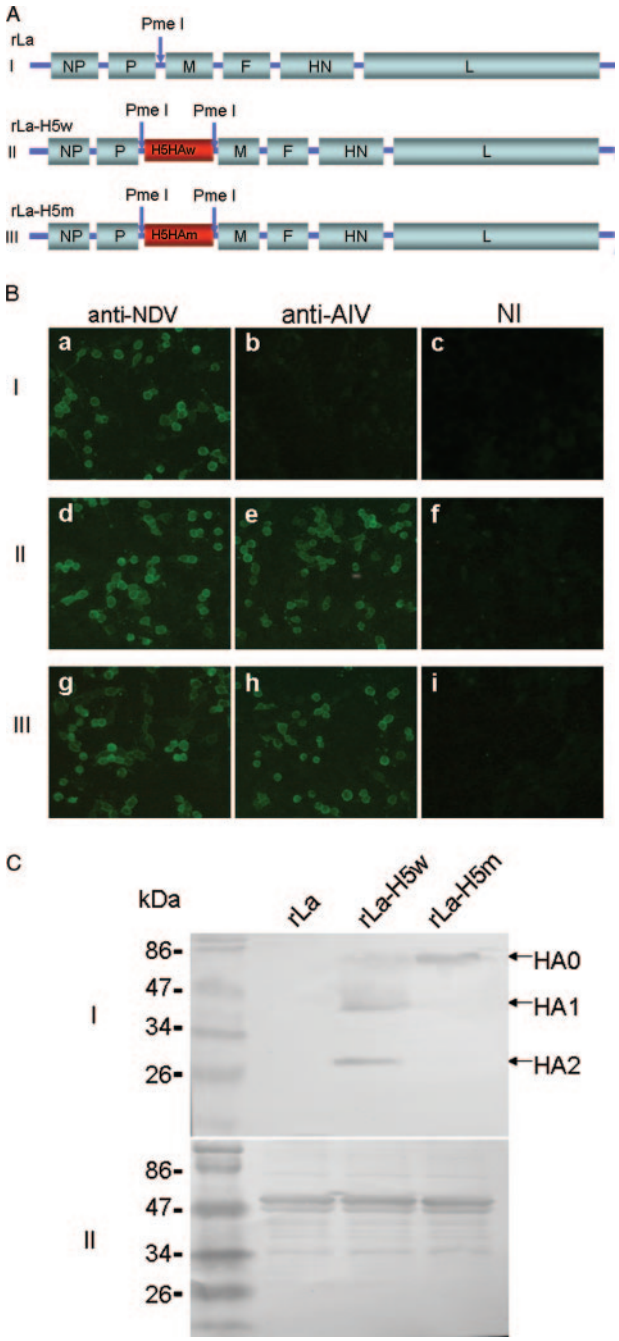

FIG. 1. Generation and characterization of H5 HA gene recombinant NDV. (A) Schematic representation of the rLa genome with the PmeI site introduced between the P and M genes (I) and the wild-type H5 AIV HA gene (II) or mutated H5 AIV HA gene (III) that were inserted at the PmeI site. (B) Immunofluorescence analysis of H5 AIV HA protein expression. Confluent BHK-21 cells were infected with rLa (I), rLa-H5w (II), or rLa-H5m (III) at a MOI of 0.2. The infected cells were fixed and probed with chicken anti-NDV antiserum (a, d, g), chicken anti-H5 AIV antisera (b, e, h), or with the noninfected (NI) SPF chicken sera (c, f, i) followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-chicken IgG (Sigma). Cells were analyzed with a Leica DMIRE2 fluorescence microscope (Leica). (C) Western blot analyses of NDV recombinants expressing AIV H5. Lysates of cells infected with rLa, rLa-H5w, or rLa-H5m were incubated with chicken H5 AIV HA-specific antiserum that was generated with H5 HA gene DNA immunization (I) or chicken anti-NDV antiserum (II). Binding was visualized with 3,3′-diaminobenzidine reagent after incubation with peroxidase-conjugated secondary antibodies. Locations of marker proteins are indicated on the left, and the uncleaved (HA0) and processed forms (HA1 and HA2) of AIV hemagglutinin are indicated on the right.

formed as described previously (19). The chicken antisera induced by the H5 AIV HA gene DNA vaccine were used as primary antibodies, and horseradish peroxidase-conjugated rabbit anti-chicken immunoglobulin G (IgG) (Sigma, St. Louis, MO) was used as a second antibody. The immunofluorescence assay was performed as described by Huang et al. (14). The BHK-21 cells in 24-well plates were infected with the rescued virus at a MOI of 0.2. The primary antibodies used were specific chicken polyclonal sera against H5N1 AIV or NDV. Secondary antibodies used were fluorescein isothiocyanate-conjugated rabbit anti-chicken IgGs (Sigma). Cells were analyzed with a fluorescence microscope.

Stability and growth properties of the recombinant viruses. To evaluate the genetic stability of the foreign gene of the recombinant virus, we passed the virus in 10-day-old embryonated SPF chicken eggs for 20 passages. Detection of the inserted gene was carried out by RT-PCR, and expression of the HA gene was confirmed by immunofluorescence assay.

To investigate if the insertion of the HA gene of H5N1 avian influenza virus affects the growth of the NDV virus, we inoculated 9-day-old embryonated SPF chicken eggs with 0.1 ml of 100 50% egg infective dose (EID_{50}) of the rescued rLa, rLa-H5w, or rLa-H5m virus. Six eggs receiving each virus were harvested and pooled at 24-h intervals and titrated in SPF eggs for the EID_{50}.

Characterization of recombinant virus in vivo. The mean death time (MDT), the intracerebral pathogenicity index (ICPI), and the intravenous pathogenicity index (IVPI) were determined to assess the pathogenicity of the recombinant viruses in embryonated SPF chicken eggs or SPF chickens as described in the OIE manual (24).

To investigate where and how well the rescued viruses replicate in chickens, groups of three 1-week-old SPF chickens were inoculated with 10^6 EID_{50} of rLa, rLa-H5m, or rLa-H5w in a 0.1-ml volume by oculonasal administration. The chickens were killed on day 3 postinoculation (p.i.), and organs including lung, liver, spleen, kidney, and brain were collected for virus titration. Swabs were also collected from the birds on day 3 p.i. for virus titration.

Vaccine efficacy in chickens. Groups of ten 1-week-old SPF chickens were inoculated by oculonasal administration with 10^6 EID_{50} of the rescued rLa, rLa-H5m, or rLa-H5w virus in a 0.1-ml volume. Sera were collected before challenge for hemagglutinin inhibition (HI) antibody detection using the OIE standard method. Three weeks p.i., the chickens were challenged with 10^3 50% chicken lethal dose (CLD_{50}) of highly pathogenic NDV F48E9 through intramuscular injection or intranasally with 10^3 CLD_{50} of highly pathogenic H5N1 GS/GD/96 and BHG/QH/05. Oropharyngeal and cloacal swabs of the chickens that were challenged with H5N1 avian influenza viruses were collected on days 3, 5, and 7 postchallenge (p.c.) for virus titration, and chickens were observed for disease signs and death for 2 weeks after challenge. Two groups of 10 chickens that received each recombinant virus were kept for 4 months to observe the HI antibody duration.

To evaluate the replication of the challenge virus in chickens, groups of nine chickens were vaccinated with rLa or rLa-H5w and challenged with GS/GD/96 or BHG/QH/05 as described above. Three chickens from each group were killed on days 3, 5, and 7 p.c., and organs including lung, heart, liver, spleen, kidney, and brain were collected for virus titration in eggs as described previously (19). Swabs were also collected for virus titration.

Mouse study. Groups of 3-week-old BALB/c mice were inoculated with 10^8 EID_{50} of rLa or rLa-H5w virus intraperitoneally in a 0.1-ml volume and then boosted 3 weeks later with the same dose and same route of inoculation. The mice were bled at 2 weeks postboost and then were challenged intranasally with 10^3 50% of the mouse lethal dose (MLD_{50}) of homologous BHG/QH/05 virus and heterologous DK/FJ/02 virus. Three mice from each group were killed on days 3 and 6 p.c., and organs, including nose turbinate, lung, spleen, kidney, and brain, were collected for virus titration in eggs as described previously (5). Five mice were observed for disease signs, body weight changes, and deaths for two weeks.

Sequence analysis. The plasmids used for virus rescue and the rescued viruses were fully sequenced to confirm the absence of unexpected mutations. Viral RNA was extracted from allantoic fluid by using an RNeasy mini kit (QIAGEN, Valencia, CA) and was reverse transcribed and amplified using specific primers (shown in Table 1). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN). Plasmids and PCR products were sequenced by

TABLE 2. Biological properties of the rescued NDV and recombinant viruses

Virus	MDT (h)	Pathogenicity in chickens		Virus titers in lungs on day 3 p.i. (log EID_{50}/g)[a]	Virus shedding on day 3 p.i. (log EID_{50}/ml)[b]
		ICPI	IVPI		
rLa	>120	0.4	0	2.0 ± 0.4	2.7 ± 0.3
rLa-H5w	>120	0	0	1.2 ± 0.4	2.0 ± 0.5
rLa-H5m	>120	0	0	1.2 ± 0.3	2.0 ± 0.8

[a] Groups of three 1-week-old SPF chickens were inoculated with 10^6 EID_{50} of the tested virus in a 0.1-ml volume by oculonasal administration, the chickens were killed on day 3 p.i., and organs were collected for virus titration. Virus was not detected from the heart, spleen, kidney, liver, and brain; therefore, only the lung titers are indicated in the table.

[b] Values are for oropharyngeal samples. Virus was not detected from the cloacal samples.

using a CEQ DTCS-Quick Start kit on a CEQ 8000 DNA sequencer (Beckman Coulter) with a set of gene-specific primers (sequences available upon request).

Serologic tests and virus titration. Hemagglutination inhibition assays were performed by following the OIE standard (24). Each swab was washed in 1 ml of cold phosphate-buffered saline (PBS), and virus titration was conducted in 10-day-old SPF embryonated chicken eggs and calculated by the method of Reed and Muench (30).

Laboratory facility. All experiments related to the HPAIV were conducted in a P3 facility.

RESULTS

Generation of recombinant NDV viruses expressing wild-type and mutant HA genes of H5N1 avian influenza virus. We constructed an infectious clone of NDV LaSota by assembling ten overlapping PCR fragments and inserting the 15,186 nucleotides of NDV LaSota and the sequences of the T7 polymerase promoter, HDV ribozyme, and T7 polymerase terminator into the plasmid pBR322. The wild-type and mutated HA genes of H5N1 AIV were inserted between the P and M genes of the NDV cDNA (Fig. 1A). The resultant recombinant viruses, rLa-H5w and rLa-H5m, were rescued from the cDNA as described in Materials and Methods, and the presence of the HA gene was confirmed by RT-PCR. Expression of influenza virus H5 HA by rLa-H5w and rLa-H5m was confirmed by immunostaining infected BHK-21 cells 28 h p.i. As expected, cells infected with rLa were not stained by chicken antisera to H5 AIV HA protein, but they were positive for immunostaining using chicken polyclonal antisera against NDV (Fig. 1B). In contrast, cells infected with rLa-H5w or rLa-H5m were stained by chicken antisera to H5 AIV HA protein as well as the anti-NDV antiserum. The expression of wild-type and mutant HA protein by the recombinant viruses was also confirmed by Western analysis. As shown in Fig. 1C, the majority of wild-type HA protein expressed by rLa-H5w was cleaved, while large amounts of the mutant HA protein lacking the cleavage site expressed by rLa-H5m was uncleaved.

Biological characterization and stability of the rescued NDV recombinant viruses. The MDT, ICPI, and IVPI tests are the internationally accepted methods for assessing the pathogenicity of NDV strains (24). Strains of NDV are categorized into three groups on the basis of their MDTs: velogenic (less than 60 h), mesogenic (60 to 90 h), and lentogenic (greater than 90 h) (24). The MDT values of the rLa, rLa-H5w, and rLa-H5m viruses are greater than 120 h, indicating that they behave as lentogenic viruses (Table 2). The ICPI value for rLa was 0.4,

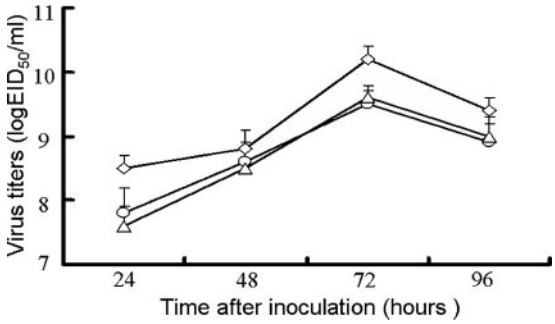

FIG. 2. Growth properties of recombinant viruses in embryonated eggs. The rescued NDV rLa (◇), rLa-H5w (△), or rLa-H5m (○) (0.1 ml of 100 EID_{50}) were inoculated into the allantoic cavities of 10-day-old embryonated eggs, and the allantoic fluid of six eggs from each group was harvested at the time points of 24, 48, 72, and 96 h postinoculation and pooled for the determination of EID_{50} in eggs; data shown were acquired from three repeats.

with two out of eight chickens that received the virus rLa intracerebrally showing disease signs on day 1 p.i and dying on day two. The chickens that received the two NDV recombinant viruses, rLa-H5w and rLa-H5m, remained healthy during the observation period, and the ICPI values of the recombinant viruses were zero. When the viruses were administrated intravenously, all of the chickens remained healthy during the observation period, and IVPI values of the viruses were zero (Table 2). These data show that the rescued recombinant viruses are attenuated in chicken embryos and chickens, suggesting that the insertion of the HA genes did not increase the virulence of the vector NDV virus.

To investigate the replication of the rescued viruses, we collected the organs and swabs of the inoculated chickens on day 3 p.i. and titrated the viruses in eggs. Virus was detected from the lungs and the oropharyngeal swabs of the chickens on day 3 p.i. but not from any other organs nor from the cloacal swabs (Table 2). These data suggest that the rescued viruses are only able to replicate in the respiratory tracts of the inoculated chickens.

To investigate whether the insertion of the H5 AIV HA gene affected the growth property of the NDV vector virus, we harvested and titrated the viruses at different time points after inoculation in eggs. As shown in Fig. 2, the two recombinant viruses, rLa-H5w and rLa-H5m, grew equally well and reached peak titers of 9.5 log EID_{50}/ml at 72 h after inoculation, though

TABLE 3. Protective efficacy of the rescued viruses against the highly pathogenic NDV challenge

Vaccine	HI antibody titers to NDV (log_2)[a]	Manifestation of chickens[b]	
		No. sick/total	No. dead/total
rLa	7.3 ± 0.4	0/10	0/10
rLa-H5w	6.6 ± 0.3	0/10	0/10
rLa-H5m	6.8 ± 0.4	0/10	0/10
PBS	<1	10/10	10/10

[a] Antiserum was collected at 3 weeks postvaccination and prior to challenge for the HI antibody test to NDV.

[b] Chickens were observed for two weeks after challenge for disease signs and deaths. The PBS control chickens died within 3 days p.c.

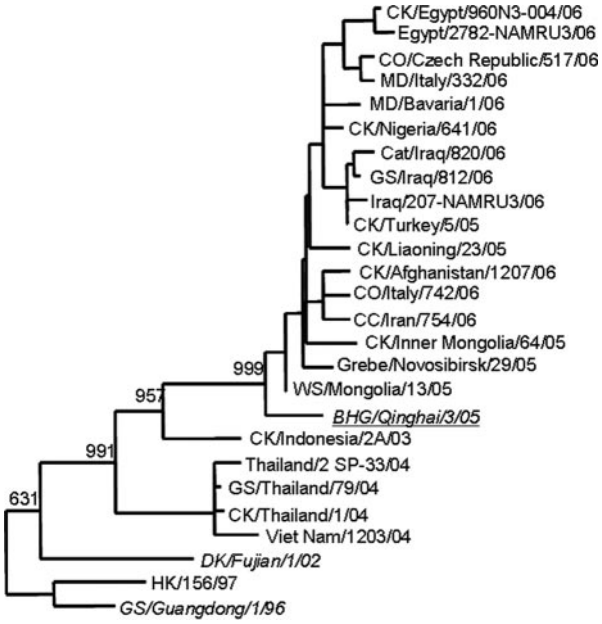

FIG. 3. Phylogenetic relationships of the hemagglutinin genes of H5N1 viruses. The tree includes avian influenza viruses that were isolated in countries from Southeast Asia during 2003 to 2005 and viruses isolated in the Middle East, Europe, and Africa during 2005 to 2006. The phylogenetic tree was generated with the PHYLIP program of the CLUSTALX software package (version 1.81) by using the neighbor-joining algorithm and based on bootstrap values of 1,000. The HA gene donor virus for the recombinant vaccine generation is underlined, and the challenge viruses used in this study are marked in italics. CK, chicken; DK, duck; GS, goose; MD, mallard; CO, *Cygnus olor*; CC, *Cygnus cygnus*; WS, whooper swan.

the titers were approximately half a log lower than that of the virus rLa.

To determine the stability of the HA gene in the recombinant viruses, rLa-H5m and rLa-H5w were passaged 20 times in 10-day-old embryonated SPF chicken eggs. RT-PCR confirmed the presence of the HA gene in the virus from each passage, and sequence analysis showed that the wild-type and mutant sequence identities of the cleavage sites in the HA genes were preserved and stably maintained even after 20 passages in chicken embryos (data not shown). The expression of the HA protein in the infected cells of each passage were also confirmed by immunofluorescence assays (data not shown). The recombinant viruses maintained similar MDT, ICPI, and IVPI values after 20 passages in chicken embryos.

Protective efficacy in chickens against NDV and H5N1 AIV challenges. The protective efficacy of the recombinant viruses against NDVs and AIVs were examined, and the potential of these recombinant viruses to serve as bivalent vaccines was investigated. To determine the protective efficacy of the recombinant virus against highly pathogenic NDV, we challenged chickens with 10^3 CLD_{50} of highly pathogenic F48E9 NDV 3 weeks postvaccination (p.v.). As shown in Table 3, chickens immunized with rLa and the recombinant viruses were completely protected from NDV challenge, showing no signs of disease or deaths for two weeks. In contrast, all of the chickens in the control groups died within 3 days.

BHG/QH/05 is an antigenic drift virus compared to the H5N1 viruses that were isolated early in China (Fig. 3). The HA gene of BHG/QH/05 shares approximately 95% identity with the HA gene of GS/GD/96 virus. To determine whether the recombinant viruses could induce protection against the homologous virus as well as heterologous H5 influenza viruses, we challenged the vaccinated chickens with the homologous virus BHG/QH/05 and the early isolate GS/GD/96. Three weeks after vaccination, we collected sera and tested their HI antibody titers to the different challenge viruses. As shown in Table 4, rLa-H5w and rLa-H5m induced mean HI antibody titers to homologous virus of 8.8 $\log_2$ and 8.9 $\log_2$, respectively. The mean HI antibody titers to the heterologous virus GS/GD/96 induced by rLa-H5m and rLa-H5w were 4.2 $\log_2$ and 4.3 $\log_2$, respectively. When we challenged the chickens with 10^3 CLD_{50} of the different viruses, the vaccinated birds were completely protected from challenge by both homologous and heterologous influenza H5 viruses. No virus shedding, clinical signs, or deaths were observed. In the control group of chickens challenged with BHG/QH/05, high titers of virus shedding were detected from both tracheae and cloacae, and the animals died on or before day 3 postchallenge. In the control chicken group challenged with GS/GD/96, all of the animals shed high titers of viruses and died before day 5 p.c. (Table 4).

Another experiment was performed to evaluate the replication of the challenge virus in the organs of chickens. As shown

TABLE 4. Protective efficacy of the recombinant vaccine against highly pathogenic H5N1 AIV challenge in chickens[a]

Challenge virus	Vaccine	HI antibody titer ($\log_2$)[b]	Virus isolation from the swabs (shedding/total [$\log_{10}$ EID_{50}])[c]				Survival/ total
			Day 3 p.c.		Day 5 p.c.		
			Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
GS/GD/96	rLa-H5m	4.2 ± 0.6	0/10	0/10	0/10	0/10	10/10
	rLa-H5w	4.3 ± 0.4	0/10	0/10	0/10	0/10	10/10
	rLa	<1	10/10 (3.1 ± 0.3)	10/10 (2.7 ± 0.4)	3/3 (2.4 ± 0.7)	3/3 (2.8 ± 0.3)	0/10
BHG/QH/05	rLa-H5m	8.8 ± 0.9	0/10	0/10	0/10	0/10	10/10
	rLa-H5w	8.9 ± 0.7	0/10	0/10	0/10	0/10	10/10
	rLa	<1	10/10 (2.8 ± 0.1)	10/10 (3.1 ± 0.4)	—[d]	—[d]	0/10

[a] Groups of 1-week-old SPF chickens were vaccinated with 10^6 EID_{50} of rLa, rLa-H5m, or rLa-H5w in a 0.1-ml volume by oculonasal administration and challenged with 1,000 CLD_{50} of GS/GD/96 and BHG/QH/05 at 3 weeks postvaccination.

[b] Chicken sera were collected at 3 weeks p.v. HI antibody titer values are the mean titers of ten chickens to the challenge viruses.

[c] Oropharyngeal and cloacal swabs were collected on days 3, 5, and 7 p.c. and titrated in SPF eggs. All of the chickens in control groups died before day 7, and no virus was detected from the vaccinated chickens; therefore, the data of day 7 are not shown in the table.

[d] All of the chickens in this group died before day 5.

TABLE 5. Replication and shedding of the challenge virus in chickens[a]

Challenge virus	Vaccine	Virus replication in organs of chickens on day 3 p.c. (log EID_{50}/g)[b]						Virus shedding on day 3 p.c. (log EID_{50}/ml)[c]	
		Lung	Liver	Spleen	Kidney	Heart	Brain	Oropharyngeal	Cloacal
BHG/QH/05	rLa-H5w	—	—	—	—	—	—	—	—
	rLa	7.8 ± 0.2	6.9 ± 0.8	6.6 ± 1.2	7.2 ± 0.7	7.6 ± 0.7	5.8 ± 0.2	3.4 ± 0.3	2.9 ± 0.3
GS/GD/96	rLa-H5w[d]	2.5	—	1.5	1.5	—	1.5	—	—
	rLa	6.5 ± 0.8	6.0 ± 0.3	5.9 ± 0.5	6.8 ± 0.7	6.0 ± 0	5.5 ± 0.3	2.9 ± 0.5	3.2 ± 0.4

[a] Groups of 1-week-old SPF chickens were vaccinated with 10^6 EID_{50} of rLa or rLa-H5w in a 0.1-ml volume by oculonasal administration and challenged with 1,000 CLD_{50} of GS/GD/96 and BHG/QH/05 at 3 weeks postvaccination.

[b] Three chickens from each group were killed on days 3, 5, and 7, and organs were collected for virus titration in SPF chicken embryos. All of the chickens in control groups died before day 5, and virus was not detected from the vaccinated chickens; therefore, the data of day 5 and day 7 are not shown in the table. —, virus was not detected from the sample.

[c] Oropharyngeal and cloacal swabs were collected on days 3, 5, and 7 p.c. and titrated in SPF eggs. All of the chickens in control groups died before day 5, and virus was not detected from the vaccinated chickens; therefore, the data of days 5 and 7 are not shown in the table. —, virus was not detected from the sample.

[d] Virus was only detected from the organs of one of three chickens in this group, and the data shown are the actual titers obtained from the organs of that chicken.

in Table 5, both the GS/GD/96 and BHG/QH/05 viruses in the rLa-vaccinated groups replicated to high titers in all of the organs tested and were detected from oropharyngeal and cloacal swabs on day 3 p.c. In the rLa-H5w-vaccinated groups, the challenge virus BHG/QH/05 was not detected from any of the organs tested, the challenge virus GS/GD/96 was detected from lung, spleen, kidney, and brain of one chicken on day 3 p.i., and the titers are over 10^4-fold lower than those in the corresponding organs of the rLa virus-vaccinated groups. Virus shedding was not detected from swabs of rLa-H5w-vaccinated chickens (Table 5). These data indicate that recombinant NDV-based vaccines expressing the H5 influenza virus HA are effective against challenge by homologous and heterologous H5N1 influenza viruses and that they are also bivalent vaccines offering protection against challenge by pathogenic NDV.

Antibody responses in chickens induced by the rescued viruses. HI antibodies are necessary to prevent AIV and NDV infection. To investigate the antibody responses induced by the rescued viruses, groups of ten 3-week-old SPF chickens that were inoculated with 10^6 EID_{50}s of the viruses were followed for four months. Serum samples from all of the chickens were collected before and after vaccination on a weekly basis for analysis of HI antibody titers to H5 AIV and NDV. The two groups of chickens inoculated with rLA-H5w or rLa-H5m displayed no differences in their antibody titers to NDV and H5N1 AIV, and therefore, the titers of the rLa-H5w virus-inoculated chickens are only shown in Fig. 4. The mean HI antibody titers to NDV and H5 AIV reached up to 2.7 and 3.8 $\log_2$ at one week p.v. The mean titer to H5 AIV reached a peak of 8.8 $\log_2$ at 3 weeks p.v. and gradually declined to 4.8 $\log_2$ at 18 weeks p.v. The HI antibody titers to NDV also reached a peak of 6.5 $\log_2$ at 3 weeks and gradually decreased to 4.2 $\log_2$ at 18 weeks p.v. These results indicate that the recombinant NDV-based vaccines induce protective anti-HA antibody responses that remain sustained over four months.

Protective efficacy in mice against H5N1 avian influenza virus challenge. Groups of mice were inoculated with two doses of 10^8 EID_{50} of the rLa or rLa-H5w virus intraperitoneally at the age of 3 weeks old and then boosted with the same dose of vaccine by the same administration route at 6 weeks old. Two weeks after the second dose of vaccine, we challenged the mice with 10^3 MLD_{50}s of the highly pathogenic H5N1 viruses BHG/QH/05 and DK/FJ/02. As shown in Table 6, virus was not detected from any organs examined from the rLa-H5w-vaccinated mice that were killed on days 3 and 6 p.c. In the rLa-vaccinated groups, virus was detected in the nose turbinate, lungs, kidneys, and brains of mice challenged with both BHG/QH/05 and DK/FJ/02 viruses on day 3 p.c, and high titers of virus were also isolated from the lungs and brains of mice challenged with both viruses on day 6 p.c. Virus was not detected from the spleens of any mice at either time point. Five mice from each group were kept for observation of clinical signs and deaths. In the rLa-vaccinated groups, the mice started to show ruffled fur and weight loss at day 3 p.c. (Fig. 5A), and all mice died within 9 days p.c. (Fig. 5B). However, the mice that received the rLa-H5w virus stayed healthy and survived during the observation period (Fig. 5; Table 6). The protective efficacy of the recombinant NDV-based vaccine observed in chickens against homologous and heterologous influenza virus challenge is also extended to mice.

DISCUSSION

We generated recombinant NDV expressing either the wild-type or mutant HA gene of an H5N1 avian influenza virus using reverse genetics and evaluated its potential use as a bivalent vaccine against pathogenic influenza infection as well as against pathogenic NDV infection. After a single immuni-

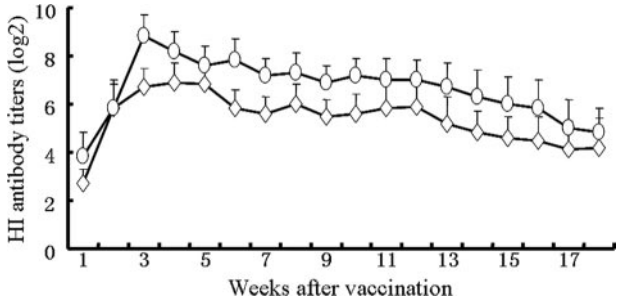

FIG. 4. HI antibody duration induced by the recombinant virus rLa-H5w in SPF chickens. One-week-old white Leghorn SPF chickens were inoculated oculonasally with 10^6 EID_{50} of the rescued recombinant virus rLa-H5w in 50 μl. Sera were collected on a weekly basis for HI antibody detection to NDV (◇) and H5N1 AIV (○). Data are represented as the means ± standard deviations.

TABLE 6. Protective efficacy of the recombinant vaccine against highly pathogenic H5N1 AIV challenge in mice[a]

Challenge virus	Vaccine	Mean HI antibody titer[b]	Virus replication in organs of mice (log_{10} EID_{50})[c]										Survival/ total
			Day 3 p.c.					Day 6 p.c.					
			NT	Lung	Spleen	Kidney	Brain	NT	Lung	Spleen	Kidney	Brain	
BHG/QH/05	rLa-H5w	160	—	—	—	—	—	—	—	—	—	—	5/5
	rLa	<10	3.0 ± 0.3	4.6 ± 0.2	—	1.3 ± 0.2	1.5 ± 0.1	—	4.2 ± 0.1	—	—	2.0 ± 0.2	0/5
DK/FJ/02	rLa-H5w	20	—	—	—	—	—	—	—	—	—	—	5/5
	rLa	<10	2.0 ± 0.3	3.3 ± 0.2	—	1.3 ± 0.1	1.2 ± 0.2	—	4.5 ± 0.2	—	—	2.2 ± 0.3	0/5

[a] Groups of 3-week-old BALB/c mice were vaccinated with 10^8 EID_{50} of rLa or rLa-H5w in a 0.1-ml volume by intraperitoneal administration and boosted with the same dose and same administration route at 3 weeks after the first dose and then challenged with 1,000 MLD_{50} of DK/FJ/02 or BHG/QH/05 at 2 weeks postboost.
[b] Mouse sera were collected at 2 weeks postboost. HI antibody titers are the mean titers of five mice to the challenge viruses.
[c] Three mice from each group were killed on days 3 and 6, and organs were collected for virus titration in SPF chicken embryos. NT, nose turbinate; —, virus was not isolated from the undiluted sample.

zation dose, the recombinant viruses expressing wild-type or mutant HA induced a strong HI antibody response to NDV and H5 AIV in chickens and protected chickens from disease signs and deaths from highly pathogenic NDV. Most importantly, the vaccinated chickens were completely protected from homologous and heterologous H5N1 virus challenges and displayed no virus shedding, signs of disease, or deaths. We also determined that the recombinant virus expressing the wild-type HA could induce complete protection in mice against lethal doses of homologous and heterologous H5N1 virus challenges. Our results demonstrated that the recombinant viruses could work as live bivalent vaccines to provide protection against infection from highly pathogenic NDV and H5 influenza virus strains.

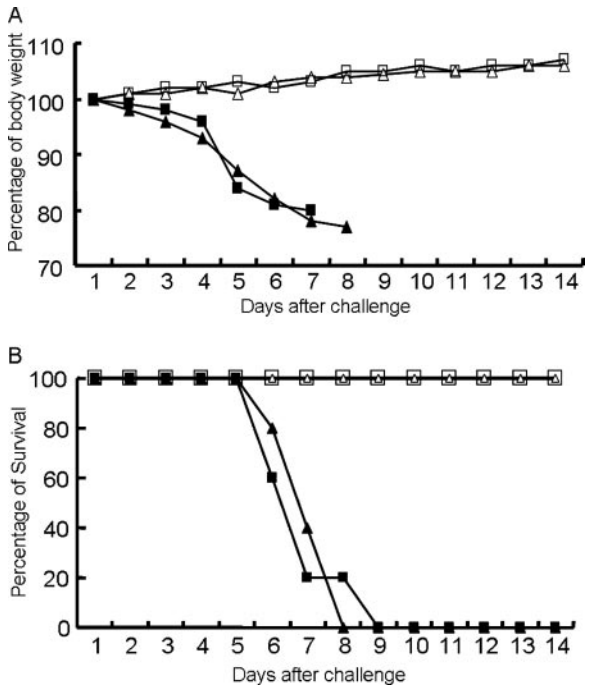

FIG. 5. Outcomes in vaccinated mice following lethal intranasal challenge with different H5N1 viruses. (A) Weight loss and (B) survival of the mice that were vaccinated with rLa (▲, ■) or rLa-H5w (△, □) and challenged by intranasal inoculation with 1,000 MLD_{50} of influenza virus BHG/QH/05 (△, ▲) or DK/FJ/02 (□, ■) two weeks after the second immunization. Mean weight loss is expressed as a percentage of original weight.

The vaccine efficacy of recombinant NDVs may be affected by several factors. The replication ability of the vector virus is an important determinant. Currently, two attenuated NDV strains, B1 and LaSota, have been used as vectors for recombinant vaccine construction. Swayne et al. (35) constructed a recombinant virus to express the HA gene of H7 avian influenza virus using the highly attenuated NDV B1 strain, and this recombinant virus only provided 40% protection against highly pathogenic H7 AIV or NDV challenge. The LaSota virus used in our experiments is less attenuated than the B1 strain, and our study as well as those of others indicate that the LaSota virus-based recombinant vaccine provides much better protection against both NDV and the targeted pathogen challenge than does the B1 strain (14, 38).

The insertion site of the foreign gene also affects the immunogenicity and vaccine efficacy of recombinant NDV. The genome of NDV contains six genes in the order of 3′-NP-P-M-F-HN-L-5′ (9). Expression levels of the proteins are attenuated in a sequential manner from the 3′ end to the 5′ end of the viral genome (17, 27). Huang et al. (14) inserted the VP2 gene of infectious bursal disease virus into the far 3′ end of the genome of NDV LaSota, and after a single dose the resultant virus only induced 90% protection against highly pathogenic Newcastle disease virus challenge, while the wild-type NDV virus induced 100% protection. We chose to insert the foreign HA gene within the NDV genome at a more 5′ position, where it would be assumed that expression of the foreign HA gene would be more attenuated than if the HA gene would have been inserted at the 3′ end of the genome, as chosen by Huang et al. These results may suggest that the overexpression of a foreign gene may impair the replication of the recombinant virus in vivo and diminish its efficacy as a vaccine.

A concern that requires examination in the construction of recombinant vaccine viruses is that the introduction of the foreign gene does not increase the virulence of the vaccine virus vector. The HA gene of avian influenza virus is a very important virulence factor for the virus, and the presence of multiple basic amino acids adjacent to the cleavage site is a prerequisite for H5 and H7 subtype AIV pathogenicity. In the present study, we generated two recombinant NDVs, one expressing the wild-type HA and the other expressing a mutant

HA containing a deletion of the multiple basic amino acids present in the cleavage site. There are no detectable differences in the biological properties between the two recombinant NDV viruses expressing the wild-type and mutant HA genes, suggesting the presence of multiple basic amino acids in the cleavage site of the HA gene does not affect the virulence of the NDV LaSota vector.

Influenza virus easily undergoes antigenic drift during circulation in nature, and the antigenic match between vaccine and the circulating viruses is one of the most decisive factors in determining the efficacy of the vaccine in preventing influenza virus replication and transmission. Therefore, it is necessary to select a virus that closely matches the prevalent virus in nature as an HA gene donor for the recombinant vaccine virus generation. We reported that several genotypes of the H5N1 viruses caused the outbreak in the wild birds in western China, and one genotype of the H5N1 virus was spread to Mongolia, the Middle East, Europe, and Africa and became the most widely distributed H5N1 virus in the world (Fig. 3). Therefore, in the present study, we selected the HA gene of the BHG/QH/05 for the generation of the recombinant virus. Our challenge studies showed that the recombinant vaccines expressing the H5 HA gene of BHG/QH/05 not only induced complete protection against the homologous virus but also induced broad cross-antibody responses and provided complete protection against the early isolate GS/GD/96, which is antigenically different.

While the manuscript was in preparation, two laboratories published results of experiments also describing the generation of NDV recombinant vaccines against influenza. Our recombinant NDV vaccines against influenza offered greater protection in chickens against homologous and heterologous influenza virus challenge than what was observed by these two laboratories, and the animals remained healthier, showing absolutely no virus shedding or symptoms of disease. Park et al. (25) generated a recombinant NDV using a modified NDV B1 strain with increased replication ability to express the ectodomain sequence of an H7 HA inserted between the P and M genes of NDV. Their recombinant NDV provided 100% protection against NDV challenge and only 90% protection against highly pathogenic H7 AIV challenge. We observed 100% protection against both NDV and pathogenic H5 AIV challenges. Again, possible differences in the replication capabilities of the different NDV strains used may have contributed to the higher level of protection observed in our experiments. Veits et al. (38) generated a H5N2 HA NDV recombinant virus using clone 30, an NDV derived from LaSota, as a vector and inserted the influenza virus HA gene between the F and HN genes of clone 30. This recombinant virus protected chickens from lethal NDV and highly pathogenic H5N2 virus challenges, though some chickens exhibited disease signs when challenged with the H5N2 virus. In addition, the level of HI antibody to H5 AIV induced by this recombinant virus was relatively low. In our study, we inserted the H5 HA genes into the NDV genome between the P and M genes, and the recombinant vaccine viruses induced high levels of HI antibody to both NDV and H5N1 AIV and provided complete protection against lethal NDV and H5N1 AIV challenge, with no animals displaying any signs of disease. It may be that the level of HA expression resulting from the more 5′ insertion position used in our recombinant vaccine may have positively influenced the replication ability of the NDV vector and its efficacy as a vaccine.

Veits et al. (38) also reported that an NDV transcription termination signal-like sequence located within the HA ORF (within the sequence of the multiple basic amino acids at the cleavage site) may limit the transcription of the HA gene and thus synthesis of HA protein, especially HA2, in the recombinant NDV. In the present study, the NDV transcription termination signal-like sequence located within the HA ORF was deleted in the rLa-H5m virus but maintained in the rLa-H5w virus. The rLa-H5m virus expressed similar amounts of HA protein as rLa-H5w, and the two recombinant viruses induced similar HI antibody responses and protection against H5 AIV in chickens. Moreover, the HA protein in the cells infected with rLa-H5w was fully expressed and cleaved into HA1 and HA2, suggesting that the NDV transcription termination signal-like sequence located within the HA may not affect the transcription of the HA gene and the synthesis and generation of HA2 protein.

The priority for the pandemic plan for protection against pathogenic influenza infection in animals and humans is the development of an effective vaccine strategy. H5N1 avian influenza virus is likely to cause the next influenza pandemic in humans. Live virus vector vaccines provided a very promising vaccine strategy against H5 influenza virus infection in humans (11, 13). Reverse genetics can allow for the rapid and abundant production of live candidate influenza vaccines, overcoming the time and quantity limitations of conventional inactivated vaccine production that could severely hinder the ability of vaccination to control the pandemic spread of avian influenza. The use of NDV as the vaccine vector for production by reverse genetics of a live virus vector vaccine against influenza has several advantages, including the ease of production, the high yield of production, the ease of widespread administration to animals in the field, and the potential for the NDV-based recombinant virus to serve as a bivalent vaccine against two viruses that can decimate bird populations. Also, the use of NDV as the vaccine backbone should prevent confusion between vaccinated birds and infected birds for surveillance purposes, which is a problematic issue with the use of whole-virus influenza vaccines. The safety and efficacy of NDV recombinants expressing protective antigens for respiratory human pathogens was recently demonstrated in two species of nonhuman primates after intranasal immunization (3). We demonstrated that the NDV recombinant virus vaccines against influenza that we created are immunogenic and provide complete protection against both homologous and heterologous lethal H5N1 virus challenges in chickens and mice. The above factors emphasize that an NDV-based vaccine should be considered as a candidate vaccine against AIV infection in animals. In addition, the advantages of an NDV-based recombinant influenza vaccine described above and its safety and efficacy in nonhuman primates make NDV-based recombinant influenza vaccines a tempting candidate vaccine to be further considered for use in humans as a potential strategy in the prevention of pandemic influenza.

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REFERENCES

1. **Alexander, D. J.** 1989. Newcastle disease, p. 114–120. *In* H. G. Purchase, L. H. Arp, C. H. Domermuth, and J. E. Pearson (ed.), A laboratory manual for the isolation and identification of avian pathogens, 3rd ed. American Association for Avian Pathologists, Inc., Kennett Square, PA.
2. **Alexander, D. J.** 1997. Newcastle disease and other avian paramyxoviridae infections. Iowa State University Press, Ames.
3. **Bukreyev, A., Z. Huang, L. Yang, S. Elankumaran, M. St. Claire, B. R. Murphy, S. K. Samal, and P. L. Collins.** 2005. Recombinant Newcastle disease virus expressing a foreign viral antigen is attenuated and highly immunogenic in primates. J. Virol. **79:**13275–13284.
4. **Capua, I., C. Terregino, G. Cattoli, F. Mutinelli, and J. F. Rodriguez.** 2003. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol. **32:**47–55.
5. **Chen, H., G. Deng, Z. Li, G. Tian, Y. Li, P. Jiao, L. Zhang, Z. Liu, R. G. Webster, and K. Yu.** 2004. The evolution of H5N1 influenza viruses in ducks in southern China. Proc. Natl. Acad. Sci. USA **101:**10452–10457.
6. **Chen, H., Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, and Y. Kawaoka.** 2006. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J. Virol. **80:**5976–5983.
7. **Chen, H., G. J. D. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. M. Peiris, and Y. Guan.** 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature **436:**191–192.
8. **Claas, E. C., A. D. M. E. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472–477.
9. **de Leeuw, O., and B. P. Peeters.** 1999. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. J. Gen. Virol. **80:**131–136.
10. **Ellis, T. M., C. Y. Leung, M. K. Chow, L. A. Bissett, W. Wong, Y. Guan, and M. Peiris.** 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. **33:**405–412.
11. **Gao, W., A. C. Soloff, X. Lu, A. Montecalvo, D. C. Nguyen, Y. Matsuoka, P. D. Robbins, D. E. Swayne, R. O. Donis, J. M. Katz, S. M. Barratt-Boyes, and A. Gambotto.** 2006. Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. J. Virol. **80:**1959–1964.
12. **Govorkova, E. A., R. J. Webby, J. Humberd, J. P. Seiler, and R. G. Webster.** 2006. Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. J. Infect. Dis. **194:**159–167.
13. **Hoelscher, M. A., S. Garg, D. S. Bangari, J. A. Belser, X. Lu, I. Stephenson, R. A. Bright, J. M. Katz, S. K. Mittal, and S. Sambhara.** 2006. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet **367:**475–481.
14. **Huang, Z., S. Elankumaran, A. S. Yunus, and S. K. Samal.** 2004. A recombinant Newcastle disease virus (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV and IBDV. J. Virol. **78:**10054–10063.
15. **Kozak, M.** 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. **196:**947–950.
16. **Krishnamurthy, S., and S. K. Samal.** 1998. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. J. Gen. Virol. **79:**2419–2424.
17. **Lamb, R. A., and D. Kolakofsky.** 2001. Paramyxoviridae: the viruses and their replication, p. 1305–1340. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott Williams & Wilkins, Philadelphia, PA.
18. **Lee, C. W., D. L. Suarez, T. M. Tumpey, H. W. Sung, Y. K. Kwon, Y. J Lee, J. G. Choi, S. J. Joh, M. C. Kim, E. K. Lee, J. M. Park, X. Lu, J. M. Katz, E. Spackman, D. E. Swayne, and J. H. Kim.** 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J. Virol. **79:**3692–3702.
19. **Li, Z., Y. Jiang, P. Jiao, A. Wang, F. Zhao, G. Tian, X. Wang, K. Yu, Z. Bu, and H. Chen.** 2006. The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J. Virol. **88:**11115–11123.
20. **Liu, J., H. Xiao, F. Lei, Q. Zhu, K. Qin, X. Zhang, X. Zhang, D. Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, and G. Gao.** 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science **309:**1206.
21. **Mase, M., K. Tsukamoto, T. Imada, K. Imai, N. Tanimura, K. Nakamura, Y. Yamamoto, T. Hitomi, T. Kira, T. Nakai, M. Kiso, T. Horimoto, Y. Kawaoka, and S. Yamaguchi.** 2005. Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. Virology **332:**167–176.
22. **Nakaya, T., J. Cros, M.-S. Park, Y. Nakaya, H. Zheng, A. Sagrera, E. Villar, A. Garcia-Sastre, and P. Palese.** 2001. Recombinant Newcastle disease virus as a vaccine vector. J. Virol. **75:**11868–11873.
23. **Neumann, G., M. A. Whitt, and Y. Kawaoka.** 2002. A decade after the generation of a negative-sense RNA virus from cloned cDNA—what have we learned? J. Gen. Virol. **83:**2635–2662.
24. **Office International des Epizooties.** 2004. OIE manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France.
25. **Park, M. S., J. Steel, A. Garcia-Sastre, D. Swayne, and P. Palese.** 2006. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. Proc. Natl. Acad. Sci. USA **103:**8203–8208.
26. **Peeters, B. P., O. S. de Leeuw, G. Koch, and A. L. Gielkens.** 1999. Rescue of Newcastle virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J. Virol. **73:**5001–5009.
27. **Peeters, B. P. H., Y. K. Cruthuisen, O. S. de Leeuw, and A. L. Gielkens.** 2000. Genome replication of Newcastle disease virus: involvement of the rule-of-six. Arch. Virol. **145:**1829–1845.
28. **Philippa, J. D., V. J. Munster, H. Bolhuis, T. M. Bestebroer, W. Schaftenaar, W. E. Beyer, R. A. Fouchier, T. Kuiken, and A. D. Osterhaus.** 2005. Highly pathogenic avian influenza (H7N7): vaccination of zoo birds and transmission to non-poultry species. Vaccine **23:**5743–5750.
29. **Qiao, C., K. Yu, Y. Jiang, C. Li, G. Tian, X. Wang, and H. Chen.** 2006. Development of a recombinant fowlpox virus vector-based vaccine of H5N1 subtype avian influenza. Dev. Biol. (Basel) **124:**127–132.
30. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
31. **Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster.** 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology **252:**331–342.
32. **Sims, L. D., Y. Guan, T. M. Ellis, K. K. Liu, K. Dyrting, H. Wong, N. Y. Kung, K. F. Shortridge, and M. Peiris.** 2003. An update on avian influenza in Hong Kong 2002. Avian Dis. **47**(Suppl.)**:**1083–1086.
33. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox.** 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science **279:**393–396.
34. **Swayne, D. E., M. Garcia, J. R. Beck, N. Kinney, and D. L. Suarez.** 2000. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine **18:**1088–1095.
35. **Swayne, D. E., D. L. Suarez, S. Schultz-Cherry, T. M. Tumpey, D. J. King, T. Nakaya, P. Palese, and A. García-Sastre.** 2003. Recombinant paramyxovirus type 1-avian influenza-H7 virus as a vaccine for protection of chickens against influenza and Newcastle disease. Avian Dis. **47**(Suppl.)**:**1047–1050.
36. **Tian, G., S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi, K. Yu, and H. Chen.** 2005. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virology **341:**153–162.
37. **van der Goot, J. A., G. Koch, M. C. M. de Jong, and M. van Boven.** 2005. Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. Proc. Natl. Acad. Sci. USA **102:**18141–18146.
38. **Veits, J., D. Wiesner, W. Fuchs, B. Hoffmann, H. Granzow, E. Starick, E. Mundt, H. Schirrmeier, T. Mebatsion, T. C. Mettenleiter, and A. Romer-Oberdorfer.** 2006. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc. Natl. Acad. Sci. USA **103:**8197–8202.
39. **Wan, X., T. Ren, K. Luo, M. Liao, G. Zhang, J. Chen, W. Cao, Y. Li, N. Jin, D. Xu, and C. Xin.** 2005. Genetic characterization of H5N1 avian influenza viruses isolated in southern China during the 2003–04 avian influenza outbreaks. Arch. Virol. **150:**1257–1266.
40. **Wyatt, L. S., B. Moss, and S. Rozenblatt.** 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology **210:**202–205.
41. **Xu, X., K. Subbarao, N. Cox, and Y. Guo.** 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology **261:**15–19.