Newcastle Disease Virus-Vectored Vaccines Expressing the Hemagglutinin or Neuraminidase Protein of H5N1 Highly Pathogenic Avian Influenza Virus Protect against Virus Challenge in Monkeys

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H5N1 highly pathogenic avian influenza virus (HPAIV) causes periodic outbreaks in humans, resulting in severe infections with a high (60%) incidence of mortality. The circulating strains have low human-to-human transmissibility; however, widespread concerns exist that enhanced transmission due to mutations could lead to a global pandemic. We previously engineered Newcastle disease virus (NDV), an avian paramyxovirus, as a vector to express the HPAIV hemagglutinin (HA) protein, and we showed that this vaccine (NDVHA) induced a high level of HPAIV-specific mucosal and serum antibodies in primates when administered through the respiratory tract. Here we developed additional NDV-vectored vaccines expressing either HPAIV HA in which the polybasic cleavage site was replaced with that from a low-pathogenicity strain of influenza virus [HA(RV)], in order to address concerns of enhanced vector replication or genetic exchange, or HPAIV neuraminidase (NA). The three vaccine viruses [NDV/HA, NDV/HA(RV), and NDV/NA] were administered separately to groups of African green monkeys by the intranasal/intratracheal route. An additional group of animals received NDV/HA by aerosol administration. Each of the vaccine constructs was highly restricted for replication, with only low levels of virus shedding detected in respiratory secretions. All groups developed high levels of neutralizing antibodies against homologous and heterologous strains of HPAIV and were protected against challenge with 2 × 107 PFU of homologous HPAIV. Thus, needle-free, highly attenuated NDV-vectored vaccines expressing either HPAIV HA, HA(RV), or NA have been developed and demonstrated to be individually immunogenic and protective in a primate model of HPAIV infection. The finding that HA(RV) was protective indicates that it would be preferred for inclusion in a vaccine. The study also identified NA as an independent protective HPAIV antigen in primates. Furthermore, we demonstrated the feasibility of aerosol delivery of NDV-vectored vaccines.

H5N1 highly pathogenic avian influenza virus (HPAIV) was first detected in human infections in 1997; previously, it had been found only in birds (11, 50). To date, this virus has been identified in 436 confirmed cases of human infection in 15 countries, 262 (60%) of which were fatal (75). The currently circulating H5N1 strains are characterized by low human-to-human transmissibility. This has been attributed, in part, to a preference for binding to -2,3-linked sialic acids that are present in high concentrations throughout the avian respiratory tract but were thought to be found primarily in the lower human respiratory tract (57), although this explanation has been questioned (48, 49). It has also been observed that mutations in the PB2 subunit of the viral polymerase are necessary to confer the ability for the virus to be spread by aerosolized nasal droplets in ferrets (72). Whatever factors may be involved, there is widespread concern that the avian virus could mutate to enhance its transmissibility among humans, possibly resulting in a global pandemic (28, 50). For the avian H9N2 virus, which also has pandemic potential, it has been demonstrated that only five amino acid changes were sufficient for the virus to gain the ability to be spread by aerosolized nasal droplets in a ferret model (60). Thus, there is an urgent need for vaccines against HPAIV.

Several vaccine strategies for HPAIV have been evaluated (reviewed in references 32 and 41), including inactivated and live attenuated vaccines. These efforts have been hampered by several factors. HPAIV strains are highly virulent for embryonated chicken eggs, the most widely used substrate for vaccine manufacture, and their rapid death following inoculation renders eggs unsuitable for efficient virus propagation. In addition, the major protective antigen, hemagglutinin (HA), administered either as a purified protein or in inactivated HPAIV virions, appears to be poorly immunogenic (69, 70). An additional factor complicating the development of HPAIV vaccines based on inactivated virus is the high cost and biohazard asso-
associated with HPAIV propagation, which must be done under enhanced biosafety level 3 (BSL-3) containment, although this problem might be addressed by the use of live attenuated reassortant influenza virus vaccines that contain the HPAIV glycoproteins on the background of an avirulent human influenza virus strain (24, 37). In addition, such reassortant strains might serve directly as live attenuated vaccines. Unfortunately, the latter approach may be limited by subtle and unpredictable incompatibility between the avian-origin glycoproteins and human-origin vaccine backgrounds acceptable for human use, which can result in overattenuation in vivo (24). There are also lingering concerns about the significant potential, with a live HPAIV vaccine, for recombination between gene segments of the vaccine virus and circulating influenza viruses, which might result in novel strains with unpredictable biological properties (63).

We and others have been evaluating Newcastle disease virus (NDV) as a general human vaccine vector for emerging pathogens, including H5N1 HPAIV (7, 18–20, 29). NDV is an avian paramyxovirus that is antigenically unrelated to common human pathogens; hence, its use in humans should not be affected by host immunity to common pathogens. The many naturally occurring strains of NDV can be categorized into three pathotypes based on virulence in chickens: velogenic strains, causing severe disease with high mortality; mesogenic strains, causing disease of intermediate severity with low mortality; and lentogenic strains, causing mild or inapparent infections (reviewed in reference 2). Lentogenic, and sometimes mesogenic, strains of NDV are in wide use as live attenuated vaccines against velogenic NDV in poultry (2). When mesogenic or lentogenic NDV was administered to the respiratory tracts of nonhuman primates as a model for the immunization of humans, the virus was highly attenuated for replication, was shed only at low titers, appeared to remain restricted to the respiratory tract, and was highly immunogenic for the expressed foreign antigen (7). We recently demonstrated that a mesogenic strain of NDV expressing the HA protein of H5N1 HPAIV (NDV/HA) elicited high titers of neutralizing antibodies in serum following combined intranasal (i.n.) and intratracheal (i.t.) delivery in a nonhuman primate model (20). Vaccination of mice with a similar NDV-vectored vaccine protected them from HPAIV challenge (29). However, results obtained with mice do not reliably predict the efficacy of an influenza virus vaccine for human use, due to the pathophysiological and phylogenetic differences between mice and humans (71). In particular, mice may produce a potent immune response to HPAIV vaccines (64) that may not be reproduced in clinical trials (38). These considerations are especially important for a vaccine based on a live viral vector platform, since its immunogenicity, and therefore its protective efficacy, is directly linked to replication, which can differ greatly in various experimental animals versus humans (reviewed in references 6 and 9). Therefore, the protective efficacy of NDV-based vaccines against HPAIV challenge in nonhuman primate models—the closest model to humans—has remained unknown.

The protease recognition sequence of the HA protein is one of the major determinants of avian influenza virus pathogenicity (62). HPAIV strains have a “polybasic” cleavage site, containing multiple basic amino acids, that is readily cleaved by ubiquitous intracellular subtilisin-like proteases, facilitating the replication and spread of the virus. In contrast, the HA cleavage site of low-pathogenicity strains contains fewer basic amino acids and depends on secretory trypsin-like proteases found in the respiratory and enteric tracts, resulting in more-localized infections (30, 62). The presence of a polybasic cleavage site in the H5 HA of any live vaccine raises some concern about the possibility of genetic exchange with circulating strains of influenza virus. It should be noted that genetic exchange involving paramyxoviruses is a rare event (14) that has been documented only once (61). However, elimination of the polybasic HA cleavage site would mitigate the effects of even this rare possibility of genetic exchange. Another concern was based on our previous finding that the HPAIV H5 HA protein is incorporated into the NDV envelope as a trimer (20), consistent with its presence in a functional form. While we previously showed that this did not enhance the pathogenicity of the NDV/HA recombinant in chickens (20), we could not rule out the possibility that it might confer an altered tropism on the NDV/HA virus in other systems. For example, a recombinant parainfluenza virus type 3 expressing the Ebola virus glycoprotein incorporated the foreign protein into its envelope, allowing cellular attachment and fusion of the vaccine virus independently of the vector’s own envelope glycoproteins (10).

In addition to the HA protein, the neuraminidase (NA) protein is also present on the surfaces of influenza virus-infected cells and virions. Antibodies specific for NA are not thought to interfere with the initial viral attachment and penetration of host cells (36, 40, 54). However, NA-specific antibodies prevent the release of virus from infected cells, thereby decreasing viral spread (35), and they increase resistance to viral infection in humans (40, 47, 54). They also provide at least some protection against viruses bearing homologous or heterologous NA proteins of the same subtype in a mouse model (12, 56). NA also appears to evolve at a lower rate than HA, suggesting that NA-specific antibodies may provide broader protection than a vaccine utilizing HA alone (39). Therefore, it was important to assess the immunogenicity and protective efficacy of the HPAIV NA independently of those of HA, which has not previously been done in a human or nonhuman primate model.

MATERIALS AND METHODS

Generation and in vitro analysis of recombinant NDV expressing HPAIV HA(RV) or NA. The sequences for HA, HA(RV) (a form of HA with reduced virulence), and NA were based on HPAIV strain A/Vietnam/1203/04 (H5N1) (30, 68) and were optimized for expression in human cells and synthesized as cDNA [HA and HA(RV)] were obtained from DNA 2.0, Menlo Park, CA, and NA was obtained from GeneArt, Regensburg, Germany] (see Fig. 1A). The construction of NDV/HA has been described previously (20). The sequence of the HA(RV) cDNA was identical to that of the previously described HA cDNA except that the HA cleavage site was from the low-pathogenicity influenza virus strain A/chicken/Mexico/3181/94. The genes were then flanked with NDV-specific gene start and gene end transcriptional signals upstream and downstream of the open reading frame, respectively. The expression cassette for each HPAIV antigen was inserted between the P and M genes, in a cDNA encoding the complete antigenic RNA of NDV strain Beaudette C, as described for NDV/HA (20). The viruses were recovered as previously described (20) and were propagated in DF-1 chicken embryo fibroblasts. For analysis of the expression of the HPAIV HA and NA proteins, DF-1 chicken embryo fibroblasts were infected with NDV, NDV/HA, NDV/HA(RV), or NDV/NA at a multiplicity of infection of 3 PFU per cell. At 24 h postinfection, cell lysates or virions, the latter purified
by sucrose gradient centrifugation as previously described (20), were analyzed by Western blotting under denaturing and reducing conditions. NDV proteins were detected using a chicken polyclonal antiserum raised against whole NDV particles (Charles River Laboratories, Wilmington, MA). HPAIV H5 HA was detected in cell lysates using a polyclonal rabbit antiserum raised against an N-terminal peptide of the HA protein (ProSci Inc., Poway, CA) and in purified virions using mouse HPAIV HA-specific monoclonal antibody clone VN04-2 (BEI Resources, Manassas, VA). HPAIV N1 NA was detected using a polyclonal rabbit antiserum raised against a conserved internal peptide of the N1 NA protein (ProSci Inc.).

Neutralizing antibody titers against HPAIV in serum were measured in a microneutralization assay in which 100 μl of medium containing 200% tissue culture infective doses (TCID50) of the homologous A/Vietnam/1203/04 strain (or an equivalent amount of strain A/Hong Kong/213/2003, H5N1, clade 1 [58], or strain A/egeg/EGipt/1162-NAMRU3/06, H5N1, clade 2), kindly provided by Ruben Donis, U.S. Centers for Disease Control and Prevention, was first mixed with 100 μl of serially diluted monkey sera and then incubated at 37°C for 1 h. A 50-μl volume of the virus-serum mixture from each serum dilution was added to approximately 150 μl of residual medium in quadruplicate wells of MDCK cells in 96-well plates. The plates were then incubated at 37°C for 72 h and were scored for virus infection (plus or minus) based on the presence of HPAIV activity in the supernatant. The titer was defined as the serum dilution resulting in complete neutralization of infection in 50% of the wells. In a modification of this assay, the serologically assayed sera were mixed with modified Harris hematoxylin, dehydrated, and mounted using permanent medium and were tested for the virus by plaque titration, and the two titers were averaged for graphical display and for calculation of group mean titer.

Vaccine immunogenicity and efficacy were assessed in a second study. AGM in groups of four were immunized by the combined i.n. and i.t. routes with 107 PFU per site of an NDV empty vector, NDV/HA, NDV/HA(RV), or NDV/NA as described previously (8). In addition, a fifth group of animals was immunized with 107 PFU of virus delivered via a nebulizer (Aerogen, Dangan, Galway, Ireland). For this immunization, an independent nebulizer unit for each animal received 2 ml of NDV/HA at 2 × 107 PFU/ml. Anesthetized animals were placed in dorsal recumbency, and then a standard infant breathing mask attached to the nebulizer respiratory circuit was held over the animal’s nose and mouth. The nebulizer was then activated until the entire 2-ml inoculum had been dispensed (approximately 2 min). Following aerosol vaccine delivery for each animal, an additional volume of input inoculum was nebulized into a collection tube—which was assayed for the titer—to assess the consistency of the titer delivered between nebulizer units. The percentages of decrease in the inoculum titer due to nebulization for the 4 animals were 31%, 35%, 29%, and 56%, respectively, suggesting that there is little variability between nebulizer units. On day 42, each of the five groups received a second immunization with the same amount of inoculum by the same route. To assess the shedding of the vaccine, NS and TL samples were taken as previously described (20).

On day 70 of the study, all animals were challenged by the combined i.n. and i.t. routes with 107 PFU per site of HPAIV A/Vietnam/1203/04. TL and NS samples were collected on days 1 to 4 postchallenge in order to assess HPAIV shedding. On day 4 postchallenge, animals were sacrificed, and tissues were collected. Tissue samples were flash frozen on dry ice, with subsequent homogenization in cell culture medium for virus titration, or were transferred to 10% neutral buffered formalin for pathology and immunohistochemistry (IHC). Comparable specimens were placed in Qiagen (Valencia, CA) RNAlater reagent and were incubated at 4°C overnight for RNA isolation.

Virological and serological assays. All virological and serological assays using HPAIV or tissue from HPAIV-infected animals were performed in an approved BSL-3 facility at the University of Maryland. The titers of NDV stocks for immunization or the titer of infectious NDV in NS and TL samples were determined by plaque titration on DF-1 chicken embryo fibroblasts, and the titer of the HPAIV stock for challenge was determined by plaque titration on Madin-Darby canine kidney (MDCK) cells. HPAIV titers in NS, TL, or tissue samples were determined by limiting dilution on MDCK cells.

To perform the hemagglutination inhibition (HI) assays, 25 μl of serum was first incubated with 50 μl of receptor-destroying enzyme (Accurate Chemical and Scientific Corp., Westbury, NY). The serum then received 25 μl of 5% sodium citrate and was heat inactivated at 56°C for 30 min. Following heat inactivation, nonspecific agglutinins were removed from each serum sample by incubation with 100 μl of pooled erythrocytes (turkey erythrocytes for NDV and chicken erythrocytes for HPAIV). The supernatant was then removed, and serial dilutions were prepared in diluted (phosphate-buffered saline [pH 7.2], 0.3% bovine serum albumin). A 25-μl volume of each serum dilution was mixed with 25 μl of diluent containing 4 hemagglutinating units of the appropriate virus, and the mixture was incubated at 37°C for 1 h. Each sample was then tested for residual hemagglutination activity by the addition of 50 μl of 0.5% (vol/vol) erythrocytes. Serum antisera specific for HPAIV HA protein were detected by an IgG isotype-specific enzyme-linked immunosorbent assay (ELISA) as previously described (20). The reported ELISA titer is the log2 of the reciprocal serum dilution at which the optical density was at least double the background and above 0.2. HA-specific antibody avidity was assessed using a modification of a published method (53), with the exception that we utilized a 4 M urea wash with a peroxidase-based ELISA, as opposed to a 7 M urea wash with a phosphatase-based ELISA as originally reported.
and one internal control (see Results) according to the manufacturer's directions. For gene amplification, we utilized a custom quantitative reverse transcription-PCR (QRT-PCR)-based gene array based on TaqMan probe chemistry (Applied Biosystems) to assess transcript levels of 23 different genes.

In the present study, we constructed a mesogenic NDV strain Beaudette C expressing the HA of the H5N1 HPAIV strain A/Vietnam/1203/04 (NDV/HA) was constructed previously (20). In the present study, the HA(RV) appeared to be incorporated into the NDV/HA(RV) vector with a similar efficiency, and the NA protein also was incorporated into the viral particles, we purified particles by ultracentrifugation through sucrose gradients and analyzed them by Western blotting. We previously estimated that the HA protein was incorporated into the NDV virion at 23% efficiency of the vector-specific proteins (20); in the present study, the HA(RV) appeared to be incorporated into the vector with a similar efficiency, and the NA protein also was incorporated into the vector particle (Fig. 1C).

Establishing a nonhuman primate model of H5N1 HPAIV infection: AGM support higher levels of HPAIV replication than RM. To evaluate the protective efficacy of the vaccine constructs in nonhuman primates, we first needed to determine the susceptibility of available species to H5N1 HPAIV infection. We compared two species of nonhuman primates, RM and AGM. Two monkeys of each species were infected with an empty NDV vector, NDV/HA, NDV/HA(RV), or NDV/NA, respectively. (Left) Proteins analyzed with anti-NDV antibodies. The major band represents the NDV F1 protein, migrating at 50 kDa. (Center) The HPAIV HA protein, detected with antibodies specific for the HA1 region of the protein. In cells infected with NDV/HA, respectively. (Right) The HPAIV NA protein, identified with NA-specific antibodies, was expressed only in cells infected with NDV/NA (lane 6), as expected. (C) Western blot analysis of purified virions of the vaccine constructs, with lanes numbered as in panel B and analyzed with the same panel of sera. The positions of Magic Mark XP protein marker bands (in kilodaltons) are shown, with amino acid differences underlined.

FIG. 1. (A) Genomic maps of the parental NDV and the vaccine construct derivatives. For NDV/HA and NDV/HA(RV), the original polybasic cleavage site and the modified site with fewer basic amino acids, respectively, are shown, with amino acid differences underlined. (B) Western blot analysis of protein expression by the NDV vectors in DF-1 chicken embryo fibroblasts in trypsin-free medium, the HA protein of NDV/HA, but not that of NDV/HA(RV), was cleaved, as expected (Fig. 1B). In addition, we constructed a virus expressing the H5N1 HPAIV NA in order to determine the contribution of NA to protection (Fig. 1A and B). To examine whether HA(RV) and NA are incorporated into the viral particles, we purified particles by ultracentrifugation through sucrose gradients and analyzed them by Western blotting. We previously estimated that the HA protein was incorporated into the NDV virion at 23% efficiency of the vector-specific proteins (20); in the present study, the HA(RV) appeared to be incorporated into the vector with a similar efficiency, and the NA protein also was incorporated into the vector particle (Fig. 1C).

RESULTS

Development and in vitro characterization of recombinant mesogenic NDVs expressing H5N1 HPAIV HA, HA bearing a modified cleavage site (HA(RV)), or NA. The recombinant mesogenic NDV strain Beaudette C expressing the HA of the H5N1 HPAIV strain A/Vietnam/1203/04 (NDV/HA) was constructed previously (20). In the present study, the HA(RV) appeared to be incorporated into the NDV/HA(RV) vector with a similar efficiency, and the NA protein also was incorporated into the vector particle (Fig. 1C).

Establishing a nonhuman primate model of H5N1 HPAIV infection: AGM support higher levels of HPAIV replication than RM. To evaluate the protective efficacy of the vaccine constructs in nonhuman primates, we first needed to determine the susceptibility of available species to H5N1 HPAIV infection. We compared two species of nonhuman primates, RM and AGM. Two monkeys of each species were infected with an empty NDV vector, NDV/HA, NDV/HA(RV), or NDV/NA, respectively. (Left) Proteins analyzed with anti-NDV antibodies. The major band represents the NDV F1 protein, migrating at 50 kDa. (Center) The HPAIV HA protein, detected with antibodies specific for the HA1 region of the protein. In cells infected with NDV/HA, respectively. (Right) The HPAIV NA protein, identified with NA-specific antibodies, was expressed only in cells infected with NDV/NA (lane 6), as expected. (C) Western blot analysis of purified virions of the vaccine constructs, with lanes numbered as in panel B and analyzed with the same panel of sera. The positions of Magic Mark XP protein marker bands (in kilodaltons) are indicated to the left.

Infectious HPAIV for AGM were higher than those for RM, except for shedding in the TL samples (Fig. 2B), and were similar to or slightly lower than published values for cynomolgus macaques (3, 55). Thus, AGM were selected as a model for the evaluation of vaccine immunogenicity and protective efficacy.

In the experiment discussed above, the animals were also monitored for disease signs. Following infection, one RM demonstrated poor appetite, moderate to severe lethargy on days 2, 3, and 4 without fever, decreased lymphocyte counts on days 1 through 4, and increased neutrophil counts on days 1 and 2.
The other RM demonstrated a small (0.6°C) increase in body temperature, an elevated neutrophil count, and decreased lymphocyte counts on days 1 through 4. One AGM demonstrated mild lethargy on day 4, similar to that observed for RM. Both AGM showed increases of 0.6 to 1.7°C in body temperature on days 1 and 2 postinfection. The lethargic animal had substantially decreased lymphocyte counts (3- to 5-fold) and mildly elevated neutrophil counts on all 4 days postinfection. The other animal demonstrated a small decrease in lymphocyte counts and elevated neutrophil counts on days 1 through 4. Thus, disease symptoms were mild, at least during the first 4 days of infection, indicating that the RM and AGM models are not suitable for evaluating HPAIV pathogenesis.

FIG. 2. Replication of H5N1 HPAIV in RM and AGM. Two monkeys of each species were infected with $2 \times 10^7$ PFU of HPAIV by the combined i.n./i.t. route. Nasal swab (NS) and tracheal lavage (TL) samples were taken on days 1, 2, 3, and 4, and, on day 4, the animals were euthanized and necropsy was performed to quantify infectious HPAIV in various tissues. (A through D) The NS (A and C) and TL (B and D) samples were assayed by limiting dilution (A and B) to measure infectious virus and by QRT-PCR (C and D) to measure HPAIV RNA. (E) Titers of infectious H5N1 HPAIV in various tissues taken at necropsy were determined by plaque titration of tissue homogenates. NT, nasal turbinates; T, trachea; LL H, left lung hilus; LL U, left lung upper lobe; LL L, left lung lower lobe; RL H, right lung hilus; RL U, right lung upper lobe; RL M, right lung middle lobe; RL L, right lung lower lobe; B LN, bronchial lymph node. The value for each tissue was calculated by titration of two replicate samples from each animal. Dashed lines indicate the limits of detection. NS and TL samples that were below the detection limit were assigned values of 1.0 $\log_{10}$ TCID$_{50}$/ml (A and B) or 1.0 $\log_{10}$ copies/ml (C and D) for calculation of the mean. For tissue samples in which no virus was detected, a value of 2.0 $\log_{10}$ TCID$_{50}$/g (E) was assigned for calculation of the mean.

FIG. 3. Shedding of the NDV vaccine constructs from the respiratory tracts of AGM following immunization. AGM (4 animals per group) were infected either with $2 \times 10^7$ PFU of the empty NDV vector, NDV/HA, NDV/HA(RV), or NDV/NA by the combined i.n./i.t. route or with $4 \times 10^8$ PFU of aerosolized NDV/HA. Infectious vaccine virus titers (mean ± standard errors) in nasal swab (NS) (A) and tracheal lavage (TL) (B) samples were determined by plaque assays. Dashed lines indicate the limits of detection. NS and TL samples in which no virus was detected were assigned a value of 0.5 $\log_{10}$ PFU/ml for calculation of the mean.

Respiratory tract delivery of the vaccines in liquid or aerosol form. We next immunized groups of 4 AGM with $2 \times 10^7$ PFU of the empty NDV vector, NDV/HA, NDV/HA(RV), or NDV/NA by the combined i.n. and i.t. routes. We also evaluated immunization with aerosolized NDV/HA produced by a nebulizer providing a mass median aerodynamic particle diameter of 2.1 $\mu$m, according to the manufacturer. It is generally accepted that particles less than 3 $\mu$m in diameter readily penetrate the small airways (reviewed in reference 45). Since the typical efficiency of delivery of aerosol particles to the lower respiratory tract in primate models is approximately 10% (13, 16), we used a 20-fold-higher dose ($4 \times 10^8$ PFU) for administration by nebulizer. NS samples taken on days 2, 4, 6, and 8 after the first vaccine dose demonstrated a lack of any significant NDV shedding from the upper respiratory tract in all groups except the NDV/HA (aerosol) group, in which modest shedding ($\leq 2 \log_{10}$ PFU/ml) was detected. In TL samples taken on the same days, we observed comparable, low levels of shedding ($\leq 2 \log_{10}$ PFU/ml) in all groups (Fig. 3). Forty-two days later, a second dose of the same vaccine was delivered to
each animal by the same route (liquid or aerosol). No NDV shedding was detected in any group, presumably due to restriction mediated by host immunity to the first dose. We did not observe any symptoms of disease after either vaccine dose in any group, as expected for this highly attenuated vector.

HPAIV- and NDV-specific serum antibody responses following immunization with the NDV vectors. Serum antibody responses to vaccination in the experiment described above were determined on days 0 (when the first vaccine dose was administered), 42 (when the second dose was administered), and 70 (Fig. 4). Substantial HA-specific responses quantitated by ELISA were detected in all three groups of animals immunized with NDV/HA or NDV/HA(RV) but were not detected with the empty NDV vector or with NDV/NA, as expected (Fig. 4A). These responses were significantly boosted by the second vaccine dose. As expected, NAI antibodies were detected only for the NA-expressing construct (Fig. 4B).

HPAIV-neutralizing antibodies were detected by a microneutralization assay (Fig. 4C). In agreement with the results of our previous study (20), little or no HPAIV-neutralizing antibodies were detected after the first dose. After the second dose, substantial levels of neutralizing antibodies were detected in all groups of animals immunized with an NDV vector expressing an HPAIV antigen, including slightly lower titers in animals immunized with NDV/NA. In this assay format, virus-serum mixtures were inoculated onto the cell monolayers and remained on the culture for the duration of the assay. Therefore, in addition to detecting antibodies that neutralize HPAIV, this assay likely would also detect antibodies that inhibit cell-to-cell spread of the virus.

We modified the microneutralization assay by replacing the virus-serum mixtures with fresh medium following the adsorption period, thereby greatly reducing the concentration of antibodies present during the subsequent time of culture. This format would be more specific to antibodies capable of blocking the initial infection. With this format (Fig. 4D), we observed similar decreases in neutralizing titers (~4-fold) for all of the groups, with the titers in the NDV/HA (aerosol) and

FIG. 4. HPAIV- and NDV-specific serum antibody responses to immunization. Continuing the experiment for which results are shown in Fig. 3, the groups of AGM that were immunized on day 0 were administered a second dose of the same virus by the same route on day 42. Serum samples were collected on days 0, 42, and 70. (A) HPAIV HA-specific antibodies analyzed by ELISA against purified recombinant H5 HA protein. (B) HPAIV NA-specific antibodies analyzed by an NAI assay. (Note that day 0 and day 70 sera were assayed for only two of the four NDV or NDV/HA animals and that the only day 42 sera assayed were for the NDV/NA animals.) (C) HPAIV-neutralizing (Neut.) antibodies measured by a microneutralization assay format that would score both neutralization of the initial infection and inhibition of spread (see Results). (D) HPAIV-neutralizing antibodies measured by a microneutralization assay format that would measure primarily inhibition of the initial infection. (E) HPAIV-specific antibodies measured by an HAI assay. (F) NDV-specific antibodies measured by an HAI assay. All antibody titers are expressed as means ± standard errors. For calculation of the mean, antibody titers below the limit of detection (dashed lines) were assigned values of 4.2 log₂ (A), 2.0 log₂ (B, C, D, and E), or 1 log₂ (F). The statistical differences between the groups were determined by a repeated-measures two-way analysis of variance with Bonferroni posthoc analysis. †, P < 0.05; ‡, P < 0.01; *, P < 0.001. Symbols directly above the vertical bars (A, B, C, D, and E) indicate statistically significant differences between the value for the group and the value for the same group at the previous time point. Symbols located above horizontal lines (A, C, D, and E) show statistically significant differences at the same time point between the two groups at the ends of each line.
NDV/NA groups only slightly exceeding the limit of detection. It should be noted that, using the modified assay, the NDV/NA group included two animals with undetectable titers and two animals with titers of 1:20. These data suggest that all vaccine constructs, including NDV/NA, induced antibodies that were able both to block the initial HPAIV infection and to prevent its spread, although the ability of NA-specific antibodies to block the initial infection was variable among animals.

HPAIV-specific antibodies were also measured by an HAI assay (Fig. 4E). Like the neutralizing antibodies, HPAIV-specific HAI antibodies were detected only after the second vaccine dose, and somewhat lower titers were detected in the NDV/HA (aerosol) and NDV/NA groups. While HAI titers are generally associated with HA-specific antibody responses, it has been reported that NA-specific antibodies also can inhibit hemagglutination (25, 40), presumably through steric hindrance of the interaction between HA and sialic acids on the surfaces of erythrocytes (40). Antibody responses to the NDV vector also were measured by an HAI assay (Fig. 4F). In contrast to the HPAIV-specific HAI antibodies (Fig. 4E), effective induction of vector (NDV)-specific HAI antibodies was observed after the first dose. Overall, these findings demonstrate that NDV/HA, NDV/HA(RV), and NDV/NA elicited HPAIV-specific antibody titers of a magnitude that could be associated with protective efficacy and that the immunogenicity of NDV/HA was similar when it was delivered in a potentially clinically feasible aerosolized form to that with i.n. and i.t. delivery.

HPAIV H5 HA lacking the polybasic cleavage site was at least as immunogenic as wild-type HA. Interestingly, most of the serological assays for which results are shown in Fig. 4 demonstrated that NDV/HA(RV), in which the H5N1 HA polybasic cleavage site was replaced with the cleavage site from a nonpathogenic H5N2 influenza virus HA, induced a greater level of H5N1 HPAIV-specific antibodies than NDV/HA, despite similar levels of vector replication in the host (Fig. 3B) and somewhat lower levels of vector-specific HAI (Fig. 4F). In particular, comparison of responses to NDV/HA(RV) versus NDV/HA shows that the levels of HA-specific antibodies to the former were increased 9.2-fold ($P < 0.01$) on day 40 and 5.7-fold ($P < 0.05$) on day 70 as measured by ELISA (Fig. 4A), 2.6-fold ($P < 0.05$) on day 70 as measured by neutralization with medium removal (Fig. 4D), and 3.5-fold ($P < 0.05$) on day 70 as measured by an HAI assay (Fig. 4E). A similar difference in neutralizing activity against heterologous strains of HPAIV was observed (see below).

Enhanced antibody affinity maturation contributes to the effective virus neutralization observed after the second vaccine dose. As mentioned above, we did not observe significant serum neutralizing antibody responses after the initial vaccine dose in any group (Fig. 4C and D), despite significant titers of HA-specific and NA-specific antibodies as detected by ELISA and an NAI assay, respectively (Fig. 4A and B). We hypothesized that the increase in neutralizing activity following the second dose might be due, in part, to increased antibody affinity maturation. Since all groups received two doses of vaccine in the present study, we used serum samples from our previous study (20), in which one group of animals received a single dose of NDV/HA on day 0 and a second group of animals received two doses of NDV/HA on days 0 and 42, respectively. Serum samples were taken on days 0, 42, and 70 to assess antibody responses. We also analyzed serum samples from the present study taken at the same time points postimmunization for comparison. Antibody avidity was measured as the proportion of ELISA antibodies that was resistant to a 4 M urea wash. The percentages of urea-resistant antibodies on days 42 and 70 were calculated based on the average results of 3 to 6 replicate assays for each animal. The values for the one-dose and two-dose groups from the previous study are plotted in Fig. 5A and B, respectively, and those for the two-dose group from the current study are shown in Fig. 5C. We also calculated the mean change in avidity between days 42 and 70 for each group (Fig. 5D). All animals, including those in the one-dose group, demonstrated increases in HA-specific antibody avidity between days 42 and 70 postimmunization (Fig. 5A, B, and C); however, the average percentage of increase in avidity over this period was 90% greater for the two-dose group than for the one-dose group ($P < 0.05$) (Fig. 5D). These data suggest that the second dose of vaccine substantially enhances antibody affinity maturation. It should be noted that the one-dose group does not develop significant neutralizing antibody titers (20), despite continuing antibody avidity maturation. Moreover, despite the robust NDV-specific antibody response to the first dose (Fig. 4F), it is likely that the second dose of the vaccine did replicate in tissues of the respiratory tract. A similar phenomenon has been demonstrated for respiratory syncytial virus replication in the respiratory tracts of virus-immune animals, which was undetectable by a plaque assay but detectable by QRT-PCR analysis of the respiratory tract tissues (4). Taken together, these results suggest that two factors likely contribute to the high neutralizing titers observed in the two-dose group: (i) the substantial increase in antibody titer elicited by the second vaccine dose, as measured by ELISA, and (ii) enhanced avidity maturation.

Vaccinations did not induce significant T-cell responses in the peripheral blood. To investigate peripheral blood T-cell responses to immunizations, we isolated peripheral blood mononuclear cells on days 0 (prior to the first immunization), 10, 42 (prior to the second immunization), 52, and 70, and we analyzed the numbers of CD4+ and CD8+ T cells secreting interleukin 2 (IL-2) and/or gamma interferon (IFN-g) (cells expressing IL-2, IFN-g, or both cytokines were considered positive events and were counted together) in response to stimulation in vitro with overlapping peptide libraries spanning the complete H5N1 HA or NA protein. Interestingly, despite the robust antibody responses noted above, cell-mediated responses were detected in peripheral blood sporadically and only in a few animals (data not shown).

Vaccination conferred a high level of protection against H5N1 HPAIV challenge. On day 70 (day 28 after the second dose), all animals were challenged with a large dose, $2 \times 10^7$ PFU, of the homologous H5N1 HPAIV strain A/Vietnam/1203/04 by the combined i.n. and i.t. routes. Following the challenge, no behavior abnormalities and no significant loss of appetite was observed in any animal. We did observe a modest increase in body temperature for all animals, which peaked on day 1 and decreased through day 4. The mean temperatures of all the vaccinated groups were lower than that of the vector-immunized control group, except for the NDV/HA-immunized animals on day 3; however, no significant difference was ob-
served due to the high animal-to-animal variability (not shown). NS (Fig. 6A) and TL (Fig. 6B) samples were collected on days 1, 2, 3, and 4 after the challenge. We detected significant infectious HPAIV titers in all specimens collected from the empty NDV vector group. In contrast, little or no infectious HPAIV shedding was detected in the groups that had been immunized with NDV/HA, NDV/HA(RV), or NDV/NA (Fig. 6A and B). Interestingly, the NDV/HA(RV) group had no detectable HPAIV challenge virus shedding in any sample, consistent with this group having the highest HPAIV-specific antibody titers (Fig. 4). On day 4, the animals were euthanized, and necropsy was performed. Analysis of tissue samples from the empty NDV vector group demonstrated moderate to high titers of infectious challenge HPAIV in nasal turbinates, tracheae, and the hilar areas and lobes of the lungs, as well as in the bronchial and mediastinal lymph nodes. In contrast, no challenge virus was detected in any immunized group, except for very low titers in sporadic tissue samples from animals vaccinated with NDV/NA (Fig. 6C).

We also performed blinded IHC evaluation of HPAIV antigens in various tissues of the respiratory tract (Fig. 7). In agreement with a recently published evaluation of HPAIV infection in cynomolgus macaques (3), abundant challenge virus antigen was detected in the epithelia of bronchi and bronchioles and in the alveolar macrophages of all four animals immunized with the empty NDV vector, as well as in type I and type II pneumocytes of two out of four animals in the same group (Fig. 7A to D). In contrast, no challenge virus antigen was detected in any tissue samples from any of the animals immunized with NDV/HA or NDV/HA(RV), and very little or no challenge virus antigen was detected in NDV/NA-immunized animals (Fig. 7E). Blinded histopathological examination of lungs of monkeys immunized with the NDV empty vector (i.e., with abundant challenge HPAIV replication) revealed a severe, diffuse bronchointerstitial pneumonia with prominent alveolar damage characterized by extensive areas of hemorrhage, fibrin deposition, edema, and type II pneumocyte hyperplasia. Animals immunized with NDV/HA(RV) demonstrated only subtle postchallenge histopathological changes, while those immunized with NDV/HA in either form (liquid or aerosol) or with NDV/NA had somewhat greater postchallenge pathology (data not shown). It should be noted, however, that the daily collection of TL samples, in which relatively large (30-ml) volumes of phosphate-buffered saline were instilled and recovered, may have contributed to the pathological changes observed in all groups. In addition, some pathological changes might reflect protective primary and secondary immune responses to the HPAIV challenge. Taken together, these data demonstrate the high protective efficacy of all vaccine constructs, with NDV/HA(RV) having the highest level of protective efficacy.

HPAIV challenge induced strong transcriptional induction of IFN-controlled genes in control animals but not in vaccinated animals. We next used QRT-PCR to compare the levels of selected host mRNAs in lung tissues collected on day 4 postchallenge, as described above, from (i) the NDV empty vector-immunized (unprotected) group, (ii) the NDV/HA-immunized (protected) group, and (iii) a group of four additional animals that had been neither immunized nor challenged with HPAIV and thus provided baseline values (Fig. 8; values are expressed as the \( \log_2 \) change relative to the level for the unimmunized/unchallenged group). The panel of genes tested included type I, type II, and type III IFNs, chemokines, and IFN-regulated antiviral factors. Compared to the unimmunized, unchallenged group, the empty NDV vector-immunized,
unprotected group (Fig. 8, black bars) exhibited a strong (11-fold) increase in the level of IFN-α3 (IL-28B, a type III IFN), which upregulates IFN-controlled antiviral genes (5, 46, 74) and plays an important role in protection against influenza virus (46). We observed modest changes in the levels of other cytokines and chemokines tested (Fig. 8A and B) in both the NDV- and NDV/HA-immunized groups. The most striking effect of HPAIV challenge was observed for empty NDV vector-immunized (unprotected) animals, which demonstrated strong, 9- to 30-fold induction of CXCL10 (Fig. 8B) and IFN-regulated antiviral genes MX1, MX2, OAS-1, OAS-2, and ISG56, which serves as a mediator of negative feedback of type I IFN and cellular antiviral responses (44) (Fig. 8C). The reduced levels of type I IFN seen in the empty NDV vector-immunized group (Fig. 8A), despite the high titers of HPAIV at the same time point (day 4) (Fig. 6C), may reflect suppression of type I IFN expression by the challenge virus NS1 IFN antagonist. Given the apparently contradictory finding that there was strong induction of mRNAs for the IFN-inducible antiviral proteins in this group (Fig. 8C, black bars), we speculate that this effect might be due to early induction of type I IFN that subsequently was suppressed by day 4 by virally expressed NS1, or that it might be mediated by some other factor that was differentially induced by challenge HPAIV infection but was not monitored here.

Antibodies induced by HA or NA expressed by recombinant NDV cross-react efficiently with heterologous H5N1 HPAIV strains. The H5N1 HPAIV strains currently circulating among birds are divided into various clades based on HA sequence relatedness, with reported human infections being associated predominantly with clades 1 and 2 (reviewed in reference 1). The A/Vietnam/1203/04 parent of the genes represented in the vaccine constructs of the present study belongs to clade 1. We evaluated the ability of sera collected from animals immunized with the panel of NDV recombinants to react with two heterologous strains of H5N1 HPAIV. The initial strain tested was A/Hong Kong/213/03 (58), which belongs to clade 1 and whose HA and NA amino acid sequences differ from those of A/Vietnam/1203/04 by 1.8% and 4.6%, respectively, not counting an internal 20-amino-acid deletion in the NA sequence for A/Hong Kong/213/03. We also tested serum reactivity against the HPAIV strain A/egret/Egypt/1162-NAMRU3/06, which belongs to clade 2.2 and whose HA and NA (the two genes were sequenced in the present study) differ from those of A/Vietnam/1203/04 by 4.0% and 3.2%, respectively. As with HAI titers against the homologous virus (Fig. 4E), we observed little or no HAI antibodies reactive with A/Hong Kong/213/03 after the first vaccine dose (day 42). However, after the second vaccine dose (day 70), A/Hong Kong/213/03-specific HAI antibodies of a magnitude similar to those observed against the A/Vietnam/1203/04 virus were detected (Fig. 9A). Antibody titers specific for the more divergent (at least based on the HA sequence) virus A/egret/Egypt/1162-NAMRU3/06 were detected on day 70 by an HAI assay (Fig. 9B). The A/egret/Egypt/1162-NAMRU3-specific HAI titers were 9.2-fold, 4.9-fold, 7.0-fold, and 1.7-fold less than those observed against the homologous A/Vietnam/1203/04 virus in animals immunized with NDV/HA, NDV/HA (aerosol), NDV/HA(RV), or NDV/NA, respectively (Fig. 9B). To further characterize the response against the A/egret/Egypt/1162-NAMRU3/06 virus, we also performed a microneutralization assay using the sera from each immunization group. Interestingly, serum neutralizing an-
tibody titers against A/egret/Egypt/1162-NAMRU3/06 were almost identical to those observed against the homologous A/Vietnam/1203/04 virus for all groups (Fig. 9C). These data indicate that vaccination by NDV expressing the HA or NA protein of the A/Vietnam/1203/04 virus is likely to be equally effective against a closely related but heterologous strain, such as A/Hong Kong/213/03, and also would be highly protective against a more divergent strain, such as A/egret/Egypt/1162-NAMRU3/06. In addition, as was observed in assessing serum antibody reactivity against the homologous virus (Fig. 4), the titers of antibodies induced by NDV/HA(RV) reactive with the heterologous viruses were consistently higher than those induced by NDV/HA. For example, the increase in the mean titer of antibodies against A/egret/Egypt/1162-NAMRU3/06 induced by NDV/HA(RV) over that induced by NDV/HA was 4.6-fold ($P < 0.001$), as determined by an HAI assay (Fig. 9B).

**FIG. 7.** IHC evaluation of AGM tissues 4 days after challenge with HPAIV. (A to D) IHC evaluation of tissues from AGM immunized with an empty NDV vector and challenged with HPAIV, all demonstrating evidence of bronchointerstitial pneumonia. Challenge virus HA antigen (stained brown) was detected with polyclonal goat anti-HA antibodies and a horseradish peroxidase detection system. (A) Prominent viral antigen in the bronchiolar epithelium (black arrow), alveolar epithelium (green arrows), and alveolar macrophages (red arrows). Captured by a 20× objective. (B) Scattered antigen-positive epithelial cells (arrows) in the terminal bronchus. Captured by a 40× objective. (C) Bronchiole (center) with intraluminal hemorrhage, inflammatory exudates, cell debris, and prominent immunostaining along the intact and denuded epithelial surface (arrow). Captured by a 20× objective. (D) Alveoli with prominent immunostaining in pneumocytes (black arrow) and alveolar macrophages (red arrows). Captured by a 40× objective. (E) Semiquantitative evaluation of HPAIV HA antigen in AGM immunized with the indicated construct and challenged with HPAIV. Antigen distribution in bronchial and bronchiolar epithelia, bronchus-associated lymphoid tissue, alveolar type I and type II pneumocytes, and alveolar macrophages was scored according to the number of antigen-positive cells per high-power field (magnification, ×400): 0, no cells; 1, 1 to 2 cells; 2, 3 to 10 cells; 3, >10 cells. Each symbol represents an individual animal and is the average from a total of four slides representing the left lung hilus, the left lung periphery, the right lung hilus, and the right lung periphery, respectively. The horizontal bars represent the mean for each group.
were expressed as the mean log2 fold change. The error bars represent

FIG. 8. Induction of cytokines (A), chemokines (B), and IFN-induced antiviral genes (C) following HPAIV challenge. The levels of mRNAs for selected host genes were measured by QRT-PCR analysis of lung tissue samples harvested on day 4 following HPAIV challenge of animals that had been immunized with the empty NDV vector (unprotected from the challenge) or NDV/HA (protected). Changes in gene expression were calculated relative to expression in four additional uninfected, unchallenged animals using the ΔΔCT method and were expressed as the mean log, fold change. The error bars represent the absolute maximum and minimum values obtained by adding the standard error of the calibrator threshold cycle (i.e., unimmunized, unchallenged animal) and the standard error of the threshold cycle for the experimental group. Statistical differences were calculated by one-way analysis of variance with a Tukey posttest. †, \( P < 0.05; \) ‡, \( P < 0.01; \)

DISCUSSION

This study describes the development of live, mucosally delivered NDV-vectored vaccines against H5N1 HPAIV and their evaluation for immunogenicity and protective efficacy against H5N1 HPAIV challenge in a nonhuman primate model. Several important conclusions can be drawn from this study. First, i.n. and i.t. immunization with two doses of NDV/HA in a liquid form induced high levels of HPAIV-neutralizing antibodies in serum and completely or almost completely protected monkeys against a high dose of challenge HPAIV. Second, these high levels of immunogenicity and protective efficacy were also observed with the modified HA(RV) protein, which lacked the polybasic cleavage site. Third, the requirement of a second dose for effective induction of HPAIV-neutralizing antibodies is not only related to the increase in antibody concentration, as measured by ELISA, but is also due to enhanced affinity maturation associated with the second dose. Fourth, expression of the NA protein alone also induced high levels of HPAIV-neutralizing serum antibodies and protective efficacy. Fifth, aerosol delivery of NDV/HA was highly immunogenic and protective, thereby providing a potentially feasible method by which lower respiratory tract delivery to a human can be achieved. Sixth, serum antibodies induced by NDV-vectored HA and NA were highly reactive against heterologous H5N1 HPAIV strains, suggesting that NDV vectors can provide broad protective immunity. These results, taken together with the observation that NDV vectors are highly restricted and attenuated in nonhuman primates (7, 18–20), indicate that NDV-vectored vaccines against HPAIV should be evaluated for safety and immunogenicity in clinical studies.

The present study showed that HA and NA are major independent protective HPAIV antigens. Evaluation of NA as an immunogen independent of HA for influenza virus in general has not been vigorously pursued, probably because of the idea that antibodies against NA would not block attachment and penetration and thus would provide incomplete immunity to replication and disease (36, 40, 54). However, NA-specific serum antibodies are associated with resistance to infection with seasonal influenza virus in humans (47). Immunization against NA achieved with DNA or baculovirus-expressed protein was protective in mice against challenge and was observed both for human influenza virus and for HPAIV (12, 34, 51, 56). Furthermore, NA-specific antibodies were more broadly protective against heterologous strains than HA-specific antibodies (34), indicating conservation of neutralizing epitopes on the NA proteins of evolving H5N1 viruses. Similarly, chickens that were immunized against H5N1 NA using baculovirus-expressed proteins, DNA, or other vectors exhibited increased survival after HPAIV challenge (52, 65). However, it remained unclear how effective immunization with the NA of H5N1 would be in a nonhuman primate with a highly attenuated vector. Thus, we were surprised to find that immunization with NA alone induced a high titer of HPAIV-neutralizing serum antibodies and almost complete protection against HPAIV challenge replication. Indeed, the immunogenicity and protective efficacy of NA approached those of HA. This indicates that NA, along with HA, should be included in vectored vaccines against influenza virus, which should result in broader, more effective protection than HA alone.

Replacement of the polybasic H5 HPAIV HA cleavage site with a cleavage site containing fewer basic residues taken from a nonpathogenic influenza virus was associated with increased immunogenicity and protective efficacy in this study. The apparently greater immunogenicity of this altered HA remains to be confirmed with a larger number of animals. Why immunogenicity might be improved by removal of the polybasic se-
sequence is unclear. This effect did not appear to be due to increased replication of the NDV/HA(RV) construct, based on the levels of vector shedding (Fig. 3A and B) and vector-specific antibody responses (Fig. 4F). One possibility is that this change affected the processing and major histocompatibility complex (MHC) presentation of the HA antigen, based on a precedent in which point mutations in the group-associated antigen (Gag) of human immunodeficiency virus greatly affected proteasomal degradation and subsequent MHC class I presentation of the protein, strongly modifying the resulting T-cell response (66). Another possibility is that the conformation of the antigen might have been modified somewhat, resulting in a difference in the repertoire of induced antibodies. In any event, the removal of the polybasic site, which should eliminate any safety concerns regarding this sequence, was associated with immune responses that were as great as or greater than those against wild-type HA. This indicates that this modified gene is the gene of choice for further evaluation.

The strong serum neutralizing antibody response detected in all animals immunized with NDV expressing HA, HA(RV), or NA likely was a major determinant of protection against the HPAIV challenge. We did not specifically measure mucosal antibody responses in the present study, but we previously demonstrated substantial HPAIV-specific antibody responses in respiratory secretions from monkeys immunized with NDV/HA (20). Therefore, it is reasonable to expect that mucosal antibodies also contributed to the protection against HPAIV challenge in the present study. In previous reports, NA-specific antibodies have been described as “infection permissive” (36), acting primarily by inhibiting virus release from infected cells and restricting cell-to-cell spread (40). Consistent with this, assays in the present study with two different microneutralization formats suggested that HA-specific antibodies appeared to be more effective at preventing the initial infection, whereas NA-specific antibodies acted primarily to inhibit virus spread (Fig. 4C and D). However, the two NA-immune animals with the highest antibody titers, as assessed by an NAI assay (Fig. 4B) and by general neutralization (Fig. 4C), also were able to neutralize the initial infection in vitro at a dilution of 1:20 (Fig. 4D). This suggests that a high titer of NA-specific antibodies can neutralize HPAIV, perhaps through steric hindrance of attachment or penetration mediated by HA. The possibility of steric hindrance of HA-mediated attachment is supported by the observation that sera from the NA-immune animals had HAI activity, presumably due to steric hindrance of the interaction between HA and sialic acid residues on the surfaces of erythrocytes by antibodies bound to NA.

Virus-specific CD8$^+$ cytotoxic T lymphocytes also are an important factor in influenza virus immunity (23, 31, 67) and may be particularly important for the control of infection with viruses that evade antibody neutralization due to antigenic drift of the envelope proteins. Influenza infection of humans induces a cytotoxic T-cell response that can be transiently detected in the peripheral blood (22); however, most of the influenza virus-specific CD8$^+$ T cells accumulate in human lung tissue rather than in the peripheral blood (17). In the present study, we did not detect a significant T-cell response in the peripheral blood (data not shown). This might reflect a reduced response due to the limited replication of NDV in the nonhuman primate lung (7) or the limited number of H5N1 antigens in the NDV vectors tested, as well as the likelihood that the cell-mediated response was localized in the lung and was not efficiently detected by sampling of peripheral blood.

NDV is highly attenuated in nonhuman primates, with very
little virus shedding, and replication appears to be restricted to the respiratory tract. These observations suggest that NDV would be a very safe vector for humans. In studies to date, we have evaluated three different NDV backbones, namely, the mesogenic Beaudette C strain (as in the present study), the lentogenic LaSota strain, and a modified LaSota strain bearing a polybasic cleavage site in the F protein derived from the Beaudette C strain. These viruses appeared to have comparable safety, immunogenicity, and protective efficacy profiles (7, 18, 19), suggesting that any of these strains would be appropriate for human use. We recently showed that NDV-vectored vaccines require delivery to the lower respiratory tracts of nonhuman primates: i.n. delivery alone was insufficient to provide sufficient virus replication to be immunogenic (19). Whether the same limitation would apply to humans is unknown, since one cannot precisely predict permissiveness in humans based on results in nonhuman primates. For example, whereas several species of monkey support moderate levels of replication of human parainfluenza viruses and metapneumovirus, replication of human respiratory syncytial virus is inexplicably more restricted (15, 21). Thus, whether i.n. administration of NDV vectors is effective for humans can be determined only by a clinical study. However, in anticipation of the possibility that lower respiratory tract delivery might also be necessary for humans, in the present study we also evaluated the alternative method of delivery by nebulizer. This employed a device that is being evaluated by the World Health Organization (43) and is based on a previous device that was used successfully to vaccinate 4 million children against measles virus (26, 27). Thus, initial clinical evaluation of an NDV construct can compare the i.n. and the aerosol route of delivery.

NDV offers a number of advantages as a vaccine vector against HPAIV. One advantage is that topical administration to the respiratory tract is likely to be particularly effective against a respiratory pathogen such as HPAIV. A second advantage is that the expression of foreign viral glycoproteins by NDV or other respiratory paramyxoviruses, such as human parainfluenza virus type 3, did not appear to alter the replication, pathogenesis, or cell tropism of the vector, indicating that the foreign gene is a silent passenger (7, 8, 10, 18, 20). In particular, we and others previously confirmed that expression of HA from a highly pathogenic H5N1 virus by NDV did not increase the replication, tropism, or pathogenesis of the NDV vector in embryonated eggs or young chickens (20, 73). The lack of effect on the vector provides for biological and environmental safety. Additional safety is provided by the use of an H5 HA lacking the polybasic cleavage site, which would restrict potential NDV infection mediated by the H5 HA to the respiratory tract of the host. Conversely, expression of the foreign protein is not involved in the replication of the vector, and thus, there is no need for a functional match with the vector proteins. As a consequence, HA and NA genes from diverse HPAIV strains can be expressed by NDV with the expectation that the properties of the vector will remain largely unaffected. This would provide for a more predictable and therefore a more rapid response against new HPAIV strains. This contrasts with chimeric reassortant influenza viruses bearing the HA and NA of HPAIV in the background of internal genes derived from attenuated human influenza virus, a situation in which the replication of the virus depends on the compatibility between the HPAIV HA/NA and the human virus internal genes. This can result in unpredictable effects on growth, including overattenuation in vivo (24, 38). A third advantage is that NDV and other nonsegmented negative-strand RNA viruses are highly refractory to genetic exchange, which greatly reduces concerns about genetic exchange between NDV-vectored vaccines and circulating viruses (14, 61). This contrasts with live attenuated influenza virus vaccines, which have the potential to exchange gene segments with circulating influenza virus strains. A fourth advantage is that NDV is used under BSL-2 conditions, providing a significant benefit in terms of safety for lab personnel as well as reduced costs for manufacture.

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