Contributions of the Avian Influenza Virus HA, NA, and M2 Surface Proteins to the Induction of Neutralizing Antibodies and Protective Immunity

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Highly pathogenic avian influenza virus (HPAIV) subtype H5N1 causes severe disease and mortality in poultry. Increased transmission of H5N1 HPAIV from birds to humans is a serious threat to public health. We evaluated the individual contributions of each of the three HPAIV surface proteins, namely, the hemagglutinin (HA), the neuraminidase (NA), and the M2 proteins, to the induction of HPAIV-neutralizing serum antibodies and protective immunity in chickens. Using reverse genetics, three recombinant Newcastle disease viruses (rNDVs) were engineered, each expressing the HA, NA, or M2 protein of H5N1 HPAIV. Chickens were immunized with NDVs expressing a single antigen (HA, NA, and M2), two antigens (HA+NA, HA+M2, and NA+M2), or three antigens (HA+NA+M2). Immunization with HA or NA induced high titers of HPAIV-neutralizing serum antibodies, with the response to HA being greater, thus identifying HA and NA as independent neutralization antigens. M2 did not induce a detectable neutralizing serum antibody response, and inclusion of M2 with HA or NA reduced the magnitude of the response. Immunization with HA alone or in combination with NA induced complete protection against HPAIV challenge. Immunization with NA alone or in combination with M2 did not prevent death following challenge, but extended the time period before death. Immunization with M2 alone had no effect on morbidity or mortality. Thus, there was no indication that M2 is immunogenic or protective. Furthermore, inclusion of NA in addition to HA in a vaccine preparation for chickens may not enhance the high level of protection provided by HA.

Avian influenza (AI) is an economically important disease of poultry worldwide. Avian influenza virus (AIV) belongs to the genus Influenzavirus A under the family Orthomyxoviridae. The genome of AIV consists of eight segments of single-stranded, negative-sense RNA that codes for 11 proteins (PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1, and NS2/NEP). The genome is surrounded by the viral envelope that has two glycoprotein spikes on its outer surface, hemagglutinin (HA) and neuraminidase (NA). The HA spikes have receptor binding and fusion functions, and NA spikes have receptor-destroying activity. The envelope also contains a third integral membrane protein, M2, which is exposed on the outer surface and functions as an ion channel, essential for uncoating. The AIV surface glycoproteins are antigenically variable and are serologically divided into 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes, whereas the nonglycosylated surface protein M2 is highly conserved (9, 43). On the basis of severity of disease in poultry, AIV strains are also classified into low-pathogenic (LP) and highly pathogenic (HP) categories. Historically, highly pathogenic avian influenza viruses (HPAIV) of subtypes H5 and H7 have caused severe disease and mortality in poultry. Recent HPAIV subtype H5N1 infections have resulted in the culling or death of more than 500 million poultry in more than 62 countries (27). Since 1997, HPAIV strains of subtype H5N1 have been found to cause disease in humans. To date, this virus has caused 436 confirmed human infections. Of these infections, 262 (60%) were fatal. Hence, HPAIV has become a major threat to both animals and humans (45). The World Organisation of Animal Health (OIE) recommends the control of HPAIV at its poultry source to decrease the viral load in susceptible avian species, thereby decreasing the risk of transmission to humans (31). The traditional method of control of HPAI has been stamping out infected flocks, which is still used in many countries, including the United States. But, due to economic reasons, culling of infected flocks is no longer considered a practical method for the control of AI in either developed or developing countries. Vaccination has been recommended by the OIE to control AI (31). Several vaccination strategies, including inactivated and live attenuated vaccines, have been evaluated for HPAIV (28). Inactivated vaccines are not commonly used because of the high cost and the difficulty in “differentiating infected from vaccinated animals” (DIVA). Live attenuated vaccines are not used because of the concern that the vaccine viruses may, through either mutation or genetic reassortment with circulating strains, become virulent (1). To overcome these difficulties, recombinant DNA technology was used to generate vectored, subunit, or DNA vaccines. Although several of these vaccines have been shown experimentally to protect against AIV, Newcastle disease virus (NDV)-vectored vaccines have shown the most promising results and also have the advantage of being bivalent vaccines against both NDV and AIV (11, 25, 32, 42). Furthermore, NDV-vectored vaccines have also been evaluated in primates.
with promising results (6). Newcastle disease (ND) is an economically important disease in poultry worldwide. The causative agent (NDV) is a nonsegmented, negative-strand RNA virus belonging to the genus Avulavirus in the family Paramyxoviridae. NDV strains vary greatly in virulence. Virulent NDV strains cause a severe respiratory and neurologic disease in poultry worldwide. Naturally occurring avirulent NDV strains are routinely used to control ND in many parts of world (30).

We recently evaluated recombinant NDV (rNDV) expressing the HA protein of an H5N1 HPAIV vaccine (rNDV-HA) in chickens (25). Chickens immunized with rNDV-HA produced NDV- and HPAIV H5-specific antibodies and were protected against clinical disease after challenge with virulent NDV or HPAIV. Furthermore, shedding of the challenge virus was not observed, indicating complete protection. Our results demonstrated that rNDV-HA is a suitable bivalent vaccine against NDV and AIV (25). To date, all NDV-vectored vaccine studies in chickens have used HA genes derived from various HPAIV strains (11, 25, 32, 42). However, in addition to the HA protein, the envelope of AIV contains two other proteins (NA and M2) on its outer surface. Although antibodies to NA are thought not to play any role in viral attachment and penetration of the host cell, they prevent the release of virus from infected cells (20) and increase overall resistance to AIV infection in humans (37). The NA gene is thought to evolve at a lower rate than the HA gene, indicating that NA-specific antibodies may increase the breadth of protection of the HA-specific antibodies (19). The other surface protein, M2, functions as an ion channel protein and also as a target for anti-HPAIV drugs. The role of M2 protein in the induction of HPAIV-neutralizing antibodies and protective immunity is not well understood. Antibodies induced by the M2e peptide corresponding to the N-terminal 24-amino-acid ectodomain (the portion present on the virus surface) displayed broad protection against influenza A viruses of both homologous (H1N1) and heterologous (H3N1) strains in vitro and in vivo (7). However, the role of entire length of the M2 protein of AIV in induction of neutralizing antibodies and protective immunity against highly pathogenic H5N1 influenza virus in chickens has not been directly evaluated. The M2 protein is conserved among all influenza A viruses and is therefore considered an attractive target for a “universal” vaccine (8). Antibodies to HA protein alone can protect against lethal AIV challenges; the inclusion of other surface proteins in the vaccine regimen may improve the protective efficacy.

In the present study, we examined the relative contribution of each of the three HPAIV surface proteins (HA, NA, and M2) to induction of neutralizing antibodies and protective immunity in chickens. Recombinant NDV vectors were constructed that individually expressed each of the three HPAIV surface proteins. They were used to immunize chickens either individually or in different possible combinations. Evaluation of the relative neutralization titers of serum antibody, shedding of challenge virus, and protection against lethal HPAIV challenge conferred by each of the NDV-vectored HPAIV surface proteins showed that HA glycoprotein was the major contributor to induction of neutralizing antibodies and protective immunity, followed by NA protein, which conferred partial protection. The M2 protein neither induced a detectable level of serum-neutralizing antibodies nor protected chickens from the HPAIV lethal challenge.

MATERIALS AND METHODS

Viruses and cells. The HPAIV strain A/Vietnam/1203/2004 (H5N1) was obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, GA). The recombinant live attenuated influenza virus (SalWF10/02/H1N1) containing the modified HA gene (deleted polybasic cleavage site) and the NA gene of virus strain A/Vietnam/1203/2004 (H5N1) was described previously (38). The recombinant version of the avirulent NDV strain LaSota was generated previously in our laboratory (14, 36). The viruses were propagated in 9-day-old, specific-pathogen-free (SPF) embryonated chicken eggs. The MDCK (Madin-Darby canine kidney), HEP-2 (human epidermoid carcinoma), and DF1 (chicken embryo fibroblast) cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). MDCK and HEP-2 cells were grown in Dulbecco’s minimal essential medium (DMEM) with 10% FBS and maintained in DMEM with 5% FBS.

Virus titration. The titers of stock preparations of rNDV were determined by a plaque assay in DF1 cells using a 0.8% methylcellulose overlay and 5% allantoic fluid. The infected cells were incubated at 37°C for 3 to 4 days until the development of plaques was apparent. The cell monolayers were then fixed with methanol and stained with crystal violet for the enumeration of plaques. Titration of rNDVs and AIVs following in vitro or in vivo growth was performed by limiting dilution in DF1 and MDCK cells, respectively, using the Reed and Muench method as described previously (13, 14). The titers are expressed as 50% tissue culture infectious doses (TCID50) units/ml. For both NDVs and AIVs, HA titers were determined using chicken red blood cells (RBC) (29). Fifty percent egg infective dose (EID50) values for rNDVs were determined by infecting five eggs per group for each 10-fold serial dilution. Following 24 h of infection, eggs were harvested for allantocoeic fluid, and the presence of virus was confirmed by an HA test. For HPAIV challenge viruses, the chicken 50% lethal dose (LC50) was determined by infecting three (5-week-old) chickens per group, and the 50% end point was determined by the Reed and Muench method (13).

Generation of rNDVs containing HPAIV HA, NA, and M2 coding sequences. The rNDV constructs were based on a full-length cDNA of the antigenic RNA of NDV strain LaSota (14, 36) that was modified to contain a unique PmeI restriction enzyme site between the P and M genes (36). The viruses were propagated in 9-day-old, embryonated eggs and HA titers were determined using chicken red blood cells (29). Fifty percent egg infective dose (EID50) values for rNDVs were determined by infecting five eggs per group for each 10-fold serial dilution. Following 24 h of infection, eggs were harvested for allantocoeic fluid, and the presence of virus was confirmed by an HA test. For HPAIV challenge viruses, the chicken 50% lethal dose (LC50) was determined by infecting three (5-week-old) chickens per group, and the 50% end point was determined by the Reed and Muench method (13).
peptide of the N1 NA protein (Prosci Inc., Poway, CA). To examine the incorporation of AIV surface proteins into NDV particles, Western blot analysis was carried out using partially purified virus from allantoic fluid of rNDV-infected eggs and the same two antisera.

Pathogenicity of rNDVs in embryonated chicken eggs. The pathogenicity of the rNDVs was determined by mean embryo death time (MDT) test in 9-day-old embryonated chicken eggs according to a standard protocol (30). Briefly, 10-fold serial dilutions of fresh infective allantoic fluid ranging from a dilution of 10⁻⁶ to 10⁻⁹ were made with sterile phosphate-buffered saline. A total of 0.1 ml of each dilution was injected into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs (Bee Eggs Company, PA) and incubated at 37°C. Each egg was examined at 12-h intervals for 7 days, and the time of embryo death was recorded. The minimum lethal dose is the highest virus dilution that causes all embryos inoculated with that dilution to die.

Growth characteristics of the rNDVs in DF1 cells. The multicycle kinetics of rNDV, rNDV-HA, rNDV-NA, and rNDV-M2 were determined in DF1 cells and embryonated eggs. DF1 cells in duplicate wells of six-well plates were infected with viruses at an MOI of 0.01 PFU. After 1 h of adsorption, the cells were washed with DMEM and then covered with DMEM containing 5% FBS and 5% allantoic fluid. The cell culture supernatants were collected and replaced with an equal volume of fresh medium at 8-h intervals until 56 h postinfection. The titers of virus in the samples were quantified by TCID₅₀ assay in DF1 cells. Whereas the growth kinetics of rNDVs in embryonated chicken eggs was performed by inoculating 100 PFU of each virus, the allantoic fluid was harvested from three eggs each at 12-h intervals until 60 h. The virus titer from each egg was determined by a TCID₅₀ assay in DF1 cells.

Immunization and challenge experiments in chickens. The immunization and challenge experiments were performed in two phases. In the first phase of the experiment, 4 groups (n = 13 per group) of 2-week-old SPF chickens were immunized by the oculonasal route with a dose of 10⁶ EID₅₀ of rNDV (empty vector), rNDV-HA, and rNDV-NA and a mixture of 10⁶ EID₅₀ each of rNDV-HA and rNDV-NA (rNDV-HA+NA). In the second phase of the experiment, 4 groups (n = 13 per group) of 2-week-old SPF chickens were immunized through the same route with a dose of 10⁶ EID₅₀ of rNDV-M2, a mixture of 10⁶ EID₅₀ each of rNDV-HA and rNDV-NA (rNDV-HA+NA+M2). The chickens were immunized with a single dose of rNDVs in a total volume of 0.2 ml (0.05 ml in each eye and nostril). Three weeks postimmunization, prechallenge serum samples were collected for serum antibody response, and the animals were challenged through the intranasal route with 100 CLD₅₀ of the homologous HPAIV A/Vietnam/1203/04 virus. Three chickens from each group were sacrificed on day 3 postchallenge for quantitation of challenge virus replication. Tissue samples were collected from the respiratory tract, including the trachea, nasal turbinates (upper respiratory tract), and lungs (lower respiratory tract), as well as from the lymphoid system (spleen) and nervous system (brain). The tissues were homogenized in cell culture medium (1 g/10 ml) and clarified by centrifugation. The virus titers in organs were determined by limiting dilution. The remaining 10 chickens in each group were observed daily for 10 days for disease symptoms and mortality following challenge. To monitor shedding of the challenge HPAIV, oral and cloacal swabs were collected on day 3 postchallenge for titration of challenge virus replication. Statistical analysis. Statistical significance differences in serological analysis of different immunized chicken groups were evaluated by one-way analysis of variance (ANOVA). The survival patterns and median survival times were compared using the log-rank test and chi-square statistics. In the log-rank test, survival curves compare the cumulative probability of survival at any specific time and the assumption of proportional deaths per time is the same at all time points. Survival data and one-way ANOVA were analyzed with the use of Prism 5.0 (GraphPad Software Inc., San Diego, CA) with a significance level of P < 0.05.

RESULTS

Generation of rNDVs expressing the H5N1 HPAIV HA, NA, and M2 surface proteins. The individual contributions of each of the three HPAIV surface proteins (HA, NA, and M2) to immunogenicity and protection were evaluated by separately expressing each protein from an addition gene inserted into the NDV-avirulent vaccine strain LaSota by reverse genetics. rNDV-HA was constructed in previous work (25), while the rNDV-NA and rNDV-M2 viruses were constructed in the present study. The HPAIV genes were derived from the H5N1 A/Vietnam/1203/2004 strain. Each ORF was placed under the control of a set of NDV transcriptional signals and inserted between the P and M genes in the NDV vector (Fig. 1A). The recombinant viruses were recovered using a previously described reverse genetic system (36). The structure of each HPAIV gene in the genome of these three viruses was confirmed by reverse transcription-PCR (RT-PCR) and nucleotide sequence analysis (data not shown).

The expression of the HPAIV surface proteins by rNDV-HA, rNDV-NA, and rNDV-M2 in infected DF1 cells was analyzed by Western blotting using a chicken antiserum generated by infection with H5N1 HPAIV (Fig. 1B, top) and a rabbit antisera specific to a C-terminal peptide of the N1 NA protein (Fig. 1B, bottom). Analysis with the polyclonal HPAIV-specific chicken antiserum detected an M2 protein band with
an apparent molecular weight of ~12,000 in lysates of cells infected with rNDV-M2 (Fig. 1B, top, lane 4), and three HA-related bands in lysates of cells infected with rNDV-HA (Fig. 1B, top, lane 2), representing (i) the inactive HA precursor HA0, with an apparent molecular weight of ~65,000; (ii) the N-terminally derived proteolytic cleavage product HA1, with an apparent molecular weight of ~40,000; and (iii) the C-terminally derived proteolytic cleavage HA2, with an apparent
molecular weight of ~25,000. Surprisingly, the postinfection chicken antiserum did not detect any apparent HPAIV-specific band in lysates of cells infected with rNDV-NA (Fig. 1B, top, lane 3). However, using the NA-specific antipeptide rabbit antiserum, the NA protein with a molecular weight of ~50,000 was detected in lysates of cells infected with rNDV-NA (Fig. 1B, bottom, lane 3).

To examine the incorporation of the HPAIV surface proteins into NDV particles, each of the rNDVs was partially purified from infected allantoic fluid by sucrose gradient centrifugation and analyzed by Western blotting (Fig. 1B, top and bottom, lanes 5 to 8). Analysis with the postinfection chicken antiserum did not detect either the NA or M2 protein in virus preparations of rNDV-NA and rNDV-M2, respectively, and detected the HA1 and HA2 proteins in the virus preparation of rNDV-HA (Fig. 1B, top, lane 6). Western blot analysis with NA-specific rabbit anti-peptide antibodies detected the NA protein in the purified virus preparation of rNDV-NA (Fig. 1B, bottom, lane 7). These results indicated that cleaved HA and NA, but not M2 or HA0, were packaged in the NDV vector particle.

Biological characterization of rNDV expressing HPAIV surface proteins. The multicycle growth kinetics of rNDV-HA, rNDV-NA, and rNDV-M2 were compared with those of the empty rNDV vector in both embryonated chicken eggs (Fig. 2A) and DF1 chicken fibroblast cells (Fig. 2B). Our results demonstrated similar kinetics of growth between the rNDV empty vector and rNDV-HA, whereas rNDV-NA and rNDV-M2 grew slightly more slowly and achieved maximum titers that were approximately 1 log_{10} lower (Fig. 2).

The virulence of these rNDVs was evaluated by the mean death time (MDT) assay in embryonated chicken eggs. The MDTs for these viruses were 110 h (rNDV-HA), 112 h (rNDV-NA), and 111 h (rNDV-M2). According to OIE guidelines, an NDV strain with an MDT value of more than 90 h is considered lentogenic or avirulent (30). Thus, the three rNDVs expressing the HPAIV surface proteins are avirulent viruses, and expression of the HPAIV protein did not increase their virulence. Indeed, the presence of each HPAIV gene marginally increased the attenuation of the NDV vector.

Evaluation of NDV- and HPAIV-specific serum antibody responses following immunization with rNDVs expressing HPAIV surface proteins. To evaluate the immunogenicity of the individual HPAIV surface proteins, groups of chickens were infected oculonasally with rNDVs individually or in several combinations. NDV-specific serum antibody responses were determined on day 21 postimmunization using an NDV-specific ELISA (Fig. 3A) and an NDV-specific HI assay (Fig. 3B). High levels of NDV-specific serum antibodies were detected by both assays, indicating replication of the rNDVs in chickens. We found only minor differences in titers between these two assays. HPAIV-specific serum antibody responses induced by the rNDVs were measured by the HI assay for HA-specific responses (Fig. 3C) and the NAI assay for NA-specific responses (Fig. 3D). Western blot analysis was performed to detect M2-specific antibody responses (Fig. 4). All four of the chicken groups whose immunization regimen included rNDV-HA had detectable AIV HI responses. The chicken group immunized with rNDV-HA alone had the highest HI titer, whereas the titer was twofold less for chickens immunized with rNDV-HA+NA and four- to eightfold less for chickens immunized with rNDV-HA+M2 or rNDV-HA+NA+M2. An NAI assay was carried out using the attenuated recombinant AIV strain 6attWF10:2H5N1 as the source of N1 NA activity. Control NAI assays performed using sera from chickens immunized with the empty rNDV vector were negative, showing that this AIV NA did not have any cross-reactivity with the NDV-specific neuraminidase of the HN protein. All four of the chicken groups whose immunization included rNDV-NA had detectable AIV NAI responses. The mean NAI antibody titer induced by rNDV-NA alone was identical to that induced by rNDV-HA+NA (Fig. 3D). However, inclusion of rNDV-M2, either in a double combination (rNDV-NA+M2) or a triple combination (rNDV-HA+NA+M2) resulted in a two- to fourfold decrease. Thus, inclusion of rNDV-M2 with rNDV-HA or rNDV-NA in a double or triple combination reduced the induction of HI or NAI antibody. Although we lacked a functional assay to detect AIV M2-specific antibodies, seroconversion to M2 protein was demonstrated by Western blot analysis. M2-specific antibodies were detected in sera of all of the chickens immunized with rNDV-M2 alone as well as in sera from other chicken groups where rNDV-M2 was included as part of a combination (Fig. 4). The antibody response to the M2 protein appeared to be substantially greater with the individual rNDV-M2 vector (Fig. 4, lanes 5 to 13) than when rNDV-M2 was combined with rNDV-NA, rNDV-HA, or rNDV-HA-NA (lanes 2, 3, and 4, respectively).

Evaluation of HPAIV-neutralizing serum antibody responses in response to rNDVs expressing HPAIV surface proteins. The ability of sera taken from chickens 21 days after immunization with rNDVs individually or in combination to...
neutralize H5N1 HPAIV was assessed by a microneutralization assay (Fig. 5). Sera from birds immunized with the rNDV empty vector or rNDV-M2 did not have detectable neutralizing antibody titers, whereas sera from birds immunized with rNDV-HA or rNDV-NA individually or together induced substantial titers of HPAIV-neutralizing antibodies, with the titer being fourfold greater for the rNDV-HA (and rNDV-HA+NA) group than for the rNDV-NA group. Inclusion of rNDV-M2 with rNDV-NA or rNDV-HA resulted in a four- or eightfold decrease in neutralizing antibody titer compared to rNDV-NA or rNDV-HA alone, respectively. These results confirmed that both HA and NA are independent neutralization antigens. In contrast, rNDV-M2 did not induce a detectable neutralizing antibody response, and inclusion of rNDV-M2 in combination with rNDV-HA and/or rNDV-NA reduced their immunogenicity.

Protective efficacy of rNDVs against HPAIV challenge virus replication. The chickens that had been immunized with the various rNDVs individually or in combination were challenged on day 21 postimmunization with a highly lethal dose (100 CLD$_{50}$) of the homologous H5N1 HPAIV A/Vietnam/1203/2004 virus. On day 3 postchallenge, three chickens from each group were sacrificed and tissue was harvested from the respiratory tract (upper trachea, nasal turbinates, and lungs), lymphoid system (spleen), and nervous system (brain) of each chicken, and the HPAIV challenge virus titers were determined by limiting dilution (Fig. 6). All of the animals in the rNDV empty vector and rNDV-M2 group died by day 2 post-
challenge (see Fig. 8). In these groups, tissue samples were harvested from the three chickens at death, rather than at day 3 postchallenge. HPAIV challenge virus replication was highest in chickens that had been immunized with the rNDV empty vector or rNDV-M2, with no apparent difference between the two groups, whereas no HPAIV replication was detected in any of the tissues from chickens immunized with rNDV-HA or rNDV-HA + M2. In the rNDV-NA-immunized group, there was a substantial reduction in challenge HPAIV titer compared to the rNDV empty vector group in the lungs (a mean reduction of 9.0 log10), trachea (a mean reduction of 7.2 log10), nostrils (a mean reduction of 4.25 log10), and spleen (a mean reduction of 7.5 log10), whereas replication in the brain was only 10-fold reduced in mean titer. The inclusion of rNDV-M2 with either rNDV-HA or rNDV-NA resulted in reduced efficacy compared to either of the latter two constructs alone. One of the chickens from the rNDV-HA + M2 group and two from the rNDV-HA + NA + M2 group lacked detectable challenge HPAIV replication, which indicated variability in the response but was consistent with HA being the most protective antigen.

Shedding of HPAIV challenge virus was monitored by taking oral and cloacal swab samples on day 3 postchallenge from the remaining 10 animals in each group. The frequency with which animals shed HPAIV challenge virus was determined by inoculation of embryonated chicken eggs and MDCK cell monolayers with swab samples, and HA assays were performed to confirm that the isolate was HPAIV. In a number of instances, embryonated eggs provided the more sensitive assay. All of the birds immunized with rNDV, rNDV-M2, rNDV-NA, and rNDV-NA + M2 were positive for both oral and cloacal shedding when the samples were assayed using embryonated eggs. No HPAIV shedding was observed from any bird in the rNDV-HA and rNDV-HA + NA groups. For chickens immunized with rNDV-HA + M2 and rNDV-HA + NA + M2, 7 and 6 out of 10 birds had HPAIV oral shedding, respectively, and 2 out of 10 birds had cloacal shedding.

The magnitude of viral shedding was determined by a limiting dilution assay of swab samples (Fig. 7). The oral shedding virus titers (Fig. 7A) were high for chickens immunized with the empty rNDV vector, as expected, and were not significantly reduced in chickens immunized with rNDV-M2, rNDV-NA, or rNDV-NA + M2. In contrast, no oral shedding was detected in the rNDV-HA or rNDV-HA + NA group. Low titers of oral shedding were detected in the rNDV-HA + M2 and rNDV-HA + NA + M2 groups, indicating that the efficacy of rNDV-HA (and rNDV-HA + NA) was reduced when combined with rNDV-M2 (Fig. 7A). The titers of cloacal shedding in the rNDV empty vector and rNDV-M2-immunized groups were higher (1 to 2 log10) than the titers of orally shed virus, indicating it had spread through the gastrointestinal tract (Fig. 7B). The rNDV-HA and rNDV-HA + NA groups had no detectable cloacal shedding, but the inclusion of rNDV-M2 resulted in detectable cloacal shedding, indicating that this inclusion resulted in a decrease in efficacy. Interestingly, whereas the level of oral shedding in the rNDV-NA- and rNDV-NA + M2-immunized chicken groups was high and indistinguishable from that of the rNDV empty control group, as noted above, the level of cloacal shedding by these two groups was very low. Thus, the ability of rNDV-NA to confer restriction of challenge virus replication was substantially greater.
against systemic spread than against replication near the site of inoculation.

Protective efficacy of rNDVs against death from HPAIV challenge. In the immunization and lethal challenge experiment described above, the remaining 10 chickens in each group were monitored following challenge to determine the total number of survivors (Table 1) and monitor the kinetics of death (Fig. 8). All of the chickens in groups immunized with rNDV empty vector or with rNDV-M2 died by day 2, with no evidence of protective efficacy associated with rNDV-M2. All of the animals immunized with rNDV-NA or rNDV-NA/H11001 died by day 5 or 6, respectively, indicating that immunity to NA extended survival but did not prevent death. In contrast, all of the chickens immunized with rNDV-HA or rNDV-HA+NA survived. The protective efficacy of these later two vaccine preparations was somewhat decreased by the further inclusion of rNDV-M2 (rNDV-HA+M2 and rNDV-HA+NA+M2), resulting in 83% and 77% survival, respectively (Fig. 8).

Comparison of pre- and postchallenge HPAIV-specific serum antibody responses by the DIVA strategy. We compared the serum antibody responses to HPAIV proteins in sera collected immediately before challenge (day 21 postimmunization) versus sera collected from survivor chickens on day 10 postchallenge. The only groups that contained survivors were

FIG. 7. HPAIV challenge virus shedding titer as determined by TCID$_{50}$ assay in oral (A) and cloacal (B) swabs collected from chicken groups immunized with rNDVs. The virus titers are expressed as means ± SEM, and the sample titers above the dotted line (1.0 log$_{10}$) were considered positive. No virus was detected in the rNDV-HA and rNDV-HA+NA groups.
TABLE 1. NDV- and HPAIV-specific serum antibody responses following immunization with rNDVs and survival of chickens after homologous HPAIV challenge

<table>
<thead>
<tr>
<th>Immunized chicken group</th>
<th>Mean NDV serum HI antibody ± SEM (log$_2$)</th>
<th>No. of birds seropositive for HPAIV/total no. of birds</th>
<th>Mean HPAIV serum HI antibody ± SEM (log$_2$)</th>
<th>No. of survivors on day 21 following challenge with the HPAIV/total no. of birds</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNDV (10)</td>
<td>5.69 ± 0.63</td>
<td>0/10</td>
<td>0</td>
<td>0/10</td>
</tr>
<tr>
<td>rNDV-HA (10)</td>
<td>6.30 ± 0.48</td>
<td>10/10</td>
<td>5.07 ± 0.95</td>
<td>10/10</td>
</tr>
<tr>
<td>rNDV-NA (10)</td>
<td>6.21 ± 0.80</td>
<td>10/10</td>
<td>4.38 ± 0.86</td>
<td>10/10</td>
</tr>
<tr>
<td>rNDV-HA+NA (10)</td>
<td>6.46 ± 0.87</td>
<td>10/10</td>
<td>0</td>
<td>0/10</td>
</tr>
<tr>
<td>rNDV-M2 (10)</td>
<td>5.30 ± 0.75</td>
<td>10/10</td>
<td>1.69 ± 1.65</td>
<td>6/10</td>
</tr>
<tr>
<td>rNDV-HA+M2 (10)</td>
<td>5.15 ± 0.68</td>
<td>10/10</td>
<td>0</td>
<td>0/10</td>
</tr>
<tr>
<td>rNDV-NA+M2 (10)</td>
<td>5.38 ± 0.65</td>
<td>10/10</td>
<td>2.84 ± 1.21</td>
<td>4/10</td>
</tr>
<tr>
<td>rNDV-HA+NA+M2 (10)</td>
<td>5.23 ± 0.83</td>
<td>10/10</td>
<td>0</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*Samples taken on day 21.*

rNDV-HA (10 survivors), rNDV-HA+NA (10 survivors), rNDV-HA+M2 (6 survivors), and rNDV-HA+NA+M2 (4 survivors), and thus the analysis was limited to these groups. We used a commercially available ELISA assay that was specific to the AIV NP protein in order to differentiate between infected birds (in which an AIV NP-specific response could occur) and birds vaccinated with an NDV-based vector (which would not stimulate antibodies against AIV NP) (Fig. 9A). This is the basis for the DIVA strategy. Pre- and postchallenge sera also were analyzed by AIV-specific HI (Fig. 9B) and NAI (Fig. 9C) assays.

Both the pre- and postchallenge sera from rNDV-HA-vaccinated birds were negative for AIV NP antibody by AIV NP ELISA (Fig. 9A), suggesting that challenge HPAIV replication did not occur or was too restricted to be immunogenic. In contrast, the postchallenge sera for 1 out of 10 chickens in the rNDV-HA+NA group, 3 out of 6 survivor chickens in rNDV-HA+M2 group, and 2 out of 4 survivor chickens in the rNDV-HA+NA+M2 group were positive for AIV NP-specific antibodies. This was indicative of breakthrough challenge HPAIV replication in these individual birds. Consistent with this, there was essentially no difference in the titers of HI antibodies in rNDV-HA-immunized chickens before and after challenge (Fig. 9B), implying a lack of HPAIV replication. In contrast, there were postchallenge increases in mean HI antibody titers of threefold, sixfold, and eightfold in the rNDV-HA+NA, rNDV-HA+M2, and rNDV-HA+NA+M2 groups, respectively, consistent with replication of the HPAIV challenge virus. Similarly, the mean postchallenge NAI titer increased fourfold and eightfold in the rNDV-HA+NA and rNDV-HA+NA+M2 groups, respectively, consistent with challenge HPAIV replication. The individual animals that had increased HI titers or NAI titers also had detectable NP antibodies. This provided further evidence of the high degree of restriction of challenge HPAIV replication afforded by rNDV-HA.

**DISCUSSION**

Circulating H5N1 HPAIV has shown increased genetic and antigenic diversity, dissemination in birds, and persistence in reservoir avian hosts. H5N1 HPAIV is also a major concern for public health due to its cross-species transmission from poultry to humans and high virulence in infected humans. Vaccination of poultry against HPAIV can play an important role in inducing immunity for infection, reducing virus shedding, and decreasing transmission to the human population. Due to the limitations of inactivated and live attenuated AIV vaccines, other approaches including nucleic acid, subunit, and vectored vaccines have been tested. Among these strategies, a promising strategy is the use of vaccine strains of NDV as a vector to express the HPAIV HA protein from an added gene (11, 25, 32, 35, 42). Newcastle disease is an economically important disease of poultry, and naturally occurring avirulent strains of NDV are widely used as live attenuated vaccines in many countries. Therefore, the use of an avirulent strain of NDV as a vector to express protective antigens of HPAIV would provide a bivalent vaccine against these two important poultry pathogens. Like HPAIV, NDV is a respiratory pathogen of chickens and has similar cellular and tissue tropisms, and thus is a highly suitable vaccine vector. Other advantages include a low price per dose, ease of administration, and the ability to...
antibody titers were expressed as the mean reciprocal log2 titer. ELISA (A) titers were measured by S/P ratio, and values above 0.3 (dotted line) were considered positive. In the AIV HI (B) and NAI (C) assays, the HPAIV-specific serum antibody responses titers were measured by AIV NP ELISA (A), HI assay (B), and NAI assay (C). The AIV NP ELISA (A) titer were measured by S/P ratio, and values above 0.2 (dotted line) were considered positive. In the AIV HI (B) and NAI (C) assays, antibody titers were expressed as the mean reciprocal log, titer ± SEM. Titers above 1.0 log, were considered positive. The statistical difference between the chicken groups were analyzed by one-way ANOVA, and the P values were <0.001 for AIV NP ELISA and <0.0001 for the AIV HI and NAI assays.

FIG. 9. Comparison of prechallenge (day 21) and postchallenge (day 31) HPAIV-specific serum antibody titers determined from surviving birds from the following immunization groups: rNDV-HA (n = 10), rNDV-HA+NA (n = 10), rNDV-HA+M2 (n = 8), and rNDV-HA+NA+M2 (n = 8). The HPAIV-specific serum antibody responses titers were measured by AIV NP ELISA (A), HI assay (B), and NAI assay (C). The AIV NP ELISA (A) titer were measured by S/P ratio, and values above 0.3 (dotted line) were considered positive. In the AIV HI (B) and NAI (C) assays, antibody titers were expressed as the mean reciprocal log, titer ± SEM. Titers above 1.0 log, were considered positive. The statistical difference between the chicken groups were analyzed by one-way ANOVA, and the P values were <0.001 for AIV NP ELISA and <0.0001 for the AIV HI and NAI assays.

distinguish between infected and vaccinated animals by assaying antibody responses to HPAIV proteins not present in the NDV-vectored vaccine.

Our goal is to develop an NDV-vectored vaccine for poultry that will produce broad and robust immunity against HPAIV H5N1 infections. We and others have previously generated NDV-vectored vaccines expressing the HA protein of H5N1 HPAIV (11, 25, 32, 35, 42). Chickens vaccinated with these vaccines were completely protected against HPAIV challenge. These initial studies focused on the HA protein, since this is considered to be the most protective AIV antigen. However, AIV also encodes two other surface antigens, namely, the NA and M2 proteins. The role of NA or M2 in immunogenicity and protection was not well understood in the context of either natural infection or a vectored vaccine. Therefore, we have used NDV as a vector to compare the relative contributions of each of the three HPAIV surface proteins (HA, NA, and M2) to immunogenicity and protection in chickens that were immunized and challenged with HPAIV. We prepared rNDVs individually expressing the HPAIV HA, NA, or M2 protein. The recombinant viruses grew efficiently in embryonated eggs and DF1 cells. Western blot analysis showed that each of the three HPAIV proteins was expressed in DF1 cells infected with the different recombinant viruses. Both the HA and NA proteins were found to be incorporated into the envelope of NDV. Similar incorporation into the vector particle had been previously noted for recombinant vesicular stomatitis virus expressing HA or NA of human influenza virus (22). Importantly, incorporation of the HPAIV surface proteins into NDV particles did not increase the virulence of the vector in a standard test in embryonated eggs, showing that the expression of HPAIV surface proteins by NDV does not pose a biosafety hazard. The fact that nonsegmented negative-strand viruses have negligible rates of genetic exchange, in contrast with attenuated influenza vaccine viruses, provides an additional safety factor.

In this study, intranasal immunization with rNDV-HA induced high levels of HPAIV-specific HI and neutralizing serum antibodies and completely protected chickens against a potent challenge with HPAIV. Antibodies to the influenza virus HA protein block virus attachment, thereby protecting cells from infection (39), and likely played a major role in the observed protective immunity. Results from the rNDV-HA-immunized animals did not support that there was any detectable challenge HPAIV replication, whether assayed by shedding, direct analysis of necropsied tissue, or postchallenge antibody increases, indicating a very high level of restriction. Furthermore, all animals were protected against illness and death. Thus, consistent with previous studies (11, 25, 32, 35, 42), the HA protein is a major independent neutralization and protective antigen, and a single immunization with an NDV-vectored vaccine expressing HA alone induced a strong serum-neutralizing antibody response and complete protection against challenge with HPAIV.

Antibodies to the NA protein can impede its receptor-degrading function, thereby preventing release of progeny virions from infected cells (39). However, the individual contribution of NA-specific antibodies to immunogenicity and protection against HPAIV remained unclear. DNA vaccines encoding NA protein provided protection against AIV challenge in mice, a nonnatural host (2, 3). McNulty et al. (24) showed that vaccination of chickens with influenza virus provided protection against challenge with a virus of the same NA subtype and an unrelated HA subtype. However, in other studies, recombinant NA protein, DNA vaccines encoding the NA protein, or alphavirus-based virus-like particles containing the NA protein provided partial protection to lethal challenge in...
chickens (40, 44). In the present study, rNDV expressing the NA protein induced high levels of HPAIV-specific NAI and neutralizing serum antibodies. It is thought that NA-specific antibodies are not typically associated with a classical neutralization of virus. However, the titer of neutralizing antibodies induced by rNDV-NA was only fourfold less than that induced by rNDV-HA. Thus, the HPAIV NA protein is a substantial, independent neutralization antigen. It may be that neutralization by NA-specific antibodies is achieved indirectly: specifically, antibodies bound to NA on the surface of the HPAIV particle might cause steric hindrance of HA-mediated attachment and penetration. Despite the induction of HPAIV-neutralizing antibodies, none of the immunized chickens were spared from the HPAIV challenge, although their survival times were prolonged by 4 to 5 days compared to nonvaccinated controls. Similar results were observed when chickens were immunized with infectious laryngotracheitis virus (ILTIV)-vectored NA vaccines: none of the chickens survived the challenge infection, but their survival times were prolonged by 1 to 2 days compared to nonvaccinated controls (33). In the present study, immunization with rNDV-NA did not prevent replication of HPAIV challenge virus, but the titers were reduced by 4.3 to 9.0 log_{10} in respiratory tract tissues and the spleen and 10-fold in the brain. In addition, while oral shedding of the HPAIV challenge virus was only modestly reduced, cloacal shedding was substantially reduced, indicating that spread through the gastrointestinal tract was reduced. These results indicate that NA-specific immunity can reduce HPAIV replication and prolong survival but cannot prevent death in a highly permissive host.

The AIV M2 protein is highly conserved among influenza A virus subtypes and has been suggested to be a candidate antigen for the development of a broadly cross-reactive “universal” influenza A virus vaccine. However, the contribution of the M2 protein to immunogenicity and protection was not clear. Antibodies to the M2 protein prevent release of progeny viral particles from infected cells \textit{in vitro} (39). Passive transfer of monoclonal antibody against M2 conferred partial protection in mice against human influenza virus (41). The AIV M2 protein was found to be weakly immunogenic and did not induce neutralizing antibodies but reduced virus replication in mice, a nonnatural host (18). Various vaccine approaches have been evaluated for development of an M2 universal vaccine, including passive transfer of M2-specific antibodies (23, 41) or immunization with conjugated M2 peptide antigens (7, 26), complete M2 protein (16), or the external domain of M2 (5, 10, 16). Although these studies demonstrated protection against influenza virus challenge in the nonnatural mouse host, the role of M2 protein in immunogenicity and protection in a natural host had not been evaluated. The rNDV-M2 vaccine elicited a good serum antibody response against M2 in all the immunized chickens, especially when administered alone, but the postimmunization sera did not neutralize the homologous virus \textit{in vitro}. Following HPAIV challenge, there was no reduction in death and no prolongation of survival. Indeed, the present challenge study showed the death of one immunized chicken before the death of nonimmunized control chickens, indicating possible exacerbation of disease. Exacerbation of disease was observed in M2-immunized pigs following challenge (12). In the present study, following the HPAIV challenge of rNDV-M2-immunized animals, there was no detectable decrease in oral or cloacal shedding or decrease in HPAIV titers in necropsied tissues, indicating that there was no detectable restriction of challenge HPAIV replication. These results suggest that the HPAIV M2 protein does not play a significant role in inducing neutralizing antibodies and protection in chickens.

In addition, the combined effect of multiple HPAIV surface proteins in immunogenicity and protection was evaluated by simultaneously immunizing chickens with mixtures of equal amounts of various combinations of rNDVs expressing HPAIV surface proteins. Western blot analysis showed that antibodies to each of the AIV proteins were produced in chickens immunized with various virus combinations, and the induction of antibodies to HA and NA was independently monitored by HI and NAI assays. Of the groups with double combinations, chickens immunized with the combination of rNDV-HA and rNDV-NA elicited good antibody responses to both HPAIV proteins and were completely protected against HPAIV challenge. Neither replication nor shedding of the challenge virus was detected, indicating that the rNDV-HA+NA combination was comparable in efficacy to rNDV-HA. However, the DIVA analysis showed that HPAIV challenge of chickens immunized with rNDV-HA+NA induced detectable antibody to the HPAIV NP protein, whereas this was not observed following challenge of animals immunized with rNDV-HA alone. This suggested that some breakthrough HPAIV replication occurred when rNDV-HA was combined with rNDV-NA, indicating that the efficacy of rNDV-HA was reduced in the combination vaccine. This differs from previous studies using recombinant fowlpox virus (rFPV) and ILTV vector systems, in which expression of NA in combination with HA increased immunogenicity in chickens (33, 34). The discrepancy in results between these studies might reflect differences in the vector systems used to express the HA and NA proteins. Alternatively, perhaps the level of immunity induced by HA was so high that the contribution made by NA was masked. We also observed reduced efficacy, as well as reduced immunogenicity, when rNDV-M2 was mixed with rNDV-HA or rNDV-NA. The inclusion of rNDV-M2 resulted in a substantial reduction in the titer of HI, NAI, and HPAIV-neutralizing antibodies induced by rNDV-HA and/or rNDV-NA. Conversely, the titer of M2-specific antibodies also decreased substantially when administered in mixtures. The inclusion of rNDV-M2 with rNDV-NA did not greatly affect the replication of challenge HPAIV or the prolongation of survival induced by rNDV-NA. However, inclusion of rNDV-M2 with rNDV-HA or rNDV-HA+NA strongly reduced the level of protective efficacy in a proportion of animals, as evidenced by increased challenge HPAIV replication and decreased survival. Furthermore, when three rNDVs were given as a trivalent vaccine, the antibodies to AIV proteins (HA, NA, and M2) were not produced at the same level. Particularly, M2 antibodies were produced at a lower level than HA and NA antibodies. Presumably this is due to either interference or competition among the three rNDVs. It is possible that rNDV-HA or rNDV-NA interfered with the replication of rNDV-M2. Alternatively, there was a competition in growth among the three rNDVs. The rNDV-HA and rNDV-NA outcompeted rNDV-M2 in growth, leading to lower antibody production against M2 protein. It is also possible that the known cytotoxicity of M2 protein (4, 17)
affected the replication and immune response of rNDV-HA and rNDV-NA. This is suggested by the observation that the NDV-specific immune response to the vector also was lower for rNDV-M2 and all combinations in which it was involved. In summary, combinations of vectors in which rNDV-HA was present retained substantial immunogenicity and protective efficacy, consistent with the status of HA as the major neutralization and protective antigen, but the inclusion of rNDV-expressing the other proteins did not provide an increase in neutralizing antibodies or protective immunity. In this study, the role of cell-mediated immunity in protection has not been evaluated. It will be interesting to perform passive antibody experiments transferring serum antibody from one chicken to another to understand the role of antibodies versus cellular immune responses generated using these rNDV vectors expressing AIV antigens.

One of the major concerns of currently available HPAIV vaccines is that they do not induce sufficient immunity to completely prevent HPAIV infections and subsequent virus shedding. Although animals may be protected from severe disease and death, they can transmit HPAIV to unvaccinated flocks and to humans. Therefore, an ideal HPAIV vaccine should completely prevent any challenge virus replication. Our study showed that rNDV-HA alone fully protected chickens and completely prevented detectable HPAIV replication and shedding. Hence, these birds would not pose a threat of transmission. In summary, the findings of this study showed that the HA protein is clearly the major neutralization and protective antigen of HPAIV and the protein of choice for inclusion in a vectored HPAIV vaccine. The NA protein induced substantial titers of neutralizing antibodies to HPAIV, but the immunity was not sufficient to protect against HPAIV challenge, although it prolonged survival. The M2 protein neither induced neutralizing antibodies nor provided protection against HPAIV challenge. These findings indicate that an NDV-vectored vaccine expressing HA was superior to a combination vectored vaccine expressing HA was superior to a combination

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