Newcastle Disease Virus Expressing Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Induces Strong Mucosal and Serum Antibody Responses in Guinea Pigs

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Human immunodeficiency virus type 1 (HIV-1) is transmitted mainly through mucosal sites. Optimum strategies to elicit both systemic and mucosal immunity are critical for the development of vaccines against HIV-1. We therefore sought to evaluate the induction of systemic and mucosal immune responses by the use of Newcastle disease virus (NDV) as a vaccine vector. We generated a recombinant NDV, designated rLaSota/gp160, expressing the gp160 envelope (Env) protein of HIV-1 from an added gene. The gp160 protein expressed by rLaSota/gp160 virus was detected on an infected cell surface and was incorporated into the NDV virion. Biochemical studies showed that gp160 present in infected cells and in the virion formed a higher-order oligomer that retained recognition by conformationally sensitive monoclonal antibodies. Expression of gp160 did not increase the virulence of recombinant NDV (rNDV) strain LaSota. Guinea pigs were administered rLaSota/gp160 via the intranasal (i.n.) or intramuscular (i.m.) route in different prime-boost combinations. Systemic and mucosal antibody responses specific to the HIV-1 envelope protein were assessed in serum and vaginal washes, respectively. Two or three immunizations via the i.n. or i.m. route induced a more potent systemic and mucosal immune response than a single immunization by either route. Priming by the i.n. route was more immunogenic than by the i.m. route, and the same was true for the boosts. Furthermore, immunization with rLaSota/gp160 by any route or combination of routes induced a Th1-type response, as reflected by the induction of stronger antigen-specific IgG2a than IgG1 antibody responses. Additionally, i.n. immunization elicited a stronger neutralizing serum antibody response to laboratory-adapted HIV-1 strain MN.3. These data illustrate that it is feasible to use NDV as a vaccine vector to elicit potent humoral and mucosal responses to the HIV-1 envelope protein.

It has been 30 years since human immunodeficiency virus type 1 (HIV-1) was first identified as the causative agent of AIDS (27). Since then, more than 60 million people have been infected with HIV around the world and nearly half of these individuals have died from HIV-related causes. Although development of new antiretroviral drugs against HIV has resulted in a dramatic decrease in mortality, antiviral drugs have disadvantages that involve high cost, compliance complications, side effects, and the occurrence of drug-resistant mutant viruses. Therefore, development of an effective vaccine is still the major goal in the effort to halt the HIV pandemic. A variety of vaccine strategies to control HIV infections have been investigated. Traditional vaccine strategies, such as those employing live attenuated and inactivated vaccines, either have been ineffective or pose safety concerns (4, 17, 54, 62). Novel vaccine strategies, including those employing DNA, recombinant proteins, peptides, and replication-competent and nonreplication-competent live viral vectors in different prime-boost combinations, have been evaluated (28, 64). Several viral vectors, such as poxvirus, adenovirus, adeno-associated virus, vesicular stomatitis virus (VSV), and herpesvirus, have been evaluated for efficacy in the delivery of HIV antigens (64). Recently, canarypox virus-vectored vaccines were tested in two phase III HIV-1 vaccine trials (63). Although those trials showed limited antibody (Ab)-based protection against HIV infection, the studies showed the potential of viral vector vaccines for HIV treatment.

It is believed that humoral and cytotoxic T cell responses, at both systemic and mucosal sites, are required for protection against diverse HIV isolates. Conceptually, antibodies would serve as the first line of defense for blocking viral infection and neutralizing released progeny virus whereas cellular responses would facilitate clearance of HIV-infected CD4+ T cells. Therefore, development of immunogens that can induce broadly reactive neutralizing antibodies (NAb) is required to provide sterilizing immunity against HIV. HIV envelope (Env) glycoprotein is the major target antigen against which neutralizing antibodies are induced. The efficacy of Env-specific neutralizing antibodies in protection against HIV-1 has been demonstrated in passive transfer studies using nonhuman primates (5, 53). It has also been shown that broadly reactive monoclonal antibodies such as b12 and VRC01, 2G12, 2F5 and 4E10, and PG9 and PG16 bind to CD4, high-mannose clusters, gp41, and the second variable loop region of Env protein, respectively, suggesting that Env represents a critical region that is
ameasurable to neutralization (6, 24, 69, 72, 73). Further, broadly reactive antibodies have been identified in a small number of HIV-1 infected individuals and those antibodies were shown to be directed mainly against the CD4-binding site of Env glycoprotein (47).

HIV Env is synthesized as a 160-kDa precursor gp160 protein that is processed by furin or related cellular proteases into its soluble attachment subunit gp120 and transmembrane subunit gp41 (3). Multiple lines of evidence suggest that gp120 and gp41 are organized on virions as trimeric spikes, with three gp120 proteins noncovalently associated with three gp41 subunits (30). Env protein undergoes disulfide bond formation, extensive glycosylation, and oligomerization in the endoplasmic reticulum. The viral envelope mediates HIV infection by establishing cell contact through a gp120-host cell CD4 interaction. This interaction also stabilizes the structure of a coreceptor binding site on gp120 that engages one of two coreceptors (CCR5 or CXCR4) that serve as natural chemokine receptors (61). The viral spike corresponds to a number of characteristics that subvert cross-reactive humoral immunity, including heavy glycosylation, conformational flexibility, and sequence variability in immunodominant domains. Therefore, significant efforts have been made to design and construct either purified Env glycoprotein immunogens or vaccine vectors that present Env glycoproteins in the form of functional trimeric complexes, thereby preferentially exposing relevant neutralizing determinants to the immune system (34, 60, 64).

In the last 15 years, reverse genetic systems for many nonsegmented negative-strand RNA viruses have been developed not only to study the pathogenesis and biology of these viruses but also to engineer them as vaccine vectors. Among these, Newcastle disease virus (NDV) is a particularly suitable vaccine vector for delivery of foreign antigens. NDV is a member of the genus Avulavirus in the family Paramyxoviridae. The genome of NDV consists of a single-stranded, negative-sense RNA of 15 kb that contains six genes (in the order 3'-N-P-M-F-HN-L-5') encoding eight proteins (18, 43). Virulent NDV strains cause an economically important disease in poultry. Based on the severity of disease, NDV strains are classified into three pathotypes: lentogenic strains, which cause mild or asymptomatic infections that are restricted to the respiratory tract; mesogenic strains, which are of intermediate virulence; and velogenic strains, which cause systemic infections with high mortality (2). Currently, lentogenic strains are widely used as live attenuated NDV vaccines for poultry throughout the world.

NDV has several properties that make it useful as a vaccine vector in humans. NDV is an avian virus that is attenuated in humans due to a natural host range restriction (10). Infection of humans is uncommon but can involve mild transient conjunctivitis (19). NDV shares only a low level of amino acid sequence identity with, and is antigenically distinct from, common animal and human pathogens; thus, vaccination would not be affected by preexisting immunity. In mouse and nonhuman primate models, NDV can infect via the intranasal (i.n.) route and has been shown to induce humoral and cellular immune responses at both the mucosal and systemic levels. NDV has been used to express antigens protective against simian immunodeficiency virus (SIV), respiratory syncytial virus, HSN1 avian influenza virus, human parainfluenza virus type 3, and severe acute respiratory syndrome (SARS)-associated coronavirus in monkeys (10, 19, 20, 22, 52, 55, 56). Recently, the ability of NDV to express HIV Gag antigens and to generate a Gag-specific immune response in mice was described (12, 49). However, NDV had not previously been explored as a viral vector for expression of the envelope glycoprotein of HIV.

In this study, we evaluated the widely used avirulent NDV vaccine strain LaSota as a vector to deliver the gp160 Env protein of the CCR5-tropic clade B HIV-1 isolate BaL.1. The ability of recombinant NDV to express gp160 trimers in vitro was characterized. Further, we investigated the potential of recombinant NDV expressing gp160 to stimulate systemic and mucosal Env-specific IgG and IgA responses in guinea pigs when administered via an i.n. or intramuscular (i.m.) route. Our results showed that vaccination by the i.n. route elicited stronger Env-specific humoral and mucosal immune responses compared to i.m. immunization. We further demonstrated that recombinant NDV (rNDV)-based vaccination stimulated Th1-biased immune responses. Additionally, i.n. immunization elicited a stronger neutralizing antibody (NAb) response to laboratory-adapted HIV-1 strain MN.3 in serum.

### MATERIALS AND METHODS

#### Cells, viruses, antibodies, and protein.

Human epidermoid carcinoma (HEp-2), chicken embryo fibroblast (DF1), Vero, and 293/T17 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The TZM-bl cell line was obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). Division of AIDS, NIAID, NIH; HEP-2, DF1, and TZM-b cell lines were grown in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS. Vero cells were grown and maintained in minimum essential medium (MEM) containing 10% FBS. 293/T17 cells were grown and maintained in reduced-serum Opti-MEM I containing 10% FBS. Recombinant and wild-type NDV strains were grown in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The modified vaccinia virus Ankara strain expressing T7 RNA polymerase was grown in primary chicken embryo fibroblast cells. HIV-1 gp120 monoclonal antibodies B12 and 2G12 and purified recombinant HIV-1 BaL gp120 protein were obtained from NIH ARRRP.

#### Construction of recombinant NDVs expressing HIV-1 gp160.

We constructed plasmid pLaSota, carrying the full-length antigenic cDNA of the lentogenic NDV vaccine strain LaSota, in previous work (35, 65). Full-length, human codon-optimized HIV gp160 cDNA (2,598 nucleotides) encoding glycoprotein gp160 was amplified by PCR using a plasmid containing a human codon-optimized HIV gp160 gene (GenBank accession number DQ104392.1) that was derived from the CCR5-tropic clade B BaL.1 strain. The forward primer 5'-AGCTTTACCTTGTTTTAGAAAAATACCGGGTAGAAACGGCGCCACTATCGCCAAGTTGCTTG-3' and reverse primer 5'-AGCTTTGTTTTAAACCTTAGAAAAATACCGGGTAGAAACGGCGCCACTATCGCCAAGTTGCTTG-3' contained PmeI sites (italicized), NDV gene end and gene start transcriptional integrity of the gp160 Env gene was confirmed by sequence analysis. The insert was digested with PmeI and cloned into pCR 2.1-TOPO vector (Invitrogen). The insert was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and sequenced to confirm the absence of any adventitious mutations.

The gp160 genomic RNA of purified virus was amplified by reverse transcription-PCR (RT-PCR) and sequenced to confirm the absence of any adventitious mutations.
Expression of HIV-1 gp160 Env protein in cells infected with recombinant virus. Expression of gp160 by rLasota/gp160 was examined by Western blot analysis. Briefly, DF1 and Vero cells were infected with rLasota and rLasota/gp160 at a multiplicity of infection (MOI) of 0.01 PFU. The cells were harvested at 48 h postinfection, lysed, and analyzed by Western blotting using a 1:10 dilution of a pool of gp120-specific monoclonal antibodies. To examine the incorporation of gp160 into NDV virions, SPF embryonated chicken eggs were infected with rLasota or rLasota/gp160 and allantoic fluid was harvested at 48 h postinfection. The allantoic fluids were clarified by low-speed centrifugation, and the viruses were concentrated by ultracentrifugation with 25% (wt/vol) sucrose in phosphate-buffered saline (PBS) at 130,000 × g for 2 h and resuspended in PBS. The viral proteins in the purified virus preparations were analyzed by Western blotting using the same antibodies.

Expression of gp160 by the recombinant virus was further examined in Vero cells by an immunofluorescence assay. Briefly, confluent monolayers of Vero cells on 4-well Lab-Tek chamber slides were infected with the recombinant viruses at an MOI of 0.1. After 24 h, the infected cells were washed with PBS and either fixed with 4% paraformaldehyde for 20 min at room temperature for detection of surface antigen or fixed in the same manner and permeabilized with 0.2% Triton X-100 in PBS for 10 min for detection of total antigen. After further washing with PBS, the cells were incubated for 30 min with 3% normal goat serum to block nonspecific binding sites and incubated for 1 h with a 1:10 dilution of a pool of gp120-specific monoclonal antibodies. The cells were rinsed with PBS and incubated with a 1:1,000 dilution of Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G antibody (Invitrogen, Carlsbad, CA) for 45 min. The cells were washed with PBS and analyzed with a fluorescence microscope.

Analysis of HIV-1 gp160 protein oligomers. The oligomeric state of gp160 expressed by the NDV recombinant was analyzed by cross-linking infected cell lysates or sucrose-purified virus followed by Western blotting. Briefly, lysates of rLasota or rLasota/gp160-infected DF1 or Vero cells or purified recombinant virus in PBS were incubated at room temperature for 30 min with a final concentration of 1 mM dithiobis(succinimidyl propionate) (DSP, Pierce), a thiol-cleavable, amine-reactive, and membrane-permeative cross-linker. After cross-linking, samples were prepared in Laemmli sample buffer (100 mM Tris pH 6.8), 2% sodium dodecyl sulfate [SDS], 15% glycerol) with and without 5% ß-mercaptoethanol and boiled for 5 min to make reduced and nonreduced samples, respectively. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed as described before.

Pathogenicity of rLasota/gp160 in embryonated chicken eggs. The pathogenicity of rLasota and rLasota/gp160 for chickens was determined by an internationally established in vivo test, the mean death time (MDT) test, for 9-day-old SPF embryonated chicken eggs. The MDT test was performed by a standard procedure (1). Briefly, a series of 10-fold dilutions of fresh allantoic fluid from eggs infected with the test virus were made in sterile PBS, and 0.1 ml of each dilution was inoculated into the allantoic cavity of each of five eggs. The eggs were incubated at 37°C and examined four times daily for 7 days. The time that each embryo was first observed dead was recorded. The highest dilution that killed all embryos was considered the minimum lethal dose. The MDT was recorded as the time (in hours) required for the minimum lethal dose to kill the embryos. The MDT has been used to classify NDV strains as velogenic (taking under 60 h to kill), mesogenic (taking between 60 and 90 h to kill), and lentogenic (taking more than 90 h to kill).
dried for 2 h at 37°C. Serial dilutions of sera or vaginal washes from immunized guinea pigs were prepared in dilution buffer (Symbiotics Corporation, San Diego, CA), added to the plates, and incubated for 2 h at room temperature. The plates were washed three times with plate-washing solution (Symbiotics Corporation) and incubated for 1 h with a 1:1,000 dilution of an isotype-specific secondary antibody, namely, horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (KPL, Gaithersburg, MD), goat anti-guinea pig IgG1, goat anti-guinea pig IgG2a (Novus Biologicals, Littleton, CO), or sheep anti-guinea pig IgA (Immunology Consultants Laboratory, Newberg, OR). The plates were washed three times and developed with ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]-di-ammonium salt) peroxidase substrate solution (Symbiotics Corporation), development was stopped by the addition of peroxidase stop solution, and analysis was performed at 405 nm using an ELx800 ELISA plate reader (BioTek, Winooski, VT). ELISA endpoint titers were defined as the highest reciprocal serum dilutions at which the mean optical density (OD) values of duplicate wells were >2-fold above the mean OD value plus 2 standard deviations (SD) for sera or vaginal washes from negative-control animals. Commercial NDV ELISA kits (Symbiotics Corporation) were used to detect antibodies against the NDV antigens.

Measuring gp120-specific antibodies in serum by Western blotting. Sera from immunized guinea pigs were assayed for gp120-specific antibodies by Western blotting. Purified BaL.1 gp120 was subjected to 10% SDS--PAGE under reducing conditions and transferred to a nitrocellulose membrane. The membrane was cut into strips and incubated with a 1:200 or 1:300 dilution of day 42 serum from an immunization group, development was stopped by the addition of peroxidase stop solution, and clarified by centrifugation and filtration using a 0.45-μm-pore-size filter. Supernatant containing pseudovirus was harvested 48 h following transfection and clarified by centrifugation and filtration using a 0.45-μm-pore-size filter. Aliquots of 1.0 ml each were made and stored at -70°C. The 50% tissue culture infectious dose (TCID50) for each pseudovirus preparation was determined by infection of TZM-bl cells as previously described (46).

Neutralization assay. Virus neutralization was measured using a luciferase-based assay and TZM-bl cells as described previously (46). This assay measures neutralization as the reduction in luciferase reporter gene expression in TZM-bl cells following a single round of virus infection. Briefly, all serum samples were heat inactivated at 56°C for 1 h before use and initially diluted 1:4 in DMEM followed by 2-fold serial dilutions performed in duplicate (96-well flat-bottom plate) in DMEM (10 μl/well). B12 and 2G12, two serially diluted monoclonal antibodies, were used as positive controls. Virus (200 TCID50) was added to each well in a volume of 40 μl and the plates were incubated for 1 h at 37°C. TZM-bl cells were then added (1.5 × 105/well in a 150-μl volume) to growth DMEM containing 10% heat-inactivated FBS and DEAE-dextran (Sigma, St. Louis, MO) at a final concentration of 15 μg/ml. Assay controls included replicate wells of TZM-bl cells alone (cell control) and TZM-bl cells with virus and no antibody (virus control). Cells were incubated for 48 h at 37°C. The medium was removed from each well and replaced with 50 μl of RPMI 1640 medium (Invitrogen) and 50 μl of Bright-Glo luciferase assay reagent (Promega, Madison, WI). The cells were allowed to lose for 2 min, then 100 μl of each cell lysate was transferred to a 96-well black solid plate, and lucinescence was measured using a Victor 3 luminometer (Perkin Elmer). The 50% inhibitory dose (ID50) titer was calculated as the serum dilution that caused a 50% reduction in relative luminescence units (RLU) compared to the level in virus control wells after subtraction of the cell control RLU value.

Statistical analysis. Statistically significant differences in data from serological analysis of different immunized guinea pig groups were evaluated by one-way analysis of variance (ANOVA) with the use of Prism 5.0 (Graph Pad Software Inc., San Diego, CA) at a significance level of P < 0.05.

RESULTS

Generation of recombinant NDV expressing HIV-1 gp160. The recombinant lentogenic NDV LaSota strain containing a unique PmeI site between the P and M genes (65) was used as a vector to express the HIV-1 gp160 glycoprotein from an added gene. The HIV-1 gp160-encoding gene was amplified by PCR from a plasmid carrying a human codon-optimized HIV gp160 gene. The gp160 ORF was placed under the control of NDV transcriptional signals and inserted at the PmeI site between the P and M genes in the NDV vector (Fig. 1). The transcription cassette was designed to satisfy the rule of six, whereby the genome nucleotide length must be an even multiple of six in order to be efficiently replicated (11, 59). A Kozak sequence was inserted before the start codon of the gp160 ORF to provide efficient translation (41). The resulting plasmid, designated pLaSota/gp160, encoded an antigenome of 17,784 nt, 2,598 nt longer than that of the parental NDV LaSota strain.

The recombinant virus, designated rLaSota/gp160, was recovered using previously described methods (35). The structure of the gp160 insert in the viral genome was confirmed by RT-PCR and nucleotide sequence analysis to be correct and free of adventitious mutations (data not shown). The recombinant viruses were propagated in embryonated chicken eggs, and the titers were determined by a hemagglutinin (HA) assay. The HA titer of the rLaSota/gp160 virus was 2 log2 lower than that of the parental rLaSota virus. This result is consistent with previous findings showing that moderate attenuation of replication can result from the insertion of a foreign gene (35, 42).

To determine the stability of the gp160 gene in rLaSota/gp160, the recovered virus was subjected to five passages in embryonated chicken eggs and five passages in chicken embryo fibroblast DF-1 cells. Sequence analysis of the gp160-encoding gene of the resulting virus preparations showed that the integrity of the gp160 gene was preserved and stably maintained even after 10 passages.

Expression of the gp160 protein by recombinant NDV. Expression of the HIV-1 gp160 protein in DF1 cells (Fig. 2A) and Vero cells (Fig. 2B) infected with rLaSota/gp160 was analyzed

FIG. 2. Western blot analysis of HIV-1 gp160 expressed by rLaSota/gp160 in DF1 and Vero cells. DF1 (A) and Vero (B) cells were infected with rLaSota or rLaSota/gp160 virus at an MOI of 0.01 PFU. After 48 h, the cells were collected and processed to prepare cell lysates. The samples were subjected to Western blot analysis using a pool of gp120-specific monoclonal antibodies. The positions of HIV gp160 precursor and N-terminal cleavage product gp120 are indicated by arrows in the right margins. Molecular masses of the marker proteins (in kilodaltons) are shown in the left margins.
To determine whether the rLasota/gp160-expressed gp160 protein was incorporated into the NDV virion. The recombinant viruses were purified through sucrose gradients, and the viral proteins were analyzed by a Western blot assay. Western blot analysis of purified viruses revealed the presence of the gp120 protein band and a predominant higher-order form, migrating slower than the monomer, was present, likely representing dimers or trimers. Under nonreducing conditions, all of the gp160 protein expressed by rLasota/gp160 migrated as a high-molecular-mass product.

Incorporation of gp160 protein into the NDV virion. It has been reported that expression of foreign envelope glycoproteins by recombimant nonsegmented negative-stranded RNA viruses can result in their incorporation into the virion with various levels of efficiency (19). Therefore, we wanted to determine whether gp160 protein was incorporated into the NDV virion. The recombinant viruses were purified through sucrose gradients, and the viral proteins were analyzed by a Western blot assay. Western blot analysis of purified viruses revealed the presence of the gp120 protein band and a predominant higher-order form, migrating slower than the monomer, was present, likely representing dimers or trimers. Under nonreducing conditions, all of the gp160 protein expressed by rLasota/gp160 migrated as a high-molecular-mass product. 

By Western blotting using a pool of gp120-specific monoclonal antibodies. Immunoblot analysis detected two bands in lysates of cells infected with rLasota/gp160: these represented (i) the gp160 precursor protein, with an apparent molecular mass of ∼160 kDa, and (ii) the N-terminal cleavage product gp120 protein, with an apparent molecular mass of ∼120 kDa. The C-terminal cleavage product gp41 was not detected, as the monoclonal antibodies used in this study do not recognize it. As expected, gp160 and gp120 proteins were not detected in DF1 and Vero cells that were infected with rLasota virus.

Expression of gp160 in Vero cells infected with rLaSota/gp160 virus was also subjected to indirect immunofluorescence analysis using a pool of gp120-specific monoclonal antibodies (Fig. 3). Intracellular expression was investigated in cells that had been fixed with paraformaldehyde and permeabilized with Triton X-100 detergent. The results showed that gp160 was expressed efficiently in the cytoplasm of Vero cells by rLasota/gp160 virus at 24 h postinfection (Fig. 3, panel b). To determine whether the rLasota/gp160-expressed gp160 protein transported to the cell surface, Vero cells were infected with the rLasota/gp160 virus, fixed with paraformaldehyde, and incubated with the gp120-specific monoclonal antibodies followed by immunostaining with Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies and analyzed by immunofluorescence. The cells were visualized using a Nikon Eclipse TE fluorescence microscope. Arrows indicate areas of positive immunofluorescence.

The results of Western blot analysis demonstrated that the gp160 protein is incorporated into the heterologous NDV envelope. The functional form of the gp160 protein is oligomeric. Further, the oligomeric structure influences the antigenicity of gp160, which is the main target of the neutralizing humoral immune response of the host. Therefore, we investigated the oligomeric state of the gp160 protein expressed by rLasota/gp160 in infected DF1 and Vero cells and in purified virus particles. Cell lysates from rLasota/gp160-infected DF1 cells (Fig. 5A) and Vero cells (Fig. 5B), in parallel with sucrose gradient-purified virus (Fig. 5C), were subjected to cross-linking with DSP, to SDS-PAGE under reducing and nonreducing conditions, and to immunoblotting with gp120-specific monoclonal antibodies. The results of Western blot analysis demonstrated that the bulk of uncleaved gp160 precursor protein and cleaved gp120 protein migrated with an apparent molecular mass consistent with the presence of monomers under reducing conditions in purified virion preparations and Vero and DF1 cell lysates. A higher-order form, migrating slower than the monomer, was also present, likely representing dimers or trimers. Under nonreducing conditions, all of the gp160 protein expressed by rLasota/gp160 migrated as a high-molecular-mass product.
The higher-order form had lower electrophoretic mobility than a 188-kDa molecular mass standard and migrated mainly as a single diffuse band, indicating that gp160 occurs as one predominant oligomeric species. These data suggest that rLasota/gp160 supports the expression of one predominant oligomeric species of gp160 protein.

**Biological characterization of rNDV expressing gp160 protein.** The multicycle growth kinetics of the rLaSota and rLasota/gp160 strains were compared using DF1 chicken fibroblast cells (Fig. 6). The results showed that replication of the rLaSota/gp160 strain was slightly retarded compared to that of the parental rLaSota virus but that the viruses achieved similar maximum titers at 64 h postinfection. The pathogenicity of rLaSota/gp160 and the parental rLaSota virus in 9-day-old embryonated chicken eggs was evaluated by the MDT test. NDV strains are categorized into three pathotypes on the basis of their MDT values: velogenic (taking less than 60 h to kill), mesogenic (taking 60 to 90 h to kill), and lentogenic (taking more than 90 h to kill). The values of MDT for rLaSota and rLaSota/gp160 were 105 h and 109 h, respectively. This test indicated that incorporation of HIV-1 gp160 protein into the NDV virion did not increase the pathogenicity of the recombinant virus in chickens. Indeed, the presence of the added gp160 gene conferred a small amount of additional attenuation to the NDV vector.

**Evaluation of anti-NDV and anti-gp160 serum antibody responses in guinea pigs immunized with recombinant NDVs.** The primary goal of this study was to evaluate the ability of strain rLaSota/gp160 to induce systemic and mucosal immune responses to HIV and to evaluate various combinations of i.n. and i.m. administration. Animals were immunized on days 0, 14, and 28 by the use of six different combinations of the i.n. and i.m. routes of inoculation, as described in Materials and Methods and shown in Fig. 7. The animals did not show any overt clinical signs of infection or any loss of body weight throughout the study (not shown), indicating that the recombinant NDVs were avirulent in guinea pigs. The induction of NDV-specific serum antibodies was measured on days 14, 28, 35, and 42 postimmunization using an NDV-specific ELISA (Fig. 8). All six animal groups exhibited high levels of NDV-specific IgG antibodies on day 14 (14 days following the first dose), and this response was boosted in all of the groups on day 28 (14 days following the second dose) and on days 35 and 42 (7 and 14 days, respectively, following the third dose). This suggested that the viruses in each of the doses replicated to some extent in the immunized animals.

Total levels of serum IgG specific to BaL.1 gp120 were measured at each time point by ELISA (Fig. 9). Responses were detected on day 14 following the initial immunization in all of the groups that received strain rLaSota/gp160 (Fig. 9).
whereas responses were not detected in either of the rLaSota negative-control groups on this or any other day (not shown). The titers were higher in the rLaSota/gp160 i.n.-i.n. and rLaSota/gp160 i.n.-i.m. groups than in the rLaSota/gp160 i.m.-i.m. and rLaSota/gp160 i.m.-i.n. groups. In the rLaSota/gp160 i.n.-i.n. group, the first boost (i.n.) increased the reciprocal geometric mean titer (GMT) from 252 to 40,637 (a 160-fold increase), and the second boost (i.n.) increased the GMT to 81,274 (a 2-fold increase). In the rLaSota/gp160 i.n.-i.m. group, the GMT increased from 318 to 12,800 (a 40-fold increase) after the first boost (i.m.), and it increased from 12,800 to 20,318 (a 1.6-fold increase) after the second boost (i.m.). In contrast, the increases in GMT were smaller when the initial immunization was administered by the i.m. route: the first boost (i.m.) after i.m. priming in the rLaSota/gp160 i.m.-i.m. group, and the first boost (i.n.) after i.m. priming in the rLaSota/gp160 i.m.-i.n. group, resulted in GMTs of 8,063 and 5,079, respectively, values that were significantly lower (10.0-fold and 16.0-fold, respectively) than those determined for the rLaSota/gp160 i.n.-i.n. group. In the rLaSota/gp160 i.m.-i.m. and rLaSota/gp160 i.m.-i.n. groups, the second boost at day 28 brought the GMTs up to 129,016 and 64,508, respectively, values that were still 2.5-fold and 5.0-fold lower, respectively, than those determined for the rLaSota/gp160 i.n.-i.n. group. The total IgG response was decreased on day 42 in all the groups. Thus, the strongest gp120-specific total IgG response was observed in the rLaSota/gp160 i.n.-i.n. group, and the next-strongest response was observed in the rLaSota/gp160 i.n.-i.m. group. We also confirmed the specificity of the response by Western blot analysis using day 42 sera from all the guinea pigs inoculated by different routes; the results showed that all of the sera reacted strongly with purified BaL gp120 protein (Fig. 10).

For a more detailed analysis of the serum antibody responses, BaL1 gp120-specific IgG1 and IgG2a responses were measured by isotype-specific ELISA (Fig. 11). BaL1 gp120-specific serum IgG1 and IgG2a responses were detected on day
14 after the first immunization in all of the groups that received rLaSota/gp160 (Fig. 11) but not in the groups that received the rLaSota-negative control (not shown). A strong IgG2a response and a weaker IgG1 response were measured on days 14, 21, 28, 35, and 42 for all the guinea pigs immunized by different routes. The IgG1 and IgG2a responses were strongest in the rLaSota/gp160 i.n.-i.n. group, followed by the responses of the rLaSota/gp160 i.n.-i.m. group, as had also been seen with the total serum IgG response. The ratio of IgG1 to IgG2a in all the groups was 1:2 on day 14; the ratio increased to 1:4 or 1:8 (depending on the group) on days 21, 28, 35, and 42. These results indicated that the humoral immune response to HIV gp160 was consistent with a Th1-biased antibody response. In addition, we measured gp120-specific IgA serum antibody levels for all the groups, but all animals gave negative results at all time points.

Evaluation of anti-gp160 mucosal antibody responses in guinea pigs immunized with recombinant NDVs. An efficient HIV vaccine probably needs to stimulate antiviral immunity in both the mucosal and systemic immune compartments, because the mucosa is the predominant portal of natural HIV-1 infection. We therefore investigated whether the immunization regimens elicited virus-specific antibody responses at mucosal surfaces. Vaginal washes were collected from each animal at each time point and evaluated by ELISA using BaL.1 gp120-coated plates (Fig. 12). Induction of BaL.1 gp120-specific total IgG was initially detected on day 21, and the GMT was highest in the rLaSota/gp160 i.n.-i.n. group followed by the rLaSota/gp160 i.n.-i.m., rLaSota/gp160 i.m.-i.n., and rLaSota/gp160 i.m.-i.m. groups (Fig. 12A). As expected, the groups that received the rLaSota-negative control did not have detectable gp120-specific antibody responses at this or any time point (not shown). After administration of the first boost to the rLaSota/gp160-immunized animals, the GMT increased by approximately 2-fold on day 28 in all the groups. After the second boost on day 28, the GMT increased by 3-fold, 4-fold, 4-fold, and 10-fold in the rLaSota/gp160 i.m.-i.m., i.n.-i.m., i.m.-i.n., and i.m.-i.m. groups, respectively. The titers decreased by day 42 in all the groups. As seen with the total serum IgG response, the total IgG response in vaginal washes was strongest in the rLaSota/gp160 i.n.-i.n. group after a second boost.

We next measured the IgG1 and IgG2a responses in the vaginal washes. As shown in Fig. 12B, BaL.1 gp120-specific IgG1 and IgG2a responses in vaginal washes were first detected 14 days after the first immunization in all of the groups. The IgG2a response was higher than the IgG1 response on
days 21, 28, 35, and 42 in all the guinea pigs immunized by the different routes. As with the responses in serum, the IgG1 and IgG2a responses in the vaginal washes were strongest in the rLaSota/gp160 i.n.-i.n. group followed by the rLaSota/gp160 i.n.-i.m. group. The ratios of IgG1 to IgG2a in the different groups ranged from 1:4 to 1:16, with the highest ratio of 1:16 being recorded for the rLaSota/i.n.-i.n. group on day 35. In agreement with the serum antibody isotype analysis described above, the isotype analysis of vaginal washes provided evidence of a Th1-biased antibody response.

BaL1 gp120-specific IgA responses in vaginal washes were also measured (Fig. 12C). A very low titer was detected for the rLaSota/gp160 i.n.-i.n. group on day 28. The level had dropped by day 35 but had not changed further by day 42. No anti-gp120-specific IgA antibodies were detected in the rLaSota/gp160 i.m.-i.m., i.n.-i.m., and i.m.-i.n. groups on days 14 or 28, but a very low level was detected on day 35 in the rLaSota/gp160 i.n.-i.m. group and on day 42 in the rLaSota/gp160 i.m.-i.n. group. The rLaSota/gp160 i.m.-i.m. group remained negative for IgA antibodies in vaginal washes throughout the study.

Evaluation of HIV-1 neutralizing antibody (NAb) responses in guinea pigs immunized with recombinant NDVs. Sera from days 35 and 42 postimmunization collected from animals immunized by the different routes were evaluated for the ability to neutralize HIV-1 strains BaL1 and MN3, which share 85% amino acid sequence identity in gp160. This evaluation was done with pseudovirus bearing the envelope protein of either strain; infection of an indicator cell line with pseudovirus results in HIV-dependent reporter gene expression, unless the pseudovirus is neutralized. Surprisingly, we were not able to detect neutralizing activity against the homologous strain
TABLE 1. Virus-neutralizing antibody activity against HIV strain MN.3 in sera collected on days 35 and 42 from guinea pigs immunized as described for Fig. 7

<table>
<thead>
<tr>
<th>Group</th>
<th>Guinea pig</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; titer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 35</td>
</tr>
<tr>
<td>i.m.-i.m.</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>160</td>
</tr>
<tr>
<td>i.n.-i.n.</td>
<td>1</td>
<td>542</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>572</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>175</td>
</tr>
<tr>
<td>i.n.-i.m.</td>
<td>1</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>138</td>
</tr>
<tr>
<td>i.m.-i.n.</td>
<td>1</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from individual animals are shown as the reciprocal dilution giving 50% neutralization (ID<sub>50</sub> titer). Titters were obtained using a TZM-bl neutralization assay with MN.3 enveloped pseudoviruses. Preimmune sera were used to establish baseline neutralizing activity for each individual guinea pig, and these values were subtracted from the values shown. No neutralizing activity was detected in sera obtained from guinea pigs immunized with rLaSota virus.

DISCUSSION

The global spread of HIV continues to be a major public health concern. The development of a safe and effective HIV vaccine is important for control of the HIV/AIDS pandemic. Several vaccination strategies to elicit protective immunity against HIV have been tried, but these efforts have not yet proven successful. Among these possible strategies, one of the most promising is the use of vaccines using live viral vectors. The major advantage of vaccines based on live viral vectors is that they do not require the use of the whole infectious pathogen and thus offer the efficacy of a live attenuated vaccine without the risk that would be associated with a live attenuated HIV vaccine. NDV has a number of characteristics that make it a promising viral vaccine vector for HIV. As an enveloped virus, it has the potential to incorporate the HIV-1 envelope protein into its envelope. In contrast to the other viral vectors that encode a large number of proteins, such as adenoviruses, herpesviruses, and poxviruses, NDV encodes only eight proteins; therefore, there is less competition for immune responses between vector proteins and the expressed foreign antigen. NDV replicates in the cytoplasm, does not involve a DNA intermediate, and does not integrate into the host cell DNA. Genetic exchange either is rare or does not occur in NDV, thus greatly reducing the possibility of genetic exchange with circulating viruses. NDV can infect via the i.n. route and induce local IgA and systemic IgG antibody and cell-mediated immune responses and does not appear to spread systematically in primates, at least for the lentogenic and mesogenic strains that have been evaluated. NDV vectors are available that are based on lentogenic strains that are already in widespread use as live vaccines and pose no danger to the poultry industry.

NDV is an avian virus but is capable of infecting nonavian species, including humans (48). NDV is highly attenuated in humans due to a natural host range restriction. NDV has been evaluated in nonhuman primates as a potential vaccine vector for human use (9, 21). NDV also has been under evaluation for a number of years as an oncolytic agent for the treatment of cancer in humans and has been shown to be safe, with very few side effects. NDV shares only minimal amino acid sequence identity with human paramyxoviruses and is antigenically distinct. Thus, prior immunity to common human viruses should not affect the replication and immunogenicity of the vector. Previous studies have demonstrated that NDV may be used as a vector to express antigens of various human pathogens such as the hemagglutinin (HA) and neuraminidase (NA) glycoproteins of highly pathogenic avian influenza virus (HPAIV) subtype H5N1, the spike glycoprotein of SARS coronavirus, the hemagglutinin-neuraminidase (HN) glycoprotein of human parainfluenza virus type 3, the GP glycoprotein of Ebola virus in monkeys, and the fusion (F) glycoprotein of respiratory syncytial virus (10, 19, 20, 22, 39, 52, 57). NDV has been used as a vector to express the Gag proteins of simian immunodeficiency virus (SIV) and HIV in mice (12, 56). Therefore, it was of interest to evaluate NDV as a vaccine vector for the envelope protein of HIV-1.

We generated rNDV expressing HIV gp160. The recombinant virus grew efficiently in embryonated eggs and in cell culture. Western blot analysis and immunofluorescence staining showed that precursor gp160 polyprotein was expressed at high levels in DF1 and Vero cells and was proteolytically cleaved and transported to the cell surface. Furthermore, a large amount of gp160 precursor and a small amount of gp120 were incorporated into the NDV virion, with essentially no effect on vector replication and pathogenicity. Incorporation of foreign glycoproteins into NDV particles has been previously noted for recombinant NDVs expressing the HA or NA glycoprotein of HPAIV subtype H5N1, the HN glycoprotein of human parainfluenza virus type 3, and the GP glycoprotein of HIV envelope with the NAb data for serum conversion correlate well with the data for neutralizing activity against HIV-1 strain MN.3.
an increase in virulence of the NDV vector, but this was not observed by MDT tests using embryonated chicken eggs, and there was no reactogenicity in guinea pigs. This indicates that expression of gp160 by NDV does not pose a biosafety hazard.

Env of HIV mediates critical functions in the virus life cycle, including attachment to the target cells and fusion of viral and cellular membranes. The Env precursor gp160 is cleaved by cellular protease into the surface subunit gp120 and the transmembrane subunit gp41, which remain associated through noncovalent interactions. The transmembrane domain within gp41 anchors an oligomeric complex comprising gp41 and gp120 to the surface of infected cells and to the surface of virions after budding. Thus, the functional form of Env is oligomeric. Previously, it was shown that soluble form of Env (gp140) expressed by Semliki Forest virus formed stable trimers that retained recognition by conformationally sensitive antibodies (26). In this study, we investigated the potential of NDV to express correctly folded gp160 oligomers. Biochemical approaches employing chemical cross-linking revealed that gp160 protein expressed in cell culture by strain rLaSota/gp160 formed homogenous higher-order oligomers that retained recognition by conformationally sensitive monoclonal antibodies against gp160. It was previously shown that Env protein associated with SIV and HIV virions was trimeric (13, 14). In the present study, we used parallel biochemical strategies to determine the oligomeric state of virion-associated gp160 protein. Immunoblotting of cross-linked sucrose cushion-purified rNDV-gp160 virions by the use of conformationally sensitive monoclonal antibodies indicated that virion-associated gp160 protein of HIV purified as one predominant oligomeric species.

HIV-1 is mainly transmitted through mucosal surfaces. Therefore, stimulation of mucosal immunity is important for protection against HIV-1 (7, 15, 16, 70). Early HIV-1 vaccine trials were focused on systemic immunity and were not designed specifically to elicit mucosal immune responses. However, other studies have shown that mucosal immunization can induce both systemic humoral and cellular immune activity against HIV (33, 45, 50). Due to the failures and limited successes of HIV-1 vaccines to date, strategies to optimize the induction of mucosal immune responses to HIV-1 are needed (8, 32, 63, 66). Approaches that have been explored include immunization via mucosal (e.g., i.n.) routes, targeting antigens to lymph nodes, and administering vaccines with mucosal adjuvants (25, 29, 31, 40, 44, 68). It was shown that i.n. immunization induced better systemic and mucosal anti-HIV antibody responses than vaginal, gastric, and rectal immunization (67).

In the present study, we evaluated the gp120-specific antibody response elicited by prime-boost immunizations with rNDVs administered by a sole i.n. or i.m. route or by several combinations of i.n. and i.m. routes. The different immunization regimens induced clear-cut differential degrees of systemic and mucosal antibody responses in guinea pigs. The first inoculation of strain rLaSota/gp160, by either the i.n. or the i.m. route, resulted in weak gp160-specific serum IgG responses. The IgG response in vaginal washes was too weak to be measured by ELISA. After a subsequent boost, the serum IgG titer was increased by 160-, 40-, 50-, and 60-fold in the rLaSota/gp160 i.n.-i.n., rLaSota/gp160 i.n.-i.m., rLaSota/gp160 i.m.-i.m., and rLaSota/gp160 i.m.-i.n. groups, respectively, and was detectable by ELISA in vaginal washes from mice in the different groups. Thus, priming and boosting by the i.n. route produced the strongest systemic and mucosal IgG responses. The antibody responses were enhanced further after the second boost in all the groups, but the increase was severalfold lower than that measured after the first boost. The three immunizations, administered either by the i.m. route or by i.m. priming followed by two i.n. boosts, did not increase the humoral or mucosal IgG responses to the same extent as observed for the rLaSota/gp160 i.n.-i.n. group. Further, we showed that the rLaSota/gp160 i.n.-i.m. group also exhibited better responses than the rLaSota/gp160 i.m.-i.m. and rLaSota/gp160 i.m.-i.n. groups. This showed that the use of the i.n. route for the initial immunization, and the use of the i.n. route for the subsequent boosts, separately enhanced immunogenicity and that the effects were additive. Thus, the route of priming and the route of boosting were important in generating potent systemic and mucosal IgG responses to rNDVs expressing HIV gp160.

In the present study, the different immunization regimens of rNDVs expressing gp160 resulted in qualitatively similar immune responses in terms of antibody isotypes. We found that immunization of guinea pigs by either the i.m. or the i.n. route resulted in a IgG2a response that was higher than the IgG1 response seen with both serum and vaginal washes, indicating a Th1-biased immune response. As seen with the total IgG response in serum and vaginal washes, the responses of IgG1 and IgG2a were strongest in the rLaSota/gp160 i.n.-i.n. group. The Th1-biased response was maintained after each of the two boosts. Our results are in agreement with a previous report indicating that immunization of mice with a recombinant Semliki Forest virus expressing gp140 trimers resulted in a Th1-biased antibody response (26). Our results further support the idea that recombinant viral vaccines generally induce Th1-biased immune responses that are stimulated due to induction of gamma interferon (IFN-γ), interleukin-2 (IL-2), and IL-12 cytokines by viral infection.

Generation of IgA antibodies is a chief criterion of mucosal immune responses. Several groups have reported the production of HIV-1-specific IgA antibodies at mucosal surfaces after i.n. immunization with peptide, naked DNA, adenovirus type 5, recombinant gp41, and whole killed HIV-1 (23, 33, 45, 50, 51). We also detected anti-HIV-1 gp120-specific IgA in vaginal washes from the rLaSota-gp160 i.n.-i.n., rLaSota-gp160 i.n.-i.m., and rLaSota-gp160 i.m.-i.m. groups, but the levels of these antibodies were low. Among the groups, the level of IgA was highest in the rLaSota-gp160 i.n.-i.m. group. In previous reports of studies using DNA- or protein-based immunogens, the level of IgA was higher, probably due to the use of multiple boosts in their vaccine protocols.

Although sera were collected on days 14, 21, 28, 35, and 42 postimmunization, we chose the day 35 and 42 samples for studying the neutralizing antibody response because the serum IgG responses were stronger on these days. Analysis of neutralizing antibody responses indicated that prime-boost rNDV immunization of guinea pigs by the i.n. or i.m. route, or by different combinations of i.n. and i.m. routes, stimulated serum neutralizing antibodies in all the groups. The neutralizing antibody titers were higher for day 35 sera than for day 42 sera in all the groups, and the neutralizing activity correlated well with the systemic IgG responses measured by ELISA. Hence, the
neutralization was able to be mediated by local IgG rather than IgA in our study. As with other studies (26), we were able to efficiently neutralize the laboratory-adapted and neutralization-sensitive HIV-1 MN.3 strain. In contrast, the primary homologous BaL1 isolate was not neutralized by the sera from any of the guinea pigs, irrespective of the immunization regimen, even though the vector expressed BaL1 gp160. A likely explanation for the resistance of the BaL1 isolate to neutralization is that the major variable V1, V2, and V3 loops of primary isolates assume tightly interfacing “closed” conformations that decrease the accessibility of many gp120 epitopes to antibodies (71). We did not evaluate the induction of CD8 T cells in addition to neutralizing antibodies due to a lack of resources. But we have established a useful platform of immunization using rNDV expressing HIV Env, and this platform should allow us to study the induction of CD8 T-cell responses in the future.

In summary, for the first time we have evaluated the potential of NDV strain LaSota as a vaccine vector for expression of the Env protein of HIV-1. Our results showed that recombinant NDV is a promising vector for use in inducing Env-specific mucosal and systemic neutralizing antibody responses. Two immunizations via the i.n. or i.m. route induced a more potent immune response than a single immunization by either route, but responses induced via the i.n. route were severalfold stronger than those induced via the i.m. route. Further, we have shown that immunization with rNDV expressing HIV Env by either of the routes induced a Th1-type response, as reflected by the strong induction of antigen-specific IgG2a in preference to IgG1. These results highlighted the possibility of using NDV-based vaccines in relevant challenge models.

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