Assembly and Biological and Immunological Properties

of Newcastle Disease Virus-Like Particles

McGinnes, Lori W., Pantua, Homer, Laliberte, Jason P., Gravel, Kathryn A., Jain, Surbhi, and Morrison, Trudy G.

Department of Molecular Genetics and Microbiology*,
Program in Immunology and Virology,
University of Massachusetts Medical School
Worcester, MA

*Corresponding Author
Telephone: 508-856-6592
Email: trudy.morrison@umassmed.edu

Trudy Morrison
Dept Molecular Genetics and Microbiology
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA, 01655

Current Addresses:
a Genentech Inc
Infectious Disease Pathogenesis
1DNA Way, B11, MS33
South San Francisco, CA 94080

b NIAID
National Institutes of Health
33 North Drive, Rm 1E13C
Bethesda, MD 20892-3210

c Department of Molecular Medicine
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655
Abstract

Virus-like particles (VLPs) released from avian cells expressing the Newcastle disease virus (NDV) NP, M, HN and F proteins, derived from NDV, strain AV, were characterized. The VLP associated HN and F glycoproteins directed attachment of VLPs to cell surfaces and fusion of VLP membranes with red blood cell membranes indicating they were assembled into VLPs in an authentic conformation. These particles were quantitatively prepared and used as immunogen, without adjuvant, in BALB/c mice. Resulting immune responses, detected by ELISA, virus neutralization, and intracellular cytokine staining, were comparable to responses to equivalent amounts of inactivated NDV vaccine virus. HN and F proteins from another strain of NDV, strain B1, could be incorporated into these VLPs. Foreign peptides were incorporated into these VLPs when fused to the NP or HN protein. The ectodomain of a foreign glycoprotein, the Nipah virus G protein, fused to the NDV HN protein cytoplasmic and transmembrane domains, was incorporated into ND VLPs. Thus ND VLPs are a potential NDV vaccine candidate. They may also serve as a platform to construct vaccines for other pathogens.
Introduction

Vaccination is the most effective means of preventing virus infection and controlling spread of a virus through a population. Most licensed viral vaccines are live, attenuated viruses or inactivated virus. Live, attenuated viruses offer long lasting and protective immunity and are considered the most effective vaccines. However, these types of vaccines may cause serious disease in immunocompromised individuals, a significant concern due to the increase in this population in recent years (reviewed in (11, 33, 34)). They can also cause disease in normal individuals, albeit at low frequency, due to reversion to virulent forms (41). It is also possible that recombination events between the vaccine virus and endemic avirulent viruses can produce a virulent virus (44). Recombinant live virus vaccines may also have unknown, novel properties and require significant amounts of testing to insure that these new viruses pose no unforeseen hazards. An additional problem with these forms of live virus vaccines is the immunogenicity of the vector virus, a complication if a human virus is used as a vector (2).

Inactivated vaccines are safer but produce poorer and shorter-lived immune responses than live virus, in part due to alteration of the immunogenicity of the viral proteins during inactivation (reviewed in (11, 33)). Inactivated virus vaccines are also thought to be less effective in stimulating cellular immune responses (11). Additionally, vaccination with some inactivated virus vaccines, notably those developed for respiratory syncytial virus and measles virus, did not protect but actually exacerbated disease upon subsequent exposure to the live virus (reviewed in (11, 33)). Some viruses are also difficult to produce in quantity because of their virulence in eggs (47) or the difficulty in growing them in tissue culture.

Other types of vaccines are subunit vaccines or DNA vaccines. Subunit vaccines are usually less effective and often require an adjuvant, which adds additional safety concerns (reviewed in (11)). DNA vaccines, while having a great deal of potential, have not yet been...
licensed for use in humans (reviewed in (7)). In human trials, immune responses are often reported to be weak without additional immunization (21).

Virus-like particles (VLPs) are increasingly being considered as potential viral vaccines (reviewed in (15, 34)) because of their safety and efficacy. Indeed, two VLP vaccines are licensed for use in humans, the papilloma vaccine and the hepatitis B virus vaccine, and a number of other VLP vaccines are in testing (15). VLPs are large particles, the size of viruses, composed of repeating structures on their surfaces and in their cores, structures that mimic those of infectious viruses (15, 34). It has been noted that just these properties account, in part, for the very potent immunogenicity of viruses (15). VLPs are formed by the assembly of the structural proteins and lipids into particles but without the incorporation of the viral genome. Thus VLPs are incapable of multiple rounds of infection typical of an infectious virus, yet they retain the superb antigenicity of virus particles.

Paramyxoviruses are enveloped, negative-stranded RNA viruses (4, 16, 19). Many members of this virus family are serious human or animal pathogens and vaccines do not exist for many of them (4, 8, 9, 12, 16). It has been reported that VLPs can be produced upon expression of structural proteins of several different paramyxoviruses (3, 5, 39, 42, 45, 46). For example, cells expressing the four major structural proteins of the Newcastle disease virus (NDV), the viral NP, M, HN and F proteins, very efficiently release particles that resemble virus particles (37). We, therefore, explored the possibility that these Newcastle disease virus-like particles (ND VLPs) could be developed as vaccines. We report that VLPs contain biologically active glycoproteins, indicating that they have retained their authentic conformation during VLP assembly. These VLPs could be quantitatively prepared and they stimulated both humoral and cellular immune responses in mice. We also explored the possibility that these VLPs could be used as a platform for assembly of sequences from other viruses. We report that the HN and F proteins from a different strain of NDV can be efficiently incorporated into these VLPs. We found that short foreign peptide sequences can be assembled into ND VLPs when fused to two
different NDV proteins. We have recently reported that the ectodomain of the RSV G protein can be incorporated into these VLPs and immunization of mice with the G protein containing VLPs stimulated protective anti-RSV immune responses (32). Here we show that the ectodomain of another glycoprotein, the G protein of Nipah virus, can be assembled into ND VLPs demonstrating the versatility of these VLPs as a platform for assembly of foreign sequences into particles.
Materials and Methods

Cells, Virus, Plasmids

ELL-0 (avian fibroblasts), obtained from the American Type Culture, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with penicillin-streptomycin and 10% fetal calf serum. NDV NP, M, F and HN protein genes derived from the AV strain of NDV were inserted into the pCAGGS expression vector as previously described (37). NDV, strain AV, and NDV, strain B1, were grown and gradient purified as previously described (29). Strain AV, a select agent, was prepared and titered by plaque assay in BSL3 conditions by authorized personnel.

HN and F protein cDNAs from the B1 strain of NDV were cloned by standard RT-PCR protocols using infected cell RNA as template as previously described (27). The resulting DNAs were sequenced in their entirety to verify that they contained the correct sequence. Addition of sequence tags, HA and FLAG, to the AV HN and F protein sequences, respectively, were accomplished by standard PCR protocols and the resulting fusion genes were verified by sequencing. That the HN protein contained the HA sequence and the F protein the FLAG sequence was verified by Western analysis and by immunoprecipitation of the expressed proteins using anti-HA antibody or anti-FLAG antibodies.

The Nipah virus G protein cDNA was obtained from Dr. Paul Rota. It was moved from pTM1 into an Xho 1, Msc1 cut pCAGGS vector using the Bam 1 (blunted with T4 DNA polymerase) and Xho 1 sites that flanked the G sequences in pTM1. Two different NDV HN-NiV protein chimera genes were constructed by ligation of PCR derived DNAs derived from pCAGGS-HN and pCAGGS-G. The HN sequences encoded amino acid 1 through 47 and the NiV G sequences encoded amino acids 71 through 601 (HN/NiV#1) or 74-601 (HN/NiV#2). Primers used to generate a DNA encoding the HN cytoplasmic (CT) and transmembrane (TM) domains were GGTTATTGTGCTGTCGACTCATTTTGGC (forward primer) and
CATACTATATGCCAGGGCGGCAGATGGCTAAG (reverse primer). This product was digested with Xho I and Not I (a site introduced without changing the amino acid sequence). The primers used to generate DNA encoding the G protein ectodomain were GGATCTATCGATCATAGCGGCCGCTCTAGCGTACAGCCAAAATTACAAAGATC (forward primer for chimera #1) or GTGATCATAGTGAAATGCGGCCGCCCTCGATCATTCCACAAGATCAACAGACAATC (forward primer for chimera #2). Both primers introduced a Not I site without changing the amino acid sequence. The reverse primer was GCCAGAAGTCAGATGGCCAAGG. The PCR products were digested with Not I and Msc I. The two DNA fragments were ligated into an XhoI-MscI digested pCAGGS vector. The resulting plasmids containing the chimera protein genes were sequenced in their entirety to verify the gene junctions (illustrated in Figure 9) and to ensure that no additional changes were introduced during the PCR reactions.

**Antibodies**

Polyclonal rabbit anti-NDV antibody was raised against UV inactivated, purified NDV as previously described (24). Polyclonal antisera specific to F protein was a mix of antibodies raised against peptides with sequences of the HR1 domain and the HR2 domain, as previously described (6, 24). Polyclonal antisera specific for the HN protein was raised against a peptide with HN protein sequences as previously described (28). Secondary antibodies utilized were goat anti-rabbit antibodies (Sigma). Anti-G protein polyclonal monospecific mouse antibody was obtained from Dr. Paul Rota. Anti-NDV M protein antibody is a monoclonal antibody obtained from Dr. Mark Peeples (10).

**Transfections**

Transfections were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. For small scale transfections, a mixture of plasmid DNA (0.5µg/35mm plate) and lipofectamine (5µl/35mm plate) in Opti-MEM media (Gibco) was incubated at room temperature for 45 minutes, and added to avian cells grown in 35mm plates and previously
washed with OptiMEM. Cells were incubated for 5h at 37°C, Opti-MEM was removed, and 2ml of supplemented DMEM were added.

For quantitative preparations of VLPs, large-scale transfections of avian cells growing in T-150 flasks (1.8 x 10^7 cells) were utilized. For each T-150 flask, plasmid DNA (8µg of each plasmid) in 1.6 ml of Opti-Mem and lipofectamine (80 µl) in 3.2 ml of Opti-MEM were each incubated for 15 min at room temperature, mixed, and further incubated for 45 min at room temperature. Opti-Mem (11.2 ml) was mixed with the DNA-lipofectamine complexes and added to cells in a T-150 flask that had been twice washed with Opti-Mem. Cells and DNA-lipofectamine complexes were incubated for 5 hours at 37°C, the complexes removed, and 15 ml of complete media were added.

**Surface Expression and Biological Activities of NDV Glycoproteins**

Quantification of surface expression of HN and F proteins expressed in tissue culture cells was accomplished by biotinylation of surfaces of avian cells transfected with cDNA encoding the HN protein or the F protein as previously described (25). Fusion activity of F-FLAG protein expressed in tissue culture cells was measured in a content mixing assay by quantifying β-galactosidase activity activated in fused cells as previously described (25). Attachment activity of HN-HA protein expressed in tissue culture cells was measured by quantifying the binding of avian red blood cells as previously described (26, 28). Neuraminidase was measured as previously described (23, 30) using N acetyl neuramin lactose as substrate.

**Metabolic Labeling of Cells and VLPs**

At 36 hours post transfection, cells (in 35mm plates) were washed with DMEM without methionine or cysteine. Media was replaced with 0.7 ml DMEM without methionine or cysteine and supplemented with 100 µCi of a (35S) methionine and (35S) cysteine mixture (NEG-722 EASYTAG express protein labeling mix; Perkin-Elmer Life Sciences, Inc.). After 4 hours of
labeling, labeling media was replaced with complete media and incubation was continued for an additional 8-12 hours. VLPs in the cell supernatant were purified as previously described (37).

**Large Scale VLP Purification**

At various times post-transfection, cell supernatants from 2-7 x 10^8 cells were collected and cell debris was removed by centrifugation at 5000 rpm (Sorvall GSA SLA-1500 rotor). VLPs in the supernatant were pelleted by centrifugation in a Type 19 Rotor (Beckman) at 18,000 rpm for 12 hours. The resulting pellet was resuspended in TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA), dounce homogenized, and layered on top of a discontinuous sucrose gradient composed of 2 ml 65% sucrose and 4 ml 20% sucrose. The gradients were centrifuged in an SW 28 rotor (Beckman) at 24,000 rpm for 6 hours. The fluffy layer at the 20-65% sucrose interface, containing the VLPs, was collected, mixed with two volumes of 80% sucrose, and placed in top of a 1 ml layer of 80% sucrose in a SW41 Beckman centrifuge tube, and then over layered with 3.5 ml of 50% sucrose and 2ml of 10% sucrose. The gradients were centrifuged for 18 hours at 38,000 rpm. The VLPs, which float to the interface of the 50% and 10% sucrose layers, were collected and concentrated by centrifugation in an SW50.1 rotor for 16 hours at 38,000 rpm. All sucrose solutions were w/v and dissolved in TNE buffer and all centrifugations were done at 4°C.

**NDV UV Inactivation**

Purified virus (strain B1) was diluted in 2 ml of PBS in a 60 mm tissue culture dish and placed on a rotating platform 10 cm from a Germicidal Lamp (G15T8, Sylvania) for 20 minutes, a time previously determined to inactivate 100% of the virus as measured by plaque assay. The efficacy of UV inactivation was confirmed in a plaque assay. Virus was inactivated prior to inoculation into animals in order to comply with USDA regulations.

**Polyacrylamide Gel Electrophoresis, Silver Staining, and Western Analysis**

To prepare cell extracts, cells were washed in cold PBS and lysed in TNE buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100 and 0.5% sodium...
deoxycholate and 2.5 mg/ml of N-ethylmaleimide (NEM). VLPs or purified virus were also lysed in TNE buffer.

Proteins in extracts, virus, or VLPs were resolved on 8% polyacrylamide gels (SDS-PAGE) as previously described (14). Silver staining of proteins in the polyacrylamide gels was accomplished as recommended by the manufacturer (Pierce). For quantification of individual proteins in the polyacrylamide gels, different concentrations of bovine serum albumin (BSA) were electrophoresed on the same gel. A standard curve based on the densitometry of the BSA (Biorad Fluor-S Multi Imager) was used to determine the concentrations of each of the proteins in the purified VLPs or virus. For Western analysis, proteins in the polyacrylamide gels were transferred to PVDF membranes and detected as previously described (14).

**Virus Neutralization**

Mouse sera were complement inactivated and then diluted in DMEM without serum. Purified NDV, strain AV, was diluted to approximately 75 to 150 pfu in 100 µl. Dilutions of mouse sera in 100 µl was added to the virus and incubated for 1 hour at 37°C. The mixture was then added to pre-washed, confluent monolayers of avian cells growing in 12 well tissue culture dishes, and the cells were incubated at 37°C for one hour. The antibody-virus mixture was removed and 2 mls of agarose overlay were added to each well as previously described. Plates were incubated for 3 days.

**Hemagglutination**

Red blood cells (RBCs) (guinea pig, Bio-link, Inc), washed three times in PBS and resuspended in PBS at a concentration of 5.3 x 10^7 cells/ml. Two fold serial dilutions of VLPs or virus in PBS were prepared and 10 µl of each dispensed into microtiter plate wells. To each well, 75 µl of the RBCs were added and the plate was incubated for 1 hour at 4°C and then photographed.

**VLP Membrane Fusion**
Hemifusion of VLPs to cell membranes was measured by dequenching of the fluorescent dye R18 (octadecyl rhodamine B, Molecular Probes) loaded into VLP membranes as has been previously described (18). Purified ND-VLPs were treated with acetylated trypsin (5 µg/ml) for 10 minutes on ice to cleave the F protein followed by the addition of soybean trypsin inhibitor (10 µg/ml). The VLPs were then incubated with R18 at room temperature in the dark for 1 hour. R18-loaded VLPs were purified away from excess R18 by sedimentation through 20% sucrose to a 20-65% interface. ND-VLPs were then incubated with chilled guinea pig erythrocytes (Bio-Link, Inc.) in 24 well plates for 1 hour on ice at 4°C in the dark. Upon incubation of plates at 37°C, R18 fluorescence (excitation 560 nm; emission 590 nm) from wells of VLP-erythrocytes was read at 7 minute intervals with a SpectraMax Gemini XS (Molecular Devices) and SoftMax Pro software (Molecular Devices). Experimental values normalized for loading of R18 and by input particles as determined by amounts of M protein. Similar results were obtained using HN protein content for normalization.

VLP Neuraminidase

Neuraminidase activities of HN proteins in purified NDV (B1 strain) and VLPs were determined in particle suspensions as previously described (23, 30) using N acetyl neuramin lactose as substrate.

Animal Immunization Protocols

Mice, 4 weeks old BALB/c male, from Taconic laboratories, were housed (groups of 5) under pathogen-free conditions in micro-isolator cages at the University of Massachusetts Medical Center animal quarters. Mice (groups of 5) were immunized and boosted by intraperitoneal (IP) inoculation of different concentrations of VLPs or UV inactivated NDV protein (10, 20 or 40 µg total particle protein/mouse) resuspended in 0.5 ml of PBS containing 30% sucrose. Boosts (10 µg total protein/mouse) were accomplished at 27 days after initial immunization. Blood was collected from tail veins by standard protocols.
Determination of Antibody Titers by ELISA

Antigens used as targets in ELISA were purified NDV (1 µg protein/well). All antigens were placed in carbonate buffer, pH 9.6, and added to microtiter plates (Costar) and incubated overnight at 4°C.

After binding of the target antigen, wells were blocked in 50 µl PBS containing 1% BSA at room temperature for 1-2 hours, washed three times in PBS, and drained. Serial dilutions of mouse sera were added to the microtiter wells in 50 µl of PBS-BSA and incubated for 1 hour at room temperature. After removing the mouse sera and washing wells three times, a biotinylated anti-mouse antibody (Sigma) in 50 µl of PBS-BSA was added and the microtiter plates were incubated for 1 hour at room temperature. The microtiter plates were then washed three times in PBS and HRP conjugated neutravidin (1:4000 dilution) (Pierce) was added in 50 µl of PBS-BSA. The microtiter plates were incubated for 1 hour at room temperature and washed four times in PBS. TMB (3,3',5,5',tetramethylbenzidene) substrate (Sigma) in 50 µl was added to each well and incubated for 15-20 min. The reaction was stopped with 50 µl 1N H₂SO₄ and the ODs were read in a plate reader (Molecular Devices).

Antibody titers were defined as the dilution of antiserum that resulted in OD values 5 times over the background.

Intracellular Cytokine Staining

At 23 days after the second immunization, mice were sacrificed, spleens recovered, and splenocytes were harvested from each spleen using standard protocols (for example (31)). Recovered splenocytes were co-cultured separately for 7 days with uninfected, UV irradiated P815 cells or UV irradiated, P815 cells that had been infected with NDV, strain B1, for 9 hours and then washed three times to remove virus. After 7 days, spleen cells were mixed with infected P815 or uninfected P815 or anti-CD3. Cells were washed, resuspended in media with GolgiPlug (BD Biosciences Pharmingen), incubated for 5 hours at 37°C, washed, incubated with
anti-CD8 or anti-CD4 for 30 min at 4°C, washed, and then incubated with Cytoperm/Cytofix (BD Biosciences Pharmingen) for 30 min at 4°C. After washing the cells were resuspended in FACS buffer (PBS containing 1% BSA and 0.02% sodium azide) and incubated with antibody specific for interferon γ for 30 min at 4°C. After washing, the percent of CD4+, IFNγ cells, the percent of CD8+, IFNγ cells, and the percent of CD3+ cells were determined by flow cytometry.
Results

Characterization of ND VLPs

For quantitative preparation of VLPs required for their use as immunogens, avian cells were transfected with cDNAs encoding the NDV HN, F, NP, and M proteins as previously described (37). A mutation was introduced into the cleavage site of the F protein (F-K115Q) in order to inhibit cytopathic effects due to syncytia formation in the transfected cells (20). VLPs released from 2 to 6 x 10^8 transfected avian cells were purified and VLP associated proteins were compared to virion proteins of two different NDV strains, AV and B1 (Figure 1). The cDNAs used to produce VLPs were derived from the AV strain of NDV, a virulent strain (reviewed in (29)). Thus the VLPs were compared to virion proteins in this strain of NDV (lanes 1 and 2). Strain B1 is a NDV vaccine strain (reviewed in (29)). The VLP proteins were also compared with this strain of NDV (lanes 3-6), which was purified in parallel. Proteins were electrophoresed in the absence (lanes 1-4) and presence (lanes 5 and 6) of reducing agent.

While the F protein in VLPs is the uncleaved F₀ protein, due to a mutation of the F protein cleavage site, the F protein in the egg grown virus, either AV or B1, is cleaved into F₁, which, in reducing gels, migrates with NP (lane 6), and F₂, which migrates off the gel (reviewed in (29)). To resolve the virion associated F protein, proteins were electrophoresed in the absence of reducing agent since the cleaved F protein is a disulfide-linked heterodimer (F₁-F₂) in nonreducing gels (Fnr) and can be separated from NP (lanes 2 and 4).

Figure 1 shows that the VLPs (lanes 1, 3, 5) contained primarily the four major NDV structural proteins, NP, M, HN and F proteins, and, like virus, there was minimal cellular protein content in the particles. That these proteins are NDV proteins is shown by their co-migration with proteins in purified virions. In addition, it has been previously shown that these proteins are specifically immunoprecipitated with anti-NDV antibodies (37). Figure 1, lanes 1 and 3, also shows that the VLP associated HN protein is a disulfide linked dimer, typical of the HN protein in NDV, strain AV (lane 2) (28). The HN protein in the B1 strain of NDV is not disulfide linked and
migrates as a monomer in nonreducing gels (lane 4). The ratios of proteins in VLPs are, in general, similar to virus, although the F protein content, relative to M protein, is increased in VLPs compared to the AV strain but similar to that in the B1 strain.

Table 1 shows a quantitative analysis of individual protein content in three different VLP preparations and in purified virus. Different preparations of VLPs contained similar ratios of viral proteins and the ratios were similar to those of the B1 virus, purified in parallel, with the exception of the relative amounts of HN protein. B1 virus contained slightly less HN protein relative to M protein. The ratio of proteins in NDV AV strain virions is also shown in Table 1 and confirms that VLPs contained an increase in F protein relative to M protein.

Glycoproteins Incorporated into ND VLPs are Functional

To determine if VLP associated glycoproteins were assembled into particles in a native conformation, the functional activities of HN and F proteins were measured. HN protein attachment activity was measured by cell binding and by hemagglutination. Figure 2 shows binding of purified, radioactively labeled ND VLPs to avian cell monolayers. Nearly 100% of the input ND VLPs (lane 1) bound to cells (lane 4). This binding was inhibited with anti-NDV antibody (lane 3), a result consistent with specific binding mediated by the HN protein. Figure 3 shows that ND VLPs are capable of hemagglutinating red blood cells, a classic property of virus particles that is the result of specific binding of the HN protein to sialic acid receptors on red blood cell surfaces (16, 19). The HA titers of equivalent amounts of VLPs and virus were similar. VLP preparations 2 and 3 had a protein concentration similar to B1 virus (Table 1) and the HA titers were similar (both with titers of 256). VLP preparation 1 had a higher protein concentration than the virus preparation and a higher HA titer (titer of 512).

The VLP associated HN protein also retained neuraminidase activity typical of virus particles. The neuraminidase activity associated with VLPs was compared to that in a dilution of NDV virions that contained equivalent amounts of HN protein as determined by silver staining.
The VLP associated neuraminidase activity was 111% (+/-31%) that of virus associated activity (average of six determinations).

The fusion activity of VLP associated F protein was assessed using a well-established fusion assay, which measures the dequenching of the fluorescent lipid, R18, incorporated into effector membranes, upon binding to target membranes (17). The effectiveness of this assay for measuring fusion directed by NDV has been previously documented (18). Fusion of R18 labeled VLP membranes with cell membranes was indicated by an increase in fluorescence signal with time at 37°C (Figure 4). That this fusion was specific to the F protein was shown by the absence of fusion of particles containing only the NDV NP, M, and HN proteins. Thus both HN and F proteins assembled into ND VLPs retained the function of virion-associated proteins.

Antibody Responses to ND VLPs

To determine if ND VLPs could stimulate immune responses in experimental animals, different concentrations of purified VLPs (10, 20 and 40 µg of total VLP protein) were injected into the intraperitoneal cavities of BALB/c mice. No adjuvant was used. In order to compare immune responses stimulated by VLPs with those stimulated by virus, the same concentrations of UV inactivated NDV were utilized as an immunogen in a second set of mice. All mice received a boost of 10 µg of either VLPs or virus at 27 days after the initial immunization. NDV, strain B1, was used as the virus control for these experiments since this is a NDV strain used as a vaccine.

The NDV specific antibody titer, with time after immunization, in serum from each mouse was determined by ELISA, as described in Materials and Methods (Figure 5). The results show that VLPs were at least as efficient in stimulating total anti-NDV antibodies as virus. The titers of antibodies to VLP proteins increased slightly faster than the titers resulting from virus immunization. The maximal titers detected from both VLP and virus immunized mice were similar. While the results shown in Figure 5 were obtained using as target antigen the proteins
in purified NDV, strain AV, the antibody titers after virus immunization were not increased when proteins from purified NDV, strain B1, were used as target antigen (not shown).

To determine if VLPs stimulated neutralizing antibodies, the ability of sera from VLP immunized mice to neutralize virus in an *in vitro* plaque reduction assay was measured. Table 2 shows that sera from mice immunized with either VLPs or virus contained neutralizing antibody. The sera derived from VLP immunized mice were at least as effective in neutralization as sera from NDV vaccinated mice.

**T cell Responses to VLP Immunization**

To determine the effectiveness of VLPs in stimulating T cell responses in vaccinated animals, splenocytes from mice immunized with two different concentrations of VLPs were harvested at 49 days post immunization and restimulated *in vitro* with irradiated, NDV infected cells. After stimulation *in vitro*, the percents of CD8+ and CD4+ T cells that were secreting INFγ were determined by flow cytometry. Figure 6, panel A, shows that comparable amounts of CD8+, INFγ+ cells were detected in spleens from mice immunized with VLPs and from mice immunized with UV inactivated virus. Similarly the percentages of CD4+, IFNγ+ T cells from both sets of mice were comparable (Figure 6, panel B). Thus VLPs were as effective in stimulating murine T cell responses as virus.

**Incorporation of Different NDV HN and F Proteins into ND VLPs**

The VLPs described above were assembled with proteins from the AV strain of NDV. To determine if the glycoproteins of another NDV strain could be assembled into these VLPs, the HN or F protein cDNAs from the B1 strain of NDV were expressed in avian cells along with the cDNAs from the AV strain. Figure 7, left panel, shows the expression of the viral proteins in transfected cells. Figure 7, right panel, shows proteins in VLPs purified from the supernatants of the transfected cells. The B1 HN protein was incorporated into ND VLPs along with the AV NP, M, and F proteins (lane 8). While incorporation of the B1 HN protein into VLPs was
reduced compared to the AV HN protein (the ratio of HN to M protein was decreased by 70% compared to the AV HN: M ratio), this reduction correlated to the reduced expression of the B1 HN protein in cells, which was also reduced by 70% compared to AV HN protein expression (panel A, lanes 3 and 5). The B1 F protein was incorporated into ND VLPs with the AV NP, M, and HN proteins (lane 9) at levels comparable to that of the AV F protein (compare lanes 7 and 9). Furthermore, both the B1 HN and F proteins were incorporated into particles with the AV NP and M proteins (lane 10). Again, incorporation of the B1 HN protein was reduced but the B1 F protein incorporation was similar to AV F protein incorporation as determined by F:M protein ratios in each VLP preparation. Thus there is not a stringent, strain specific requirement for the assembly of glycoproteins into these particles and any reduction in incorporation likely relates to expression levels.

Incorporation of Foreign Peptide Sequences into ND VLPs

Incorporation of foreign sequences into the VLPs would expand their use for presentation of antigens. To determine if foreign peptide sequences could be specifically incorporated into ND VLPs, three approaches were explored. First, short peptide sequences were fused to the carboxyl terminus of the HN or F protein. To test this approach, the HA sequence tag was fused to the carboxyl terminus of the HN protein, and the FLAG sequence tag was fused to the carboxyl terminus of the F protein. Table 3 shows quantification of the surface expression and biological activities of these proteins compared to the wild type proteins. Neither sequence tag inhibited the expression of the glycoproteins (Figure 8, panel A, left). The surface expression of F-FLAG was very similar to the wild type protein (Table 3). The HN-HA protein surface expression was reduced, on average, by 23%. The FLAG sequence at the carboxyl terminus of the F protein had no effect on the fusion activity of the F protein while the HA tag did decrease the binding and neuraminidase activities of the HN protein even if these activities were normalized to surface expression. However the HA tag did not depress the
fusion promotion activity of the HN protein, but surprisingly enhanced it. The mechanisms involved in this fusion enhancement are a subject of current investigation.

The incorporation of these tagged proteins into VLPs is shown in Figure 8, panel A, right. The FLAG tagged F protein was not incorporated into particles. This result suggests that a foreign sequence fused to the cytoplasmic tail of the F protein can block the assembly of F protein into particles. Inhibition of F protein assembly had no effect on assembly of the HN protein into VLPs. The HA tagged HN protein was, however, incorporated into particles. There was a reduction in amount of detected HN-HA in VLPs compared to HN protein. However this reduction was likely due to a decreased expression of M protein in transfected cells in this experiment. Other experiments did not show this reduction. Indeed, the HN-HA: M protein ratio in VLPs shown in Figure 8 was identical to the HN: M protein ratio and the ratios were very similar in two other separate experiments. Thus, fusion of foreign sequences to the carboxyl terminus of HN protein is a potential approach for the introduction of additional epitopes into NDV particles.

In a second approach to incorporation of foreign epitopes into NDV VLPs, a sequence tag was fused to the amino terminal end of the NP, HA-NP, or separately to the carboxyl terminus, NP-HA. The HA sequence tag fused to either the amino terminus or the carboxyl terminus of the NP had little effect on the incorporation of NP into the VLPs (Figure 8, panel B). The sequence tag also had little effect on incorporation of the glycoproteins.

In a third approach to incorporation of foreign sequences into NDV VLPs, the hypothesis that the entire ectodomain of a foreign glycoprotein could be incorporated into NDV VLPs was tested using the ectodomain of the Nipah virus G protein. The approach was to fuse the ectodomain sequence to the transmembrane (TM) and cytoplasmic domain (CT) of a NDV glycoprotein. Since the Nipah virus G protein and the NDV HN protein are both type 2 glycoproteins, the ectodomain sequence of the G protein was fused to the TM and CT domain...
sequences of the HN protein as diagramed in Figure 9. Two different chimera proteins were constructed as shown in Figure 9.

To determine if the resulting HN/G chimera proteins were expressed, avian cells were transfected with cDNAs encoding the chimera proteins as well as the wild type G protein and the wild type HN protein. Figure 10, panel A, lanes 1-6, shows the precipitation of radioactively labeled proteins from cell extracts using anti-NDV antibody (lanes 1 and 2) or anti-Nipah virus antibody (lanes 3-6). The chimera proteins were expressed at levels similar to the wild type G protein. To determine if the chimera proteins were expressed on cell surfaces, surfaces of radioactively labeled cells expressing these proteins were biotinylated. Following cell lysis, the biotinylated proteins were sequentially precipitated with neutravidin-agarose and then with either anti-NDV or anti-Nipah virus G protein antibody. Figure 10, panel A, lanes 7-12, shows the proteins in the final precipitates. Clearly both the chimera proteins were expressed on cell surfaces at levels comparable to the wild type protein.

To determine if a chimera protein could be incorporated into ND VLPs, cells were transfected with cDNA encoding HN/G#1 chimera and the cDNAs encoding the NDV NP and the NDV M proteins. Another set of cells was transfected with the HN/G#1 chimera cDNA only. In addition, to determine if inclusion of one or both NDV glycoproteins had any effect on the assembly of the HN/G chimera protein into VLPs, cDNAs encoding the F protein, the HN protein, or both glycoproteins were included in parallel transfections. VLPs were purified from radioactively labeled cells, solublized in lysis buffer, and the chimera protein present in the VLPs was immunoprecipitated with anti-Nipah virus G protein antibody. Any particles in the supernatant of cells transfected with the chimera protein alone were purified and associated proteins immunoprecipitated in parallel. Figure 10, panel B, shows that the chimera protein was incorporated into particles in the presence of NP and M proteins (lane 2). No chimera protein was detected in the particles purified from cells expressing only the chimera protein (lane 1). Inclusion of F protein inhibited incorporation of the chimera protein slightly (lane 3).
HN protein, in the absence or presence of F protein, inhibited incorporation of the chimera protein (lanes 4, 5). Thus the entire ectodomain of a foreign glycoprotein can be incorporated into ND VLPs in the presence of the NDV NP and M protein.
Discussion

Results presented here describe the characterization of ND VLPs as a vaccine and as a vaccine platform for presentation of sequences from other pathogens. The results showed, first, that ND VLPs could be quantitatively prepared from cells transiently transfected with cDNAs encoding the NDV HN, F, NP, and M proteins. Second, VLP associated glycoproteins retained their functional activities of attachment and fusion. Third, immunization of mice with these VLPs stimulated antibody and T cell responses comparable to those stimulated by virus. Fourth, foreign sequences, including the entire ectodomain of a foreign glycoprotein, could be incorporated into these VLPs raising the possibility that these VLPs can serve as a platform for the construction of vaccines for a number of pathogens.

VLPs as Vaccines

Virus-like particles have a great deal of potential as viral vaccines (reviewed in (11, 15, 34)) since this type of vaccine may offer significant advantages over more commonly used vaccines. Two VLP vaccines have been licensed for use in humans, but no VLP vaccine is currently used in animals (11). The hepatitis B vaccine is a particle produced in yeast and is composed of membrane and the HBV surface antigen (22). VLPs composed of virus capsid proteins have been approved as a papilloma virus vaccine (13). VLPs composed of structural proteins of numerous other viruses have been reported (reviewed in (34)). Many of these VLPs have been tested in animal model systems as vaccines with generally positive results (summarized in (15, 34)). VLPs based on paramyxovirus structural proteins have been reported (3, 5, 37, 39, 42, 45, 46) but none have been explored as a potential vaccine.

ND VLPs as a NDV Vaccine

Consideration of ND VLPs as a vaccine candidate for Newcastle disease illustrates the potential of VLP vaccines. Newcastle disease virus (NDV), avian paramyxovirus 1 (APMV-1), is an avian pathogen with a significant economic impact worldwide (1). There are many different
strains of NDV, which are grouped according to their virulence in birds (1, 43). Most commercially raised chickens in the United States are vaccinated with an infectious, avirulent strain of NDV. The currently used vaccines are not, however, ideal. First, live vaccine viruses often cause mild respiratory or gastrointestinal disease (1). As a result, vaccinated birds have lower body weights, lower egg production, and increased susceptibility to other pathogens compared to unvaccinated birds (1). A second problem is failure of these vaccines to stimulate an immune response that is protective against all strains of virus. Flocks of vaccinated chickens can be susceptible to virulent or "exotic" strains resulting in outbreaks of the disease in the US (35, 36). A third potential problem with live virus vaccination is the reported recombination between vaccine viruses and circulating wild viruses (40). A fourth problem with the currently used vaccines, either live virus or inactivated virus, is the difficulty in discriminating between birds that have been vaccinated and those that have been infected with a wild virus, a discrimination important in monitoring outbreaks of the disease in vaccinated flocks.

ND VLPs as a vaccine for Newcastle disease could overcome most of the problems with currently used NDV vaccines. ND VLPs are incapable of a spreading infection thereby eliminating negative effects of live virus immunization. ND VLPs have no genome, eliminating the possibility of recombination with endemic NDV strains. We also demonstrated that VLPs could be constructed with glycoproteins from two different strains of NDV raising the possibility that broad-spectrum VLP vaccines can be developed. Assembly of the NDV glycoproteins into VLPs requires the cytoplasmic domains of the proteins, which are likely for specific interactions with the viral NP and/or M protein (McGinnes, et al, in preparation). In addition, the specific transmembrane domain of the glycoproteins is critical for assembly into ND VLPs (McGinnes, et al. in preparation). There are minor sequence differences between the CT and TM domains of different strains of NDV. For example, the HN proteins from the B1 and AV viruses vary by 3 and 6 residues in the CT and TM domains, respectively. While the assembly of the B1 HN protein into ND VLPs composed of AV NP, F, and M proteins is less efficient than assembly of...
the AV HN protein, this reduction can be completely accounted for by the reduced expression of
the B1 HN protein in cells. The F proteins of the B1 and AV strains of NDV vary by 4 residues
and 1 residue in the TM and CT domains, respectively. These residues had no effect on
assembly of the B1 F protein into VLPs. Thus the minor sequence differences between the TM
and CT domains of proteins from different strains of virus have minimal effects on their
assembly into VLPs, suggesting that these ND VLPs can be constructed with glycoproteins from
different strains of NDV. We also showed here that VLPs can be sequence tagged, which would
facilitate discrimination between vaccinated and previously infected birds.

For use as a vaccine, ND VLPs must be quantitatively and efficiently produced. Indeed,
quantitative amounts of ND VLPs could be produced from 2-6 x 10^8 avian cells. They could be
purified using protocols utilized for virus purification and the purified VLPs showed minimal cell
protein contamination. Furthermore, we found previously (37) and confirmed here that the ratios
of viral proteins were similar to those in virus particles. Most importantly, in order for VLPs to be
an effective immunogen, the proteins, and particularly the glycoproteins, assembled into the
particles must be in an authentic conformation. The most stringent test of the conformation of a
glycoprotein is the retention of the biological activities typical of glycoproteins associated with
virus. Indeed, we showed that the ND VLPs HN protein mediated cell binding and possessed
neuraminidase activity. F protein in these particles could direct the fusion of the VLP membrane
with red blood cell membranes.

The effectiveness of VLPs as an immunogen was demonstrated in a mouse model. The
immune responses were compared to those stimulated by a vaccine strain of NDV. Soluble
antibodies, characterized by ELISA, resulting from ND VLP immunization were at least as good
as those resulting from immunization with inactivated virus. Neutralizing antibody responses
were also as good as the responses to vaccine virus. Furthermore, ND VLPs stimulated T cell
responses at levels slightly better than those stimulated by a vaccine virus. Thus ND VLPs
stimulated immune responses in an animal model that have all the characteristics of an effective
vaccine. Their effectiveness in generating protective immune responses in chickens will be tested in future experiments.

**ND VLPs as a Vaccine Platform**

Because of the efficient ND VLP preparation and the potent immune responses that they stimulated, ND VLPs have the potential to be utilized as a platform for the construction of vaccines for other viruses including human viruses. This hypothesis was tested in several ways. First, incorporation of peptide sequences into ND VLPs could expand their use as vaccines. For example, if a small domain of a protein has been identified as a domain that stimulates neutralizing antibody responses, incorporation of this domain into the VLPs could stimulate these antibodies. Incorporation of T cell epitopes could enhance the ability of the VLPs to stimulate cell-mediated immune responses to specific pathogens. To test the feasibility of incorporation of foreign sequences into ND VLPs, the HA sequence tag was added to the amino terminus or the carboxyl terminus of the NDV NP protein or to the carboxyl terminus of the HN protein. All three approaches resulted in the successful incorporation of the sequence tag into VLPs and suggest that these approaches could be utilized to expand the scope of immune responses to the ND VLPs. In contrast, a sequence tag fused to the carboxyl terminus of the F protein inhibited its incorporation into particles. This sequence had no effect on F protein fusion activity or surface expression. This result is consistent with an important role of the F protein CT domain in assembly and suggests that extra sequences at the end of this F protein domain can interfere with necessary interactions required for F protein assembly.

In an alternative approach to extending the use of ND VLPs as vaccines, we hypothesized that specific, efficient incorporation of foreign proteins into ND VLPs could be achieved by constructing chimera protein genes composed of foreign proteins fused the transmembrane (TM) domain and cytoplasmic (CT) domain of the appropriate NDV glycoprotein. The NDV glycoprotein TM and CT domains should specifically interact with the NDV M and NP proteins resulting in efficient incorporation of the chimera protein into VLPs. To
test this hypothesis, we have reported that a chimera protein resulting from the fusion of the
respiratory syncytial virus G protein ectodomain with the HN protein CT and TM domains can be
incorporated into ND VLPs (32). Further we demonstrated that these VLPs stimulated anti-RSV
immune responses, responses that were protective upon RSV challenge (32). Here we have
demonstrated that the sequence encoding the Nipah virus G protein ectodomain, fused to the
CT and TM domains of the NDV HN protein, could also be incorporated into ND demonstrating
that RSV G protein is not a special case and that ectodomains of different type 2 glycoproteins
could be incorporated into these VLPs using similar approaches. This same approach could
also be used to assemble into VLPs the ectodomain of a type 1 glycoprotein by fusing the
ectodomain to the TM and CT domains of the NDV F protein, a type 1 glycoprotein. Indeed,
such an approach has been used to incorporate the influenza HA protein ectodomain into NDV
virions (38). Using this approach, the respiratory syncytial virus F protein ectodomain has been
assembled into ND VLPs (McGinnes, et al, in preparation). Thus ND VLPs have potential for
development of vaccines for other paramyxoviruses and, indeed, for other human pathogens.
Acknowledgements

This work was supported in part by grants AI30572 (TM) and U19AI057319 (awarded to UMass Center for Translational Research on Human Immunology and Biodefense) from the National Institutes of Health, and by grants from the Massachusetts Technology Transfer Center and the Worcester Foundation. We thank Dr. Paul Rota for cDNA and antibody specific to the NiV G protein and Dr. Mark Peeples for anti-M protein antibody.
References


Legends to Figures

Legend to Figure 1: Proteins in ND VLPs.
Proteins in purified ND VLPs (VLPs) and egg grown, purified NDV (virus) were separated on polyacrylamide gels and visualized by silver staining. Lanes 1, 3, 5 show proteins in VLPs from three different preparations. Proteins in purified virus are shown in lanes 2 (strain AV), 4 (B1 strain), and 6 (B1 strain). Lanes 1-4 show proteins electrophoresed in the absence of reducing agent (-βME). Lanes 5 and 6 show proteins electrophoresed in the presence of reducing agent (+ βME). HN, hemagglutinin-neuraminidase protein, either dimer or monomer; Fo, uncleaved fusion protein; NP, nucleocapsid protein; M, membrane protein; Fnr, nonreduced fusion protein (mix of uncleaved Fo and disulfide linked F1+F2). Asterisk along side lane 2 indicates BSA that co-purifies with some preparations of virus.

Legend to Figure 2: Attachment activity of ND VLPs.
Different amounts (1x, 0.5x, and 0.1 x) of radioactively labeled ND VLPs were added to avian cell monolayers on ice and incubated for 30 minutes. Unbound particles were removed, the cells washed with PBS, and the cells lysed. Viral proteins in the resulting cell extracts were electrophoresed on polyacrylamide gels and visualized by autoradiography. Lane 1, input VLPs (1x); lane 2, negative control (particles purified from supernatants of vector transfected cells); lane 4, 1x VLPs added to cells; lane 5, 0.5x VLPs added to cells; lane 6, 0.1x VLPs added to cells. Lane 3 shows the binding of 1x VLPs to cells in the presence of anti-NDV antiserum. The relative ratios of proteins in VLPs shown in Figure 2 appear different than those shown in Figure 1 due to the variation in methionine and cysteine residues in the NDV proteins.

Legend to Figure 3: Hemagglutination activity of ND VLPs
Hemagglutination was titered as described in Materials and Methods. Two fold dilutions of the three preparations of ND VLPs described in Table 1 or purified NDV (strain B1) were...
added, in duplicate, to wells of a microtiter plate. RBCs were added to each well. Shown is a
digitally acquired photo of the plate.

Legend to Figure 4: Membrane fusion activity of ND VLPs.

Purified VLPs formed with NP, HN, F, and M proteins, VLPs formed with NP, HN, and M
proteins (37), and VLPs formed with F, NP, and M proteins (37) were loaded with R18 as
described in Materials and Methods. The VLPs were added to RBCs on ice and warmed to 37°C.
Figure shows increase in fluorescence with time after transfer to 37°C. The data were
normalized for the R18 loading in each VLP as determined by fluorescence after chemical
dequenching induced by addition of Triton X-100. Shown are the averages of three
experiments and error bars indicate standard deviations.

Legend to Figure 5: ELISA Titers of NDV specific antibodies after immunization with ND
VLPs or NDV.

Groups of five mice were immunized with the total protein concentrations of VLPs or
virus indicated at the bottom of each panel. All mice received a boost of 10 µg of total VLP or
virus protein at day 26. Serum was collected at 10, 20, 37, and 49 days post immunization.
Figure shows the titer of antibodies using as target antigen total NDV proteins in purified NDV,
strain AV. Titer was defined as the reciprocal dilution of antibody that resulted in an OD that
was 5 fold over background. Panel A, titers obtained after ND VLP immunization; Panel B, titers
obtained after immunization with UV inactivated NDV.

Legend to Figure 6: Activation of T cells by ND VLPs.

Activation of CD8 or CD4 T cells to secrete INFγ was measured by intracellular cytokine
staining. Splenocytes from groups of four mice immunized with different concentrations of ND
VLPs or UV inactivated virus (shown at the bottom of each panel) were stimulated in vitro as
described in Materials and Methods. The percents of total cells that were CD8+, IFNγ+ (panel
A) or CD4+,INFγ+ (panel B) were determined by flow cytometry. The percent of cell stimulated
with anti-CD3 antibody is shown as a positive control. Mean and SEM, calculated using Prism
Graph Pad software, are show for each set of mice.

Legend to Figure 7: Incorporation of HN and F proteins from NDV, strain B1, into ND
VLPs.

Avian cells were transfected with the cDNAs encoding the NP and M proteins from strain
AV as well as various combinations of cDNAs encoding HN and F proteins from either strain AV
or strain B1, as indicated at the top of the figure. Two sets of cells were pulse labeled with $^{35}$S
methionine and then chased with nonradioactive media as described in Materials and Methods.
Radioactively labeled proteins in cell lysates of one set of cells prepared at the end of the pulse
label are shown in the left panel. VLPs harvested at the end of the non-radioactive chase in the
other set of cells were purified and the proteins present in extracts and VLPs were precipitated
with a cocktail of antibodies (anti-NDV antibody, anti-HN protein antibody, anti-F protein
antibodies, and anti-M protein). Proteins in the precipitate were resolved on polyacrylamide
gels and detected by autoradiography.

Legend to Figure 8: Incorporation of peptides into ND VLPs

Avian cells were transfected with cDNAs encoding the NDV M protein as well as various
combinations of untagged and HA tagged HN protein, untagged and FLAG tagged F protein,
and untagged and HA tagged NP protein as indicated at the bottom of each panel. F protein
cDNA containing a mutation in the cleavage site (20), as described above, was used in order to
resolve the F protein, on polyacrylamide gels, from NP. Panel A: VLPs were radioactively
labeled as described in Materials and Methods and legend to Figure 7. Proteins present in the
pulse labeled cell extracts (panel A) or in purified VLPs (panel B), harvested from supernatants
of cells subjected to a radioactive pulse and a non-radioactive chase, were detected by
autoradiography of polyacrylamide gels containing proteins precipitated using a polyclonal
antibody cocktail. Panel B: Avian cells were transfected with the cDNAs indicated at the bottom
of the panel. VLPs were harvested at 24 hours post transfection. The figure shows the proteins
in purified VLPs detected by Western analysis (WB) using an antibody cocktail (described in Legend to Figure 7) (top panel) or anti-HA antibody (bottom panel). M, marker NDV infected cell extract.

**Legend to Figure 9: Construction of HN/NiG chimera protein**

The diagram shows the locations of the cytoplasmic domain (CT), transmembrane domain (TM), and ectodomains domain of the NDV HN protein and the NiV G protein and the domains present in two NDV HN-NiV G chimera proteins. Below the bars, the sequences at the junctions of the TM and ectodomains of the two wild type proteins as well as two different chimera proteins, HN/NiG#1 and HN/NiG#2, are shown.

**Legend to Figure 10: HN/NiG Expression and Assembly into ND VLPs**

Panel A: Lanes 1-6 show the total protein immunoprecipitated from radioactively labeled extracts of cells expressing the HN protein, the NiV G protein, or the two chimera proteins. HN protein was precipitated with anti-NDV antibodies (lanes 1, 2). The NiV G protein and the chimera proteins were precipitated with anti-NiV antibody (lanes 3-6). Lanes 7-12 show biotinylated surface expressed in cells transfected with the HN protein, NiV G protein, or chimera protein cDNAs. Biotinylated proteins were sequentially precipitated with neutravidin-agarose and then anti-NDV antibodies (lanes 7, 8) or anti-NiV G protein antibody (lanes 9-12).

Panel B shows radioactively labeled NiV G protein sequences in VLPs prepared from cells transfected with the cDNAs indicated at the bottom of the panel for each lane. Purified VLPs were lysed and the proteins precipitated with anti-NiV G protein antibody and proteins detected by autoradiography.
Table 1: Protein Concentrations and Ratios in VLPs and Virus

<table>
<thead>
<tr>
<th>Protein</th>
<th>VLP#1</th>
<th>VLP#2</th>
<th>VLP#3</th>
<th>VLPs</th>
<th>NDV (B1)</th>
<th>NDV (AV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>HN</td>
<td>177</td>
<td>109</td>
<td>92</td>
<td>1.89+/-0.37</td>
<td>50</td>
<td>0.66</td>
</tr>
<tr>
<td>F</td>
<td>120</td>
<td>85</td>
<td>53</td>
<td>1.29+/-0.32</td>
<td>80^c</td>
<td>1.07</td>
</tr>
<tr>
<td>NP</td>
<td>140</td>
<td>98</td>
<td>92</td>
<td>1.65+/-0.18</td>
<td>100^c</td>
<td>1.33</td>
</tr>
<tr>
<td>M</td>
<td>72</td>
<td>63</td>
<td>60</td>
<td>1.0</td>
<td>75</td>
<td>1.00</td>
</tr>
<tr>
<td>Total Protein</td>
<td>509</td>
<td>355</td>
<td>297</td>
<td>1.0</td>
<td>305</td>
<td></td>
</tr>
</tbody>
</table>

^a Determined by densitometer scans of silver stained polyacrylamide gels. Values assume that all proteins bind silver stain equally.

^b from 3 dozen infected eggs

^c determined using nonreducing gels

^d values from a single stock prepared in 20 dozen eggs, standard deviations show variations in 3 to 5 determinations of protein ratios.
Table 2: Virus Neutralization

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Serum Dilution</th>
<th>NDV Titer After incubation with serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.91 x 10^{10}</td>
</tr>
<tr>
<td>No serum</td>
<td>----</td>
<td>1.49 x 10^{10}</td>
</tr>
<tr>
<td>Pre immune sera</td>
<td>1:4</td>
<td>1.49 x 10^{10}</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>1.65 x 10^{10}</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>1.41 x 10^{10}</td>
</tr>
<tr>
<td>VLP 20 µg</td>
<td>1:4</td>
<td>&lt;5 x 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>5.00 x 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>3.00 x 10^{8}</td>
</tr>
<tr>
<td>VLP 40 µg</td>
<td>1:4</td>
<td>&lt;5 x 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>&lt;5 x 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>&lt;5 x 10^{7}</td>
</tr>
<tr>
<td>NDV 20 µg</td>
<td>1:4</td>
<td>1.50 x 10^{8}</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>7.00 x 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>1.65 x 10^{9}</td>
</tr>
<tr>
<td>NDV 40 µg</td>
<td>1:4</td>
<td>&lt;5 x 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>1.10 x 10^{9}</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>2.90 x 10^{9}</td>
</tr>
<tr>
<td>rabbit anti-NDV antibody</td>
<td>1:8</td>
<td>&lt;5 x 10^{7}</td>
</tr>
</tbody>
</table>

*a Sera from mice in each group (five mice) were pooled for this analysis. Each titer is the average of two separate determinations.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Surface Expression</th>
<th>Attachment</th>
<th>Neuraminidase</th>
<th>Fusion Promotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HN-HA</td>
<td>77 (+/-33)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 (+/-12)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>48 (+/-14)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>164 (+/-3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>100</td>
</tr>
<tr>
<td>F-FLAG</td>
<td>105 (+/-22)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>na</td>
<td>na</td>
<td>110 (+/-2)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

na = not applicable

<sup>a</sup> average of eight determinations

<sup>b</sup> average of six determinations

<sup>c</sup> average of four determinations
Reciprocal of Dilution of Particles

<table>
<thead>
<tr>
<th>PBS</th>
<th>VLP#1</th>
<th>VLP#1</th>
<th>VLP#2</th>
<th>VLP#2</th>
<th>VLP#3</th>
<th>VLP#3</th>
<th>NDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>512</td>
<td>1024</td>
<td>2048</td>
<td>4096</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[Image of a 96-well plate with different dilutions]