

Newcastle Disease Virus-Like Particles Containing Respiratory Syncytial Virus G Protein Induced Protection in BALB/c Mice, with No Evidence of Immunopathology[∇]

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Respiratory syncytial virus (RSV) is the leading cause of serious respiratory infections in children as well as a serious cause of disease in elderly and immunosuppressed populations. There are no licensed vaccines available to prevent RSV disease. We have developed a virus-like particle (VLP) vaccine candidate for protection from RSV. The VLP is composed of the NP and M proteins of Newcastle disease virus (NDV) and a chimeric protein containing the cytoplasmic and transmembrane domains of the NDV HN protein and the ectodomain of the human RSV G protein (H/G). Immunization of mice with 10 or 40 µg total VLP-H/G protein by intraperitoneal or intramuscular inoculation stimulated antibody responses to G protein which were as good as or better than those stimulated by comparable amounts of UV-inactivated RSV. Immunization of mice with two doses or even a single dose of these particles resulted in the complete protection of mice from RSV replication in the lungs. Immunization with these particles induced neutralizing antibodies with modest titers. Upon RSV challenge of VLP-H/G-immunized mice, no enhanced pathology in the lungs was observed, although lungs of mice immunized in parallel with formalin-inactivated RSV (FI-RSV) showed the significant pathology that has previously been documented after immunization with FI-RSV. Thus, the VLP-H/G candidate vaccine was immunogenic in BALB/c mice and prevented replication of RSV in murine lungs, with no evidence of immunopathology. These data support further development of virus-like particle vaccine candidates for protection against RSV.

Human respiratory syncytial virus (RSV), a member of the *Paramyxoviridae* family, is the primary cause of serious lower respiratory tract infections in infants and young children and is an important pathogen in elderly and immunocompromised populations worldwide (15, 16, 23, 42). RSV infections can induce a wide spectrum of respiratory diseases, ranging from common cold-like symptoms to more serious disease, such as bronchiolitis or pneumonia (16, 57). Despite the significance of this pathogen, no vaccine is available. Strategies utilizing traditional subunit vaccines or attenuated virus preparations as well as live virus vectors and DNA vaccines have not resulted in a licensed vaccine (reviewed in reference 42). Complicating RSV vaccine development are previous vaccine trials of a formalin-inactivated vaccine (FI-RSV), which predisposed infants to more severe disease upon natural exposure to live virus. These studies have raised concerns about the safety of all subsequently developed RSV vaccines (reviewed in references 15 and 42).

Both soluble and cell-mediated immune responses have

been proposed to be important for protection from RSV infections (3, 13–15, 29, 42, 67). The RSV F protein, one of the two major antigens expressed on virion surfaces (15), is thought to be the most important target of neutralizing and protective antibodies (15, 25, 72). Indeed, monoclonal antibodies specific for the RSV F protein are used clinically for RSV disease prophylaxis in high-risk infants (4, 61). The F protein is also a major target of CD8 T cells in mice (12), but the association between cell-mediated immunity and protection from RSV disease has not been established (62). The role of the G protein, the other major antigen on virion surfaces, in stimulating protective immune responses is less clear, although it is thought that antibodies to this molecule do have a role in protection (54, 68). No CD8 T-cell epitopes have been reported for this protein. The G protein is unlike other paramyxovirus glycoproteins. Its ectodomain is heavily glycosylated by N-linked and, primarily, O-linked carbohydrates (77). The estimated 24 or 25 O-linked carbohydrate side chains and 4 N-linked side chains increase the molecular mass of the protein, as synthesized in Vero cells, from 32.5 kDa to approximately 90 kDa (15, 16). This extensive glycosylation may help to mask the underlying polypeptide backbone from immune recognition (15).

A previous RSV vaccine, FI-RSV, resulted not in protection but in disease enhancement upon subsequent live virus infection (37, 38). Many subsequent studies have attempted to define the reasons for this response. These studies have consis-

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tently shown that enhanced disease is characterized by unbalanced Th2-biased cytokine responses, weak CD8 T-cell responses, pronounced eosinophilia, and induction of low-affinity and nonneutralizing antibodies (20, 21, 63, 64, 75). It is less clear which precise properties of the FI-RSV vaccine led to these results (reviewed in reference 42). The absence of these characteristics of enhanced disease is now one of the benchmarks for development of a successful RSV vaccine. Thus far, no vaccine approach reported has resulted in both the absence of enhanced disease upon RSV challenge and adequate, long-lasting protective responses in animal models (42).

A virus-like particle (VLP) vaccine strategy has not been reported for RSV. VLPs are large particles, the size of viruses, composed of repeating structural arrays on their surfaces and in their cores, and these structures mimic those of infectious viruses (reviewed in references 36 and 56). 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 the multiple rounds of infection typical of an infectious virus, yet they retain the superb antigenicity of virus particles. Native viral antigens arrayed on VLP surfaces and in their cores likely contribute to potent humoral responses, CD4 T-cell proliferation, and expansion of cytotoxic CD8 T cells, unlike less immunogenic subunit vaccines, which are often comprised of individual purified viral proteins (9–11, 27, 41, 43, 66, 70). The potential of VLPs as safe, effective vaccines for viral disease is increasingly being recognized. Indeed, two VLP vaccines are now licensed for use in humans, namely, the papillomavirus vaccine and the hepatitis B virus vaccine, and a number of other VLP vaccines are being evaluated in preclinical and clinical trials (reviewed in reference 36). Therefore, VLPs expressing one or both RSV glycoproteins may be an attractive strategy for designing an effective RSV vaccine.

There is only one report of VLPs formed with RSV proteins (73). These particles have not been well characterized, nor is their efficiency of release known. Furthermore, their detection requires incorporation of a minigenome. However, we have previously reported that the expression of the four major structural proteins of Newcastle disease virus (NDV), an avian paramyxovirus, results in the very efficient release of particles that structurally and functionally resemble virus particles (60; L. W. McGinnes et al., unpublished data). Furthermore, we have found that these particles (ND VLPs) stimulate potent anti-NDV immune responses in mice, including neutralizing antibody responses (McGinnes et al., unpublished data). These results led us to test the hypothesis that ND VLPs could serve as a platform for the expression of antigens from human viruses, including RSV G and F proteins, and that these particles could serve as an effective RSV vaccine.

In this study, we report that the ectodomain of the RSV G protein, fused to the cytoplasmic tail (CT) and the transmembrane (TM) domain of the NDV hemagglutinin-neuraminidase (HN) protein, can be incorporated efficiently into VLPs containing the NDV NP and M proteins and that these particles can be prepared quantitatively and used as an immunogen. We demonstrate that immunization with these particles stimulated robust soluble immune responses. Furthermore, these particles conferred protection in BALB/c mice, characterized by increased viral clearance in lung tissue, after live RSV challenge. Importantly, infectious RSV challenge of mice fol-

lowing VLP-H/G immunization did not result in the enhanced lung pathology typified by FI-RSV immunization (17, 18, 55).

MATERIALS AND METHODS

Cells, virus, and plasmids. ELL-0 cells (avian fibroblasts) were obtained from the American Type Culture Collection, as were Vero cells, COS-7 cells, and HEP-2 cells. ELL-0 cells and HEP-2 cells were maintained in Eagle's minimal essential medium (MEM) (Gibco) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, vitamins, penicillin, streptomycin, and 10% fetal calf serum. Vero cells were grown in DMEM supplemented with penicillin, streptomycin, and 5% fetal calf serum. RSV strain A2 was obtained from Ralph Tripp.

The RSV G protein cDNA was a synthetic gene obtained from Novavax, Inc. NDV NP, M, F, and HN protein genes, as well as the RSV G protein gene, were inserted into the pCAGGS expression vector as previously described (46, 47).

An NDV HN protein-RSV G protein chimeric gene was constructed by ligation of PCR-derived DNAs derived from pCAGGS-HN and pCAGGS-G. The HN sequence encoded amino acids 1 through 47, and the RSV G sequence encoded amino acids 65 through 298. The primers used to generate a DNA encoding the HN CT and TM domains were GGTATATTGTGCTGTGCACT ATTTGGC (forward primer) and CATACTATATGCCAGGGCGGCCGCA GAGATGGCTAAG (reverse primer). This product was digested with XhoI and NotI (a site introduced without changing the amino acid sequence). The primers used to generate DNA encoding the G protein ectodomain were CTCCC TCATCATTGCAGCGCCGCTCTTGCCTACTCTGCGAATCATAAGGTC (forward primer which introduced a NotI site without changing the amino acid sequence) and GCCAGAAGTCAGATGGCCAAGG (reverse primer). The product was digested with NotI and MscI. The two DNA fragments were ligated into an XhoI-MscI-digested pGAGGS vector. The resulting plasmid containing the chimeric protein gene was sequenced in its entirety to verify the gene junctions (Fig. 1A) and to ensure that no additional changes were introduced during the PCRs.

Antibodies. Polyclonal rabbit anti-NDV antibody was raised against UV-inactivated, purified NDV as previously described (48). Polyclonal goat anti-RSV antibody (Biodesign) and mouse monoclonal anti-G antibody (MyBiosource) were used in Western blots. Anti-RSV F monoclonal antibody (clone 131-2A; Chemicon) was used in plaque assays. Secondary antibodies utilized were anti-goat antibody (Sigma), anti-mouse antibody (Sigma), and anti-rabbit antibody (Sigma).

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

For quantitative preparations of VLPs, large-scale transfections of cells growing in T-150 flasks were utilized. For each T-150 flask, plasmid DNA (8 μ g of each plasmid) in 1.6 ml of OptiMEM and Lipofectamine (80 μ l) in 3.2 ml of OptiMEM were each incubated for 15 min at room temperature, mixed, and further incubated for 45 min at room temperature. OptiMEM (11.2 ml) was mixed with the DNA-Lipofectamine complexes and added to cells in a T-150 flask that had been washed twice with OptiMEM. Cells and DNA-Lipofectamine complexes were incubated for 5 h at 37°C, the complexes were removed, and 15 ml of complete medium was added.

Polyacrylamide gel electrophoresis, silver staining, and Western analysis. Proteins in extracts, virus, or VLPs were resolved in 8% polyacrylamide gels as previously described (60). 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 in the same gel. A standard curve based on the stain of the BSA (using a Bio-Rad densitometer to measure the intensity of staining) was used to determine the concentration of each of the proteins in the purified VLPs or virus. For Western analysis, proteins in the polyacrylamide gels were transferred to polyvinylidene difluoride (PVDF) membranes (PerkinElmer) by dry transfer (iblot; Invitrogen). Proteins were detected in the blots as previously described (39).

VLP purification. At 24 h posttransfection, heparin was added to the cells at a final concentration of 10 μ g/ml. At 72 h posttransfection and again at 96 h posttransfection, cell supernatants were collected and cell debris was removed by centrifugation at 5,000 \times g (Sorvall GSA SLA-1500 rotor). VLPs in the super-

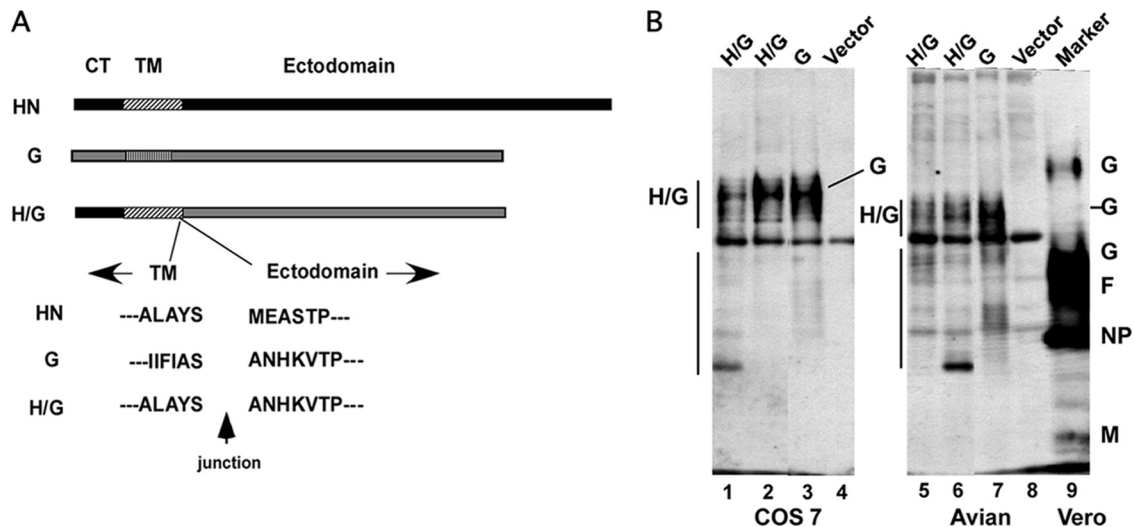


FIG. 1. Construction and expression of NDV HN-RSV G protein chimera. (A) The diagram shows the locations of the cytoplasmic domain (CT), transmembrane domain (TM), and ectodomain of the NDV HN protein and the RSV G protein and the domains present in the NDV HN-RSV G chimeric protein (H/G). Below the bars, the sequences at the junctions of the TM domain and ectodomain in the three proteins are shown. The HN protein junction is between amino acids S47 and M48. The G protein junction is between amino acids S64 and A65. The H/G chimeric protein contains amino acids 1 to 47 of the HN protein and amino acids 65 to 298 of the G protein. (B) Avian cells and COS-7 cells were transfected with cDNAs encoding the proteins shown at the top of the figure. Proteins present in extracts of cells at 48 h posttransfection or in extracts of RSV-infected cells (marker) prepared after 4 days of infection were detected by Western analysis using anti-RSV antibody. Lanes 1 to 4, transfected COS-7 cell extracts; lanes 5 to 8, avian cell extracts; lane 9, RSV-infected Vero cell extracts. Lanes 1 and 2 and lanes 5 and 6 are independent extracts prepared after transfection with the plasmid encoding the H/G protein.

nant were pelleted by centrifugation in a type 19 rotor (Beckman) at $28,000 \times g$ for 12 h. 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 SW28 rotor (Beckman) at $80,000 \times g$ for 6 h. The fluffy layer at the 20 to 65% sucrose interface, containing the VLPs, was collected, mixed with 2 volumes of 80% sucrose, placed on top of a 1-ml layer of 80% sucrose in an SW41 Beckman centrifuge tube, and then overlaid with 3.5 ml of 50% sucrose and 2 ml of 10% sucrose. The gradients were centrifuged for 18 h at $175,000 \times g$. The VLPs, which floated to the interface of the 50% and 10% sucrose layers, were collected and concentrated by centrifugation in an SW50.1 rotor for 16 h at $140,000 \times g$. All sucrose solution concentrations are given as weights per volume, all sucrose solutions were dissolved in TNE buffer, and all centrifugations were done at 4°C .

RSV purification. RSV (multiplicity of infection [MOI] of 0.1) in 5 ml of DMEM without serum was added to confluent Vero cells growing in T-150 flasks. Cells were incubated with virus for 2 h at 37°C , and then 15 ml of DMEM with 5% fetal calf serum was added. Infected cells were incubated for 3 to 4 days at 37°C . The cells were then scraped into the cell supernatant, and the suspended cells were frozen at -80°C and then thawed. The resulting cell lysates were clarified by centrifugation at $1,200 \times g$ (Sorvall) for 20 min. Virus in the supernatant was precipitated using polyethylene glycol 8000 (PEG 8000; 50% [wt/vol]), which was added to the supernatant to a final concentration of 10% and incubated for 90 min with stirring at 4°C . The precipitated virus was pelleted by centrifugation at $3,000 \times g$ for 20 min at 4°C , snap-frozen, and stored at -80°C . The thawed virus pellet was resuspended in 10% sucrose in TNE, homogenized, and layered on top of a discontinuous sucrose gradient composed of 1 ml of 60%, 3 ml of 45%, and 4 ml of 30% sucrose (all dissolved in TNE buffer), and the gradient was centrifuged at $160,000 \times g$ in an SW41 rotor for 90 min. The visible virus band between the 30% and 45% sucrose layers was collected. The viral protein content (M, NP, F, and G) of purified RSV was determined as described above, using known amounts of BSA included in the same gel.

RSV UV inactivation. Purified virus was diluted in 2 ml of phosphate-buffered saline (PBS) in a 60-mm tissue culture dish and placed on a rotating platform 10 cm from a germicidal lamp (G15T8; Sylvania) for 20 min, a time previously determined to inactivate 100% of the virus, as measured by plaque assay. The efficacy of UV inactivation was determined in a plaque assay.

FI-RSV. Formalin-inactivated RSV (FI-RSV) was prepared by a modification of the method of Prince et al. (64). Virus was harvested from infected HEP-2

cells, clarified by centrifugation ($1,000 \times g$ for 20 min at 4°C), filtered through a $0.8\text{-}\mu\text{m}$ filter, formalin treated by the addition of a 1/10 volume of a 1:400 dilution of 37% formaldehyde in water, and incubated for 3 days at 36°C with gentle stirring. The formalin-treated virus was pelleted at $100,000 \times g$ for 60 min at 10°C and resuspended in a 1/20 volume of EPES MEM and a 1/10 volume of SPG (2 M sucrose, 110 mM potassium phosphate, pH 7.1, 5 mM monosodium glutamate). The resuspended virus was centrifuged at $1,000 \times g$ for 15 min at 4°C , and the supernatant was incubated with 4 mg/ml aluminum hydroxide (Alhydrogel) overnight at room temperature. The virus-alum mixture was centrifuged at $1,000 \times g$ for 15 min, and the aluminum hydroxide pellet (adsorbed virus) was resuspended in a one-half volume of HMEM and a 1/10 volume of SPG and stored in 0.5-ml aliquots at 4°C . The amount of virus bound to the alum was estimated to be 44% by measuring the amount of total protein in the formalin-inactivated virus sample before and after alum adsorption. The amount of virus adsorbed to the alum was calculated to be equivalent to 6.1×10^8 PFU/ml. This estimate was based on the known virus titer prior to formalin treatment, the volume of the virus prior to formalin inactivation, the percent protein bound to the alum, and the concentration factor for each step in the procedure.

RSV immunoplaque assays. Vero cells (1.5×10^5 /well) were added to 24-well tissue culture plates (Costar) and incubated overnight at 37°C or until confluent. After removal of the medium, serial dilutions (in 200 μl of DMEM without serum) of RSV in virus stocks or in lung tissue clarified supernatants were adsorbed to monolayers that had been washed with DMEM without serum, in duplicate. Plates were incubated at 37°C for 2 h, the supernatant was removed, and each well received 1 ml of methylcellulose overlay (1 volume of $2 \times$ DMEM containing 10% fetal calf serum and 2% penicillin-streptomycin and 1 volume of 2% methylcellulose [Sigma]). After 3 to 6 days of incubation at 37°C , the overlay was removed, the cells were washed with PBS, and the monolayers were fixed with 1 ml of ice-cold acetone-methanol (60:40) for 10 min. After air drying, plates were blocked in 5% nonfat dry milk in PBS for 10 min at room temperature. Anti-F monoclonal antibody (1:800 dilution) was added to wells and incubated for 2 hours, followed by a 1-hour incubation with secondary anti-mouse immunoglobulin G (IgG) antibody conjugated to alkaline phosphatase. Antibodies were diluted in 5% nonfat milk, and plates were incubated at 37°C . Plates were washed twice with PBS containing 0.5% Tween 20 (Sigma) after each antibody incubation step. Individual plaques were developed using a DAB substrate kit (Vector Laboratories) as recommended by the manufacturer.

Antibody neutralization. Mouse sera were complement inactivated or not and then diluted in DMEM without serum. Purified RSV was diluted to approximately 75 to 150 PFU in 100 μ l. Dilutions of mouse sera in 100- μ l aliquots were added to the virus and incubated for 1 h at 37°C. The mixture was then added to prewashed, confluent monolayers of Vero cells growing in 24-well tissue culture dishes, and the cells were incubated at 37°C for 1 hour. The antibody-virus mixture was removed, and 1 ml of methylcellulose overlay was added to each well as described above. Plates were incubated for 3 to 4 days, and plaques were stained as described above.

Animal immunization and challenge protocols. Three-week-old BALB/c mice, from Jackson Laboratories or Taconic Laboratories, were housed in groups of five under pathogen-free conditions in microisolator cages at the University of Massachusetts Medical Center animal quarters. All protocols requiring open cages were accomplished in biocontainment hoods. Mice were immunized by intraperitoneal (i.p.) or intramuscular (i.m.) inoculation of different concentrations of VLPs or UV-inactivated RSV in 0.5 ml (i.p.) or 0.05 ml (i.m.) of PBS containing 30% sucrose. Other groups of mice were lightly anesthetized with isoflurane and then infected by intranasal (i.n.) inoculation of RSV (1×10^6 to 3×10^6 PFU/mouse in 50 μ l). Mice that received an immunization boost were injected i.p. or i.m. with 10 μ g of VLPs or UV-inactivated RSV/mouse or received 1×10^6 to 3×10^6 PFU of live RSV (i.n.). (The RSV protein concentration in 1×10^6 to 3×10^6 PFU was, at most, 400-fold less than the protein concentration in the VLP or UV-RSV preparation.)

Mice challenged with live RSV were lightly anesthetized as described above and infected i.n. with 1×10^6 to 3×10^6 PFU of virus.

Detection of virus in lung tissue. Mice were anesthetized with isoflurane and exsanguinated after severing of the right caudal artery. Lungs were removed aseptically, placed in 0.5 ml of 30% sucrose in PBS, and stored at -80°C . Upon thawing, lungs were weighed and then homogenized using a pestle (Kontes). The homogenate was centrifuged at 12,000 rpm for 15 min, and the virus titer in the supernatant was determined by plaque assay as described above.

Determination of antibody titers by ELISA. Antigens used as targets in enzyme-linked immunosorbent assays (ELISAs) were RSV-infected Vero cell extracts or extracts from 293T cells transfected with pGAGGS-G. To prepare cell extracts, cell monolayers 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. All antigens were placed in carbonate buffer, pH 9.6, added to microtiter plates (Costar), and incubated overnight at 4°C. The amount of transfected extract added to each well was adjusted so that the amounts of G protein were comparable, as determined by Western blotting.

After binding of the target antigen, wells were blocked in 50 μ l PBS containing 1% BSA at room temperature for 1 to 2 h, washed three times in PBS, and drained. Different dilutions of mouse sera were added to the microtiter wells in 50 μ l of PBS-BSA and then incubated for 1 h at room temperature. After removal of the mouse sera and washing of the wells three times, a biotinylated anti-mouse antibody (1:4,000 dilution) (Sigma) in 50 μ l of PBS-BSA was added, and the microtiter plates were incubated for 1 h at room temperature. The microtiter plates were then washed three times in PBS, and horseradish peroxidase (HRP)-conjugated neutravidin (1:4,000 dilution) (Pierce) was added in 50 μ l of PBS-BSA. The microtiter plates were incubated for 1 h at room temperature and washed four times in PBS. TMB (3,3',5,5'-tetramethylbenzidine) substrate (Sigma) in a 50- μ l volume was added to each well and incubated for 15 to 20 min. The reaction was stopped with 50 μ l 1 N H_2SO_4 , and the optical density was read in a plate reader (Molecular Devices).

Pulmonary histology of RSV-infected mice. For histological analysis of lung tissue, mice were anesthetized with isoflurane and exsanguinated after severing of the right caudal artery. The lungs were then fixed via infusion through the trachea with 4% formalin, removed, immersed in 4% formalin for 24 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff stain (PAS) by the University of Massachusetts Core Facility. Six sections per mouse were obtained. Sections from each mouse were scored blindly for the degree of inflammation (H&E stains) of blood vessels, airways, or interstitial spaces, on a scale of 0 to 3, as previously described (52). For sections stained with PAS, the percentage of airways positive for PAS in 25 randomly selected airways was determined.

Statistical analysis. Statistical analyses of data were accomplished using Graph Pad Prism 5 software.

RESULTS

Construction and expression of NDV HN/RSV G chimeric protein gene. To incorporate significant levels of RSV G pro-

tein into ND VLPs, the ectodomain of the RSV G protein was fused to the NDV HN protein cytoplasmic and transmembrane domains (Fig. 1A) to create a chimeric H/G protein. The sequences at the junction between the HN and G protein domains are shown in Fig. 1A.

The chimeric protein was expressed in COS-7 cells and avian cells, and its expression was compared to the expression of the wild-type RSV G protein in transfected cells as well as the RSV G protein in RSV-infected Vero cell extracts (Fig. 1B). The chimeric protein (lanes 1, 2, 5, and 6) was made in amounts similar to those of wild-type protein (lanes 3 and 7) and comigrated with the wild-type protein made in transfected cells. Both the wild-type and chimeric proteins were heterogeneous in size, as indicated by the vertical lines on the left side of each panel, a phenomenon likely due to inefficient glycosylation. The heterogeneity also varied somewhat between monolayers, as illustrated in Fig. 1B, lanes 1, 2, 5, and 6. The maximal sizes of both the wild-type and chimeric proteins varied with the cell type, as previously reported (26, 50). The G protein made in infected (or transfected [not shown]) Vero cells (Fig. 1B, lane 9) as well as in 293T cells (not shown) migrated the slowest, while the G protein or H/G chimeric protein made in transfected COS-7 cells (Fig. 1B, lanes 1 to 3) (or HEP-2 cells [not shown]) was slightly smaller. The proteins expressed in avian cells (Fig. 1B, lanes 5 to 7) were the smallest.

Incorporation of H/G chimeric protein into ND VLPs. To determine the optimal combination of NDV proteins for the incorporation of the H/G protein into ND VLPs, avian cells were transfected with the H/G chimeric protein cDNA, the NDV NP and M protein cDNAs, and various combinations of the NDV F and HN protein cDNAs. VLPs were pelleted from cell supernatants, and their protein content was determined by Western blotting, as shown in Fig. 2A. The NDV NP present in the VLPs is shown in the top panel, while the bottom panel shows the H/G protein content. Clearly, maximal incorporation of the chimera into particles was achieved in the presence of only the NDV NP and M proteins. Inclusion of the NDV F protein had little effect, but inclusion of HN protein inhibited incorporation of the chimeric protein.

Preparation of these VLPs for use as an immunogen was accomplished by transient transfection of avian cells with the NDV NP and M protein and the H/G chimeric protein cDNAs. Particles released from these cells were purified as described in Materials and Methods. The total protein content of the purified VLPs is shown by silver staining of a polyacrylamide gel containing VLP proteins (Fig. 2B, lane 2). Purified NDV (Fig. 2B, lane 1) is shown to mark the positions of the NP and M proteins. In addition, material released from cells transfected with vector DNA and purified in parallel is shown in lane 3. The silver stain indicated inclusion of the heterogeneous H/G protein, marked by vertical bars alongside the panel. The presence of the H/G protein was confirmed by Western analysis of the proteins in the purified VLPs (Fig. 2B, right panel, lane 5). Purified RSV is also shown (lane 6).

It should be noted that we found that release of these particles from transfected cells was enhanced 8- to 10-fold by inclusion of heparin in the culture supernatant (not shown). It is known that the RSV G protein binds to glycosaminoglycans (GAGs) and that heparin blocks this binding (31, 32). Thus, we

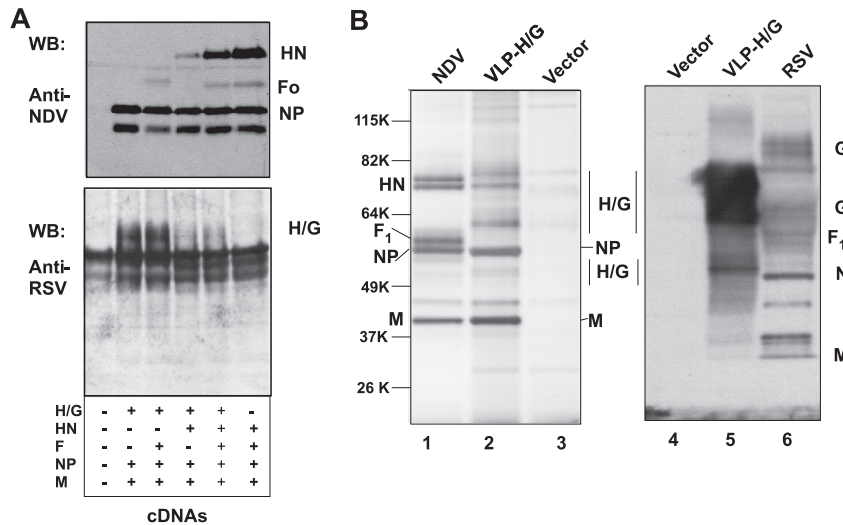


FIG. 2. Incorporation of H/G protein into ND VLPs. (A) Avian cells were transfected with the cDNAs shown at the bottom of the panel (0.5 μ g/35-mm plate). VLPs in the supernatant were collected by being pelleted through a layer of 30% sucrose. Proteins in the particles were detected by Western analysis (WB) using anti-NDV (top) or anti-RSV (bottom). The antibodies used do not detect the NDV M protein. HN, hemagglutinin-neuraminidase protein; F₀, uncleaved NDV fusion protein; NP, NDV nucleocapsid protein; H/G, HN-G protein chimera. (B) Avian cells were transfected with cDNAs encoding the NDV M protein, NDV NP, and the H/G chimeric protein (lanes 2 and 5) or with vector DNA only (lane 3). Particles in the supernatant were collected and purified as described in Materials and Methods. Proteins present in the purified particles were electrophoresed in polyacrylamide gels. Lanes 1 to 3 show silver staining of the proteins in the gel, while lanes 4 to 6 show a Western blot (WB) of the gel, using anti-RSV to detect G protein sequences in the VLPs. Proteins in purified NDV (lane 1) or RSV (lane 6) were included as markers. NP, NDV nucleocapsid protein; M, NDV or RSV membrane protein; H/G, HN-G protein chimera; G, RSV G protein; F₁, cleaved NDV or RSV fusion protein; N, RSV nucleocapsid protein.

hypothesized that released VLPs with the RSV G protein ectodomain might rebind to GAGs on surfaces of cells, decreasing the release of particles into the cell supernatant. Heparin should compete with this binding, enhancing recovery of VLPs from cell supernatants.

Densitometer analysis of silver-stained gels containing several preparations of VLP-H/G similar to that shown in Fig. 2B showed that the H/G protein represented 15 to 20% of the total viral protein in these particles. The amount of HN protein in ND VLPs is approximately 30% of the total VLP protein (McGinnes et al., unpublished data). Thus, the efficiency of incorporation of the H/G chimeric protein is slightly less than that of HN protein incorporation into VLPs.

Murine immune responses to VLP-H/G. We first determined if VLP-H/G could stimulate immune responses to the RSV G protein. Increasing amounts of VLP-H/G were delivered by i.p. inoculation, and the antibody responses specific to RSV proteins, over time, were determined by ELISA, using RSV proteins in infected Vero cell extracts as the target antigens. Figure 3A shows that antibody levels increased with time after immunization and increased with the dose of antigen. Thus, immunization with the H/G chimeric protein delivered in the VLP-H/G stimulated RSV-specific antibody responses. Immunization even with 1 μ g total VLP protein (0.15 to 0.2 μ g H/G protein) stimulated a detectable response by 14 days postimmunization.

Because the size of the avian virus-derived RSV G protein was different from that of the mammalian virus-derived RSV G protein, it was important to compare immune responses to the G protein delivered in VLPs with responses to the G protein in virus released from mammalian cells. For this comparison, we

utilized gradient-purified UV-inactivated RSV delivered by i.p. inoculation as the immunogen, as well as infectious RSV delivered i.n. For comparisons with UV-RSV, the amounts of virus and VLPs that had comparable amounts of G protein were utilized as immunogens. The concentrations of the G protein in VLP-H/G and UV-RSV were determined as described in Materials and Methods, and the volume of the VLP-H/G preparation was adjusted so that the concentration of H/G protein was the same as the concentration of G protein in virus. Figure 3B shows that the normalized preparations of UV-RSV and VLPs contained similar amounts of RSV G protein (compare lanes 5 and 6).

Mice (groups of 5) were immunized by i.p. inoculation with 30 μ g total VLP-H/G protein or a comparable amount of UV-irradiated, purified RSV or were given a single i.n. dose of infectious RSV. The antibody titers were determined by serial dilution of the serum (Fig. 3C), using G protein made in human 293T cells transfected with a plasmid encoding the RSV G protein as the target antigen. In addition, titers were determined using sera obtained 14 days after a boost of antigen at day 28. The antibody titers to VLP-H/G increased with time, and titers at 28 or 42 days postimmunization were similar to titers obtained after UV-RSV immunization or infectious RSV immunization. For comparison, the titers of serum antibodies after immunization with VLP-H/G, using G protein from purified RSV as the capture antigen, are also shown. These titers were quite similar to those obtained using G protein in transfected 293T cells as the target antigen. Thus, the antibody levels obtained after i.p. immunization with avian cell-derived VLP-H/G were comparable to levels obtained after

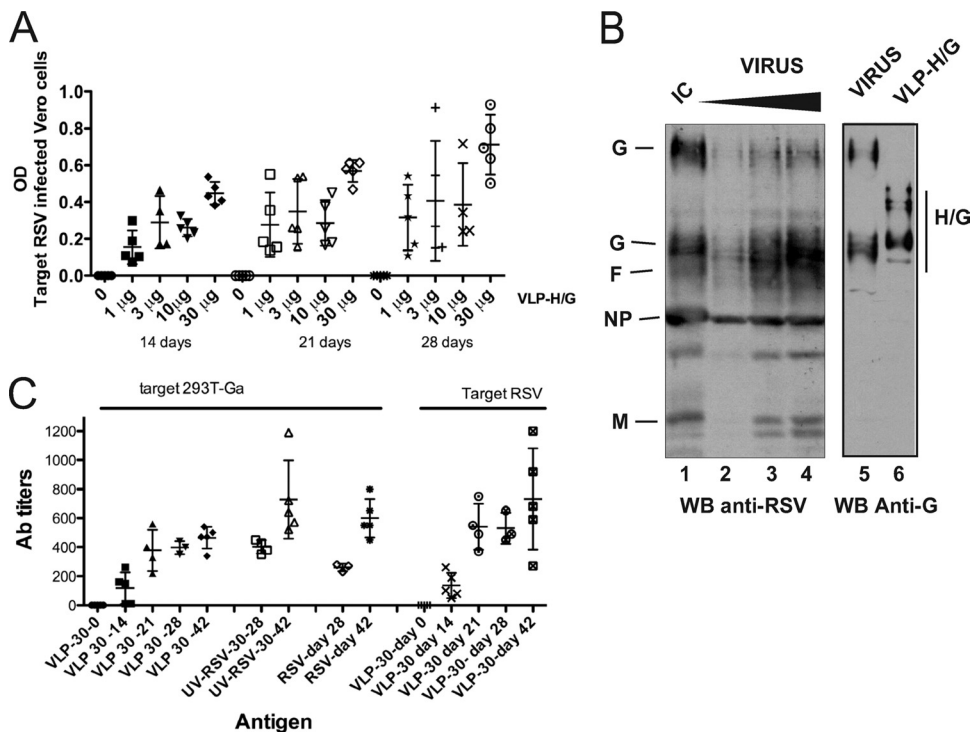


FIG. 3. Serum antibody responses to VLP-H/G delivered by intraperitoneal inoculation. (A) Mice (groups of 5) were immunized by i.p. inoculation of 0, 1, 3, 10, or 30 µg of total VLP-H/G protein. Serum was collected at 14, 21, and 28 days postimmunization. Antibody present in a 1:100 dilution of each serum sample was detected by ELISA as described in Materials and Methods, using RSV-infected cell proteins as the target antigens. The panel shows the optical density (OD) for each sample. The mean and standard deviation within each group are indicated by the horizontal line and the vertical line, respectively. (B) RSV was harvested and purified as described in Materials and Methods. The increasing amounts of proteins in the purified virus were electrophoresed in polyacrylamide gels, and the proteins were detected by Western blotting using anti-RSV antibody (lanes 2 to 4). Proteins in extracts from infected Vero cell extracts (IC) were included as marker proteins (lane 1). Based on these results as well as silver stains of the gel, the concentration of the VLP-H/G stock was adjusted such that the amount of G protein present was equivalent to the amount of G protein in virus stocks. Equivalent volumes of the VLP-H/G and virus stocks were electrophoresed in polyacrylamide gels, and the G protein sequences were detected using a monoclonal antibody specific to the G protein (lanes 5 and 6). NP, RSV nucleocapsid protein; M, RSV membrane protein; F, RSV fusion protein, G, RSV G protein; H/G, HN-G protein chimera. (C) The antibody titers in serum samples obtained 14, 21, or 28 days after immunization of mice with 30 µg of total VLP-H/G protein (i.p.) or with infectious RSV (3×10^6) (i.n.) were determined by ELISA with serial dilutions of the serum samples. (The amount of RSV protein in a dose of infectious virus was at least 400-fold less than that in UV-RSV preparations.) The target antigen was G protein in extracts of 293T cells transfected with DNA encoding the G protein or G protein in purified RSV, as indicated at the top of the panel. The titer was defined as the reciprocal of the dilution of serum that gave an OD of threefold over the background. Titers for individual mice are shown. The mean is shown by a horizontal line, and the standard deviation is shown by a vertical line. At 28 days postimmunization, VLP-H/G-immunized mice were boosted with 10 µg VLP-H/G protein. The UV-RSV-immunized mice were boosted with UV-RSV containing amounts of G protein equivalent to that in 10 µg of VLP-H/G, and RSV-immunized mice were boosted with infectious virus (3×10^6) (i.n.). Antibody titers in serum samples obtained 42 days after the initial immunization were also determined as described above.

i.p. immunization with UV-RSV or intranasal infection with infectious RSV.

Protection of mice from RSV replication after intraperitoneal immunization. To determine if the immune responses to VLP-H/G could protect BALB/c mice from RSV challenge, mice which had been immunized with 30 µg of total VLP-H/G protein and then boosted with 10 µg of total VLP-H/G protein were challenged i.n. with infectious RSV. As controls, mice previously infected with infectious RSV and boosted with infectious RSV were challenged with infectious RSV. Another set of mice, which had not received any immunogen, were also infected. At 4 days postinfection, the time previously determined to yield maximal virus titer (53), the virus titers in the lungs were determined. Figure 4A shows that RSV was detected in the lungs of unimmunized mice but that no virus was detected in the lungs of mice previously immunized with VLP-

H/G or, as expected, mice previously infected with live RSV. Thus, immunization with VLP-H/G protected mice from RSV replication in lungs.

Neutralizing antibody responses. One correlate of protective immune responses is the generation of neutralizing antibodies. To determine if immunization with VLP-H/G stimulated neutralizing antibodies, the sera were characterized in an *in vitro* plaque reduction assay. Because neutralization of virus by anti-G protein monoclonal antibodies has been attributed, in part, to a complement-dependent mechanism (19), neutralization was assayed using complement-containing sera as well as complement-inactivated sera. The reductions in titer after incubation with different dilutions of each set of sera are shown in Fig. 4B. Sera from animals immunized with VLP-H/G did stimulate neutralizing antibody, but, as expected, the titers were lower than those in sera resulting from immunization

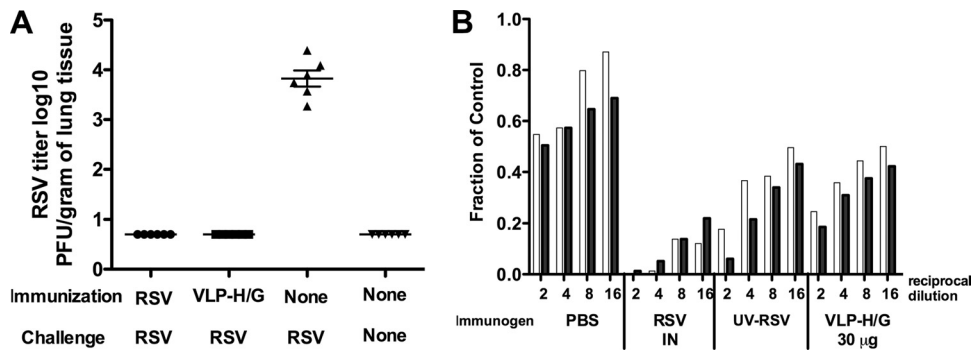


FIG. 4. Protective responses of immunized mice. (A) Protection from RSV replication in lungs after RSV challenge. Groups of five mice were immunized with infectious RSV (i.n.; 3×10^6 PFU/mouse) or VLP-H/G (i.p.; 30 µg/mouse). At 48 days postimmunization, mice were boosted with RSV (i.n.; 3×10^6 PFU/mouse) or VLP-H/G (i.p.; 10 mg/mouse). After 12 days, both the RSV- and VLP-H/G-immunized mice were infected by intranasal inoculation of infectious RSV (3×10^6 PFU/mouse). Unimmunized control mice (five animals) were infected with RSV (i.n.; 3×10^6 PFU/mouse). Another group of unimmunized mice was left unchallenged. After 4 days of infection, lungs were removed and virus in lung tissue was titrated by plaque assay as described in Materials and Methods. Titers shown for unchallenged mice are the limit of detection. (B) Neutralization of virus in an *in vitro* plaque reduction assay. Sera from animals immunized as described in the legend to Fig. 3C were pooled. Half of the serum was incubated at 56°C for 30 min to inactivate complement (– complement). The other half was left untreated (+ complement). Plaque reduction by incubation of virus with different dilutions of sera was determined as described in Materials and Methods. The graph shows the fraction of input virus (the amount of input virus was 107 PFU, which is the average of four determinations) detected after incubation with each dilution of serum. Each point is the average of two determinations. Open bars, + complement; filled bars, – complement.

with infectious RSV, since infectious RSV will also stimulate anti-F-protein antibodies. The titer of anti-RSV neutralizing antibody has been defined as the reciprocal of the dilution of serum that results in a 60% reduction in titer (33, 52). On this basis, and using the titer of input virus without incubation with preimmune serum as 100%, the titers of sera from VLP-H/G-immunized animals were approximately 10, while the titers of sera from infectious RSV immunization were >16. The presence or absence of complement had little effect on neutralization. Interestingly, UV-RSV immunization resulted in sera with neutralization activity comparable to that after VLP-H/G immunization.

Intramuscular immunization with VLP-H/G. Because immunization in humans is most often performed via i.m. inoculation, the immune responses of mice following this route of VLP-H/G immunization were determined. Also included in this study was FI-RSV, which served as a positive control for abnormal immune responses to vaccine preparations, as previously described. A group of mice immunized i.n. with infectious RSV was included as a positive control. Figure 5A shows that i.m. immunization with 10 µg and, particularly, 40 µg of VLP-H/G protein resulted in detectable antibodies specific to the RSV G protein, at levels that continued to increase for up to 38 days postimmunization. In contrast to the results shown in Fig. 3C, immunization by a single i.n. inoculation of infectious RSV resulted in barely detectable levels of serum antibodies. However, these mice received nearly three times less virus than did mice in the groups described for the experiment shown in Fig. 3. Sera from mice immunized with FI-RSV also contained barely detectable levels of RSV G protein-specific antibodies (Fig. 5A). All antibody levels, however, increased significantly following antigen boosting, as shown in Fig. 5A and B.

To determine the protective effects of i.m. immunization, mice that had received only one dose of immunogen were challenged with infectious RSV 38 days after immunization.

The titers of virus in lungs of these mice after 4 days of infection are shown in Fig. 6A. Remarkably, mice immunized with a single dose of either 10 µg or 40 µg of total VLP-H/G protein were protected from virus replication, as were mice that had previously been infected with live RSV. However, mice immunized with FI-RSV were not protected from virus replication.

The neutralizing antibody titers in sera prior to boost and after an antigen boost were determined in a plaque reduction assay as described for Fig. 3B. While sera obtained prior to a boost had detectable virus neutralization activity (Fig. 6B), the neutralization activity was significantly enhanced by a booster immunization (Fig. 6C). The presence of complement had little effect on neutralization. However, in spite of the low levels of neutralizing antibodies detected prior to the boost, a single immunization offered significant protection from RSV replication upon challenge with infectious virus.

Lung pathology after RSV challenge. A key property required of an effective RSV vaccine is the absence of abnormal immune responses upon challenge with infectious RSV. In experiments utilizing i.p. inoculation, lungs from each group of mice were examined for inflammation 4 days after infection with RSV. No excess inflammation was observed in mice immunized with VLP-H/G (not shown) compared to mice immunized with infectious RSV. To maximize any potential abnormal responses due to VLP immunization, mice immunized i.m. with 10 or 40 µg of VLP-H/G protein were boosted with 10 µg of VLP-H/G protein 38 days after immunization and infected i.n. with RSV 7 days later. Lungs were harvested after 6 days after infection. Three control groups of mice included unimmunized, unchallenged mice, mice subjected to a primary RSV infection, and immunized, boosted mice who were then challenged with infectious RSV. Positive controls for abnormal responses were mice immunized i.m. and boosted with FI-RSV and then challenged with infectious RSV. Figure 7A to H show representative fields of lung sections, stained with H&E, from each group of mice. Lungs from mice previously immunized

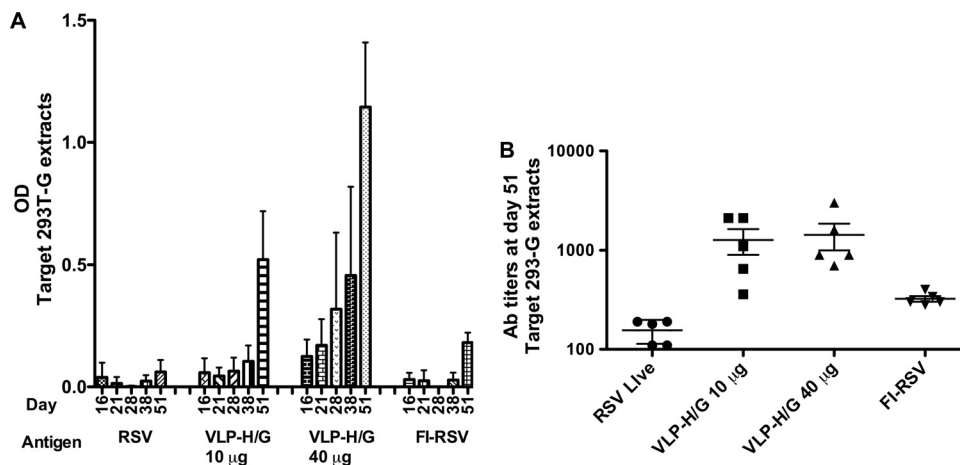


FIG. 5. Immune responses to VLP-H/G delivered by intramuscular inoculation. (A) Mice (groups of 5) were immunized by i.m. inoculation of 10 or 40 µg of total VLP-H/G protein. Another group of mice received FI-RSV by i.m. inoculation (equivalent of 5×10^6 PFU/mouse). Control groups of mice received infectious RSV (1×10^6 PFU/mouse by i.n. inoculation) or no immunogen. Serum was collected at 16, 21, 28, 38, and 51 days postimmunization. A boost (10 µg VLP-H/G, 1×10^6 PFU infectious RSV, or 5×10^6 PFU equivalent of FI-RSV) was delivered at day 38. Antibody present in a 1:50 dilution of each serum sample was detected by ELISA, as described in Materials and Methods, using proteins from 293T cells transfected with cDNA encoding the RSV G protein as target antigens. The panel shows the optical density (OD) for each sample. The mean and the standard deviation within each group are indicated by the horizontal line and the vertical line, respectively. (B) Mice (groups of 5 animals), immunized as described in the legend to panel A, were boosted with the same antigen 38 days after the initial immunization (RSV at 1×10^6 PFU/mouse i.n., 10 µg VLP-H/G/mouse i.m., or FI-RSV at the equivalent of 5×10^6 PFU/mouse i.m.) and then were challenged with RSV (2.9×10^6 PFU/mouse) 6 days later. Serum was collected 6 days after challenge (51 days post-initial immunization), and the antibody titers in serum samples were determined by ELISA with serial dilutions of the serum samples. The titer was defined as the reciprocal of the dilution of serum that gave an OD of threefold over the background, using proteins in extracts from 293T cells transfected with cDNA encoding the RSV G protein as target antigens. Antibody titers for individual mice are shown. The mean is shown by a horizontal line, and the standard deviation is shown by a vertical line.

with infectious RSV, mice with a primary RSV infection, and VLP-H/G-immunized mice showed some influx of lymphocytes. However, the FI-RSV-immunized mice showed a massive influx of lymphocytes around both blood vessels and airways and in the interstitial spaces, with formation of circumferential or near-circumferential cuffs around blood vessels and small airways. Lung sections from all mice were scored for inflammation around blood vessels and airways and in interstitial spaces, as described in Materials and Methods, and the results are shown in Fig. 8A to C, respectively. While immunization with FI-RSV recapitulated the previously documented abnormal histology of lungs after infectious virus challenge, the lungs from mice immunized with VLP-H/G did not show this abnormal pathology. Rather, the inflammation scores for the VLP-H/G-immunized mice showed no statistical differences from scores for mice immunized and then challenged with infectious RSV or mice subjected to primary RSV infection alone. In contrast, the differences between the scores of lungs from FI-RSV- and VLP-H/G-immunized mice were statistically significant.

The lung sections of FI-RSV-immunized mice also showed abnormal cells lining airways, consistent with increased intracellular mucus production (Fig. 7H), while cells lining airways of VLP-H/G-immunized mice did not show this type of morphology (Fig. 7G). To explore this observation, lung sections were stained with PAS to visualize material consistent with increased mucus production. Representative sections of lungs from VLP-H/G-immunized mice and FI-RSV-immunized mice as well as control mice are shown in Fig. 7I to N. These sections were scored for the percentage of airway linings showing PAS

staining, and the results are shown in Fig. 8D. VLP-H/G-immunized mice showed significantly less PAS staining than did FI-RSV-immunized mice ($P = 0.0014$). Thus, comparisons of the lungs of RSV-challenged mice indicate that VLP-H/G-immunized mice do not show the abnormal pathology associated with the FI-RSV vaccine.

DISCUSSION

The results presented here describe a novel vaccine candidate for respiratory syncytial virus, namely, a virus-like particle that contains an RSV surface protein. The approach takes advantage of the very efficient production of VLPs by the structural proteins of Newcastle disease virus (60). The results presented here demonstrated, first, that the ectodomain of the RSV G protein could be incorporated into particles formed with the NDV NP and M proteins and that these particles could be produced quantitatively. Second, VLP-H/G stimulated soluble immune responses specific to the RSV G protein. Third, immunization of mice with two doses or even a single dose of these particles resulted in the protection of mice from RSV replication in murine lungs. Lastly, upon RSV challenge of VLP-H/G-immunized mice, there was no increased pathology in the lungs compared to lungs of mice immunized by natural infection.

Generation of ND VLPs containing the RSV G protein. While VLPs are increasingly being considered as vaccines and two have been licensed for use in humans (36), VLPs have not been explored as a potential RSV vaccine. We chose to utilize ND VLPs as a platform for the presentation of RSV proteins

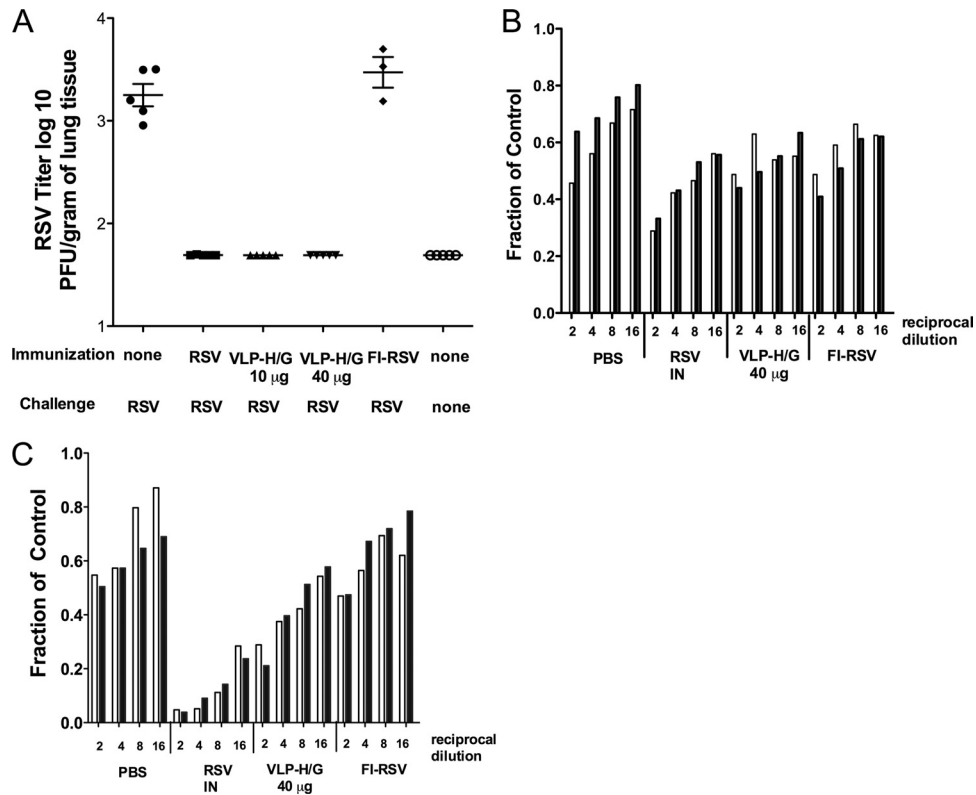


FIG. 6. Protective responses of immunized mice. (A) Virus titers in lungs after challenge of immunized animals. Mice (groups of 5) were immunized by i.m. inoculation of 10 or 40 μg of total VLP-H/G protein. Another group of mice received FI-RSV by i.m. inoculation (equivalent of 5×10^6 PFU/mouse). Control groups of mice received infectious RSV (1×10^6 PFU/mouse by i.n. inoculation). After 38 days, all mice were challenged with infectious RSV (1.6×10^6 PFU/mouse). One group of mice received no immunization and no RSV challenge. Four days after infection, lungs were harvested and the virus titers were determined by plaque assay as described in Materials and Methods. Titers shown for uninfected mice are the limit of detection. (B and C) Neutralization of virus in an *in vitro* plaque reduction assay. Sera from animals immunized as described in the legend to panel A were pooled. Half of the serum was incubated at 56°C for 30 min to inactivate complement (– complement). The other half was left untreated (+ complement). Plaque reduction by incubation of virus with different dilutions of serum was determined as described in Materials and Methods. The graph shows the fraction of input virus (the amount of input virus was 107 PFU) detected after incubation with each dilution of serum. Each point is the average of two determinations. Open bars, + complement; filled bars, – complement. Panel B shows results with sera obtained at day 38, prior to a boost. Panel C shows results with sera obtained at day 51 (postboost).

in a VLP for several reasons. First, only one study of the assembly of VLPs with RSV proteins has been published (73), and the requirements for their formation are relatively undefined. Our preliminary studies showed that the release of RSV VLPs is significantly less efficient than release of ND VLPs (unpublished data). Second, ND VLPs stimulate robust soluble and cellular immune responses, and the antibody responses are neutralizing (unpublished data), indicating that these VLPs are potent immunogens. Third, since NDV is an avian pathogen (2), there is no preexisting immunity to NDV proteins in human populations. Fourth, the possibility that RSV antigens could be incorporated into ND VLPs was suggested by the results of our previous studies on the requirements for assembly of ND VLPs.

Wild-type foreign glycoproteins can be incorporated into ND VLPs due to the phenomenon of phenotypic mixing (7), but at a very low frequency (unpublished observations). However, specific, quantitative assembly of a glycoprotein requires only the cytoplasmic tail and transmembrane domains of an NDV glycoprotein, likely due to specific interactions with the core proteins (unpublished data). Since both the NDV HN

protein (45) and the RSV G protein (15, 76) are type 2 glycoproteins, we chose to make a chimeric protein with the NDV HN protein CT and TM domains fused to the RSV G protein ectodomain. We have also reported that release of ND VLPs requires the M protein. Furthermore, the efficient assembly of the NDV HN protein into ND VLPs requires both the NP and M proteins (60). Thus, our VLP-H/G particles were built using these two NDV proteins.

Immune responses to RSV G protein upon immunization with VLP-H/G. As previously noted by others (50), we found that the maximal sizes of the RSV G protein and the H/G chimeric protein varied with the type of cell in which they were expressed, a result likely due to cell type variations in glycosylation. The least glycosylated protein was produced in avian cells, and it was this cell type that was utilized to produce the VLPs. However, the underglycosylated protein stimulated antibodies that would bind to more heavily glycosylated proteins present in mammalian cells (Fig. 3A and C). The titers of these antibodies were similar to titers of antibodies raised against comparable amounts of the more fully glycosylated G protein. Thus, the lower levels of glycosylation in the VLP-associated

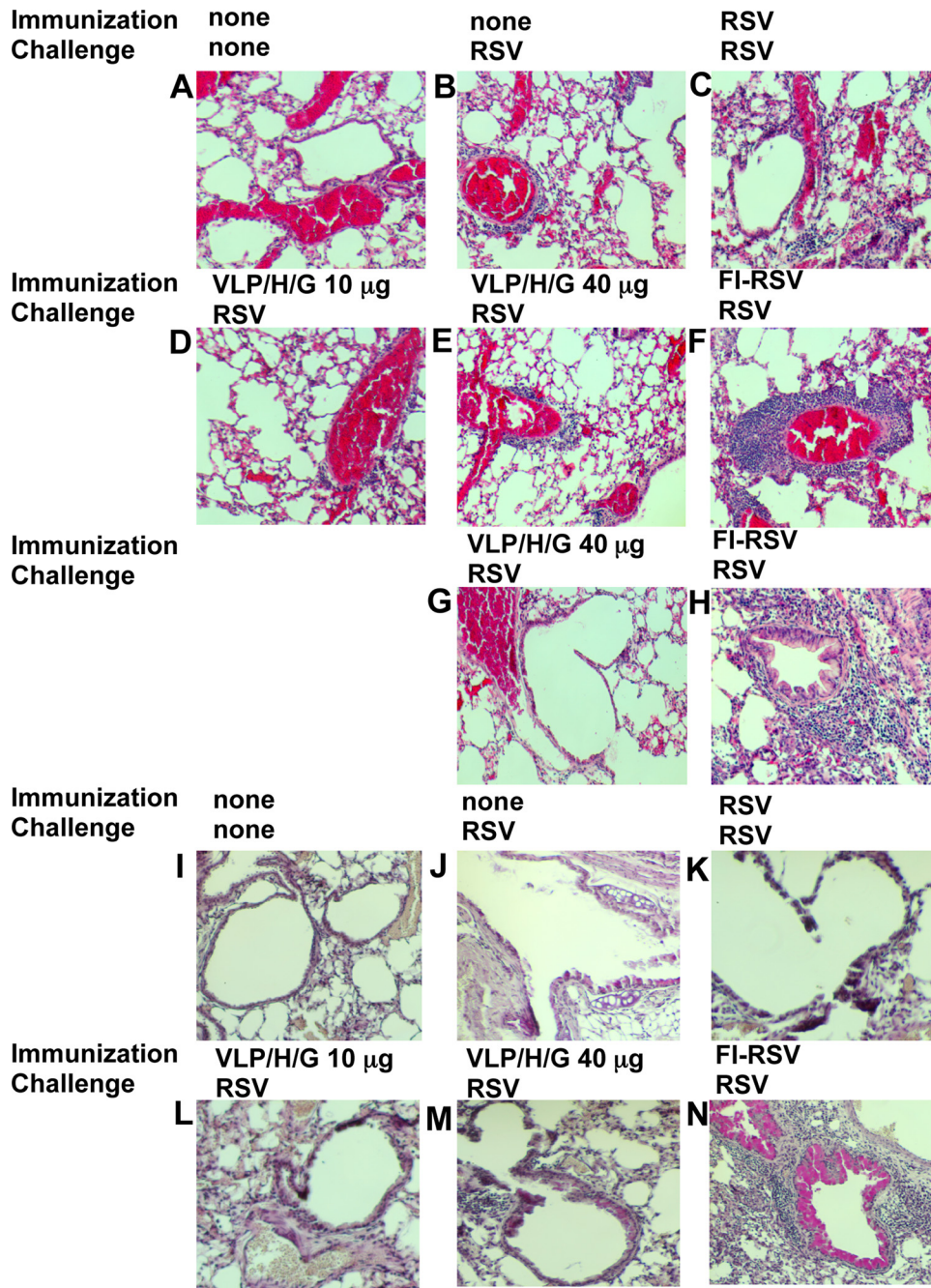


FIG. 7. Comparisons of pulmonary histopathology after VLP-H/G and FI-RSV immunization. Groups of mice were immunized with infectious RSV (1×10^6 PFU/mouse i.n.), 10 µg VLP-H/G (i.m.), or 40 µg VLP-H/G (i.m.), boosted with the homologous antigen (1×10^6 PFU RSV/mouse or 10 µg of VLP-H/G/mouse), and challenged with RSV (2.88×10^6 PFU/mouse). After euthanasia at day 51, lungs were prepared for tissue sections, as described in Materials and Methods, and were stained with H&E (A to H) or PAS (I to N). Panels are labeled at the top with the immunogen followed by the challenge. (A and I) No immunogen, no challenge; (B and J) primary RSV infection; (C and K) RSV-immunized mice challenged with RSV; (D and L) VLP-H/G (10 µg) immunization and RSV challenge; (E, G, and M) VLP-H/G (40 µg) immunization and RSV challenge; (F, H, and N) FI-RSV immunization and RSV challenge. All images were acquired using the same camera settings. Images were acquired using a Zeiss Axiovert 100 microscope at a magnification of $\times 20$, using an attached Canon EOS 30D digital camera. Images were adjusted for contrast using Adobe Photoshop, using the same settings for all images.

H/G protein did not adversely affect the levels of anti-G-protein antibodies after immunization.

Our results also showed that while the VLP-H/G delivered by either i.p. inoculation or i.m. immunization resulted in the

production of antibodies to the G protein, antibody levels increased faster and peaked sooner after i.p. immunization than after i.m. immunization. However, after an i.m. boost, the antibody levels after i.m. immunization increased significantly,

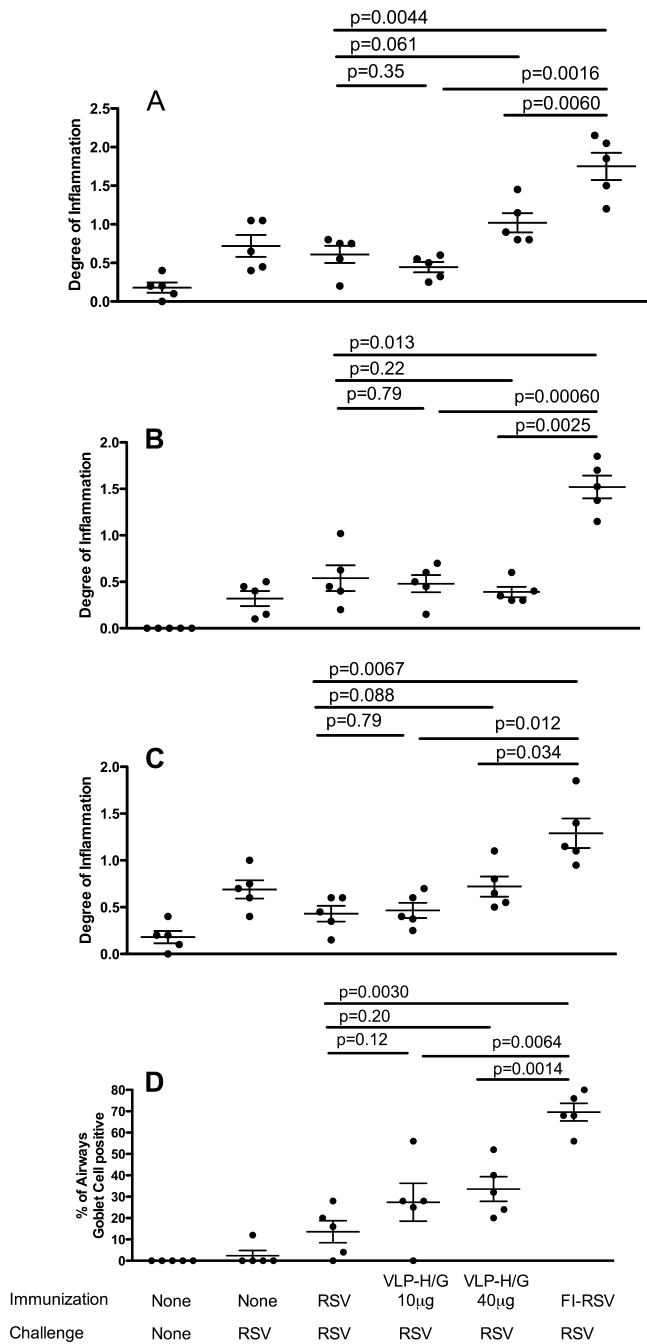


FIG. 8. Scores for pulmonary inflammation and bronchiolar mucus production. Tissue sections stained with H&E, obtained from each mouse and illustrated in Fig. 7, were scored for inflammation on a scale of 0 to 3 as described in Materials and Methods. (A) Blood vessels; (B) airways; (C) interstitial spaces. (D) Tissue sections stained with PAS were scored as the percentage of 25 random airways positive for PAS. P values obtained by comparisons of all combinations of two sets of animals are shown in the figure.

to levels higher than those seen after boosts delivered i.p. (compare Fig. 3C and 5B).

Protection against RSV challenge is provided by VLP-H/G immunization. The role of the RSV G protein in stimulating protective immune responses is not entirely clear. Some G

protein subunit vaccines containing a sequence of the G protein induced protection in animals, although others have resulted in enhanced lung pathology or short-lived responses (reviewed in reference 42). Virally vectored vaccines containing the G protein gene have been shown to be protective in some systems but not in others (reviewed in reference 42). DNA vaccines containing the G protein gene have demonstrated some protection (reviewed in reference 42). However, in many cases, the mechanisms involved in induction of protective responses are unclear.

VLP-H/G immunization protected mice from RSV replication in murine lungs after i.n. challenge with infectious RSV. Protection was observed after i.p. immunization with 30 µg total VLP protein followed by a boost of 10 µg. Protection was also observed after a single i.m. injection of VLP-H/G. In addition, protection was obtained after a single dose of 10 µg of total VLP-H/G protein, an amount that corresponded to approximately 1.5 to 2 µg of G protein per animal. Thus, VLP-H/G was a very potent protective immunogen.

The mechanisms responsible for this protection are not yet clear. Soluble antibody may be involved. However, the anti-G antibodies in the murine sera were only weakly neutralizing in an *in vitro* plaque reduction assay. However, there are studies that suggest a protective role for anti-G-protein antibodies which is unrelated to virus neutralization, as measured by virus neutralization *in vitro*. Several studies have shown that despite weak neutralization activity, anti-G antibodies do confer protection in BALB/c mice (19, 49, 51, 65, 69, 71, 74). For example, Miao et al. (51) described a role for G protein-specific MAb 131-2G in the clearance of virus from the lungs of passively immunized mice. They suggested that antibody-dependent cytotoxicity, mediated by an interaction of antibodies bound to virus with Fc receptors on leukocytes, might be involved. Furthermore, Corbeil et al. (19) demonstrated that passive immunization of BALB/c mice with the nonneutralizing anti-RSV G monoclonal antibody 18A2B2 conferred protection against virus replication following live RSV challenge. These authors suggested that complement-mediated cytotoxicity contributed to protection. Thus, there is evidence that nonneutralizing or weakly neutralizing antibodies directed against the RSV G protein play a role in protection, although the mechanisms underlying this process remain to be determined. Therefore, it is possible that protection observed after VLP-H/G immunization was due to anti-G-protein antibody responses.

It is also possible that cell-mediated immune responses have a role in protection. While no CD8 T-cell epitopes have been described for the G protein, there is evidence that suggests a role for G protein in establishing a cytotoxic CD8 T-cell response (8, 24), although a potential mechanism for these responses has not yet been described. The roles of antibodies as well as cellular responses in providing protection after VLP-H/G immunization are the topics of future investigations.

Enhanced disease after RSV challenge. Major complications in RSV vaccine development are the results of early vaccine trials using formalin-inactivated RSV as an immunogen. FI-RSV immunization resulted in enhanced disease upon RSV infection, and enhanced disease was characterized by heightened pulmonary cellular infiltrate and increased airway hyper-reactivity, indicative of Th2 allergy-like responses (37, 59).

Enhanced disease has been mimicked in animal models, including the BALB/c mice used here, and is characterized by an increased inflammation of respiratory tissue, likely due to an influx of lymphocytes and eosinophils, enhanced mucous secretion, and an unbalanced immune response (21, 55, 59). Increased inflammation associated with FI-RSV immunization was reproduced here, as indicated by increased numbers of immune cells around blood vessels and airways and in interstitial spaces in FI-RSV-immunized mice. Airway hyperreactivity is often associated with a strong Th2-biased response and the presence of goblet cells. Goblet cells are implicated in disease pathogenesis because they can produce large quantities of mucin, which contributes to airway obstruction, further worsening disease (1, 22). Previous studies have shown that RSV can induce goblet cells, a condition referred to as goblet cell hyperplasia (GCH), in the lungs of BALB/c mice and that Th2-sensitized mice exhibit extensive GCH following RSV infection (5, 6, 44). Indeed, the results presented here indicate that FI-RSV-immunized mice following RSV challenge exhibited significant GCH, a metaplastic process resulting in nearly total replacement of epithelial cells by goblet cells in most airways.

Many studies over decades have sought to clarify the mechanisms for this enhanced disease. Early reports suggested that immunization with G protein alone or vaccinia virus-delivered G protein elicits an unbalanced, Th2-dominated immune response (30, 34, 58). It has also been suggested that disease enhancement is related to two mucin-like domains (GCRR) in the G protein ectodomain (28, 40). This GCRR domain includes a CX3C chemokine motif that has been hypothesized to affect leukocyte trafficking to lungs (35), indirectly promoting Th2 responses by inhibiting important immune pathways that support Th1 responses. In contrast, it was suggested recently that enhanced disease, at least that caused by formalin-inactivated RSV vaccines or any nonreplicating vaccine, is not due directly to the G protein. Rather, it was suggested that these immunogens are poor activators of innate immunity and, as a result, fail to stimulate a strong acquired immune response (20).

To determine if VLP-H/G immunization stimulated symptoms of enhanced disease in mice, inflammation of the lungs of immunized and challenged mice was scored as previously described (52), and airways were scored for GCH. There were no statistically significant differences between VLP-H/G- and infectious RSV-immunized mice. Thus, VLP-H/G did not induce increased pulmonary inflammation and GCH compared to natural infection. In contrast, the FI-RSV-immunized mice showed dramatic inflammation that was significantly different from that observed for VLP-H/G-immunized mice. FI-RSV-immunized mice also displayed heightened GCH that was significantly enhanced over that observed in the VLP-H/G- or RSV-immunized mice. These results suggest that a VLP vaccine strategy may not predispose animals to enhanced RSV-induced disease upon challenge.

In sum, our results demonstrate that the RSV G protein ectodomain was efficiently incorporated into virus-like particles based on Newcastle disease virus proteins and that these particles stimulated very effective immune responses in mice, which prevented replication of RSV in lung tissue. Furthermore, these particles did not result in the enhanced

pulmonary inflammation typified by FI-RSV or UV-RSV immunization.

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