VIRAL IMMUNOLOGY
Volume 20, Number 3, 2007

Pp. 441–452
DOI: 10.1089/vim.2007.0027

A Novel Intranasal Virus-Like Particle (VLP) Vaccine Designed to Protect against the Pandemic 1918 Influenza A Virus (H1N1)

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ABSTRACT

We have prepared a virus-like particle (VLP) vaccine bearing the surface glycoproteins HA and NA of the 1918 influenza A virus by infecting Sf9 cells with a quadruple recombinant baculovirus that expresses the four influenza proteins (HA, NA, M1, and M2) required for the assembly and budding of the VLPs. The presence of HA and M1 in the purified VLPs was confirmed by Western blot, and that of NA by a neuraminidase enzymatic assay. For *in vivo* studies, the 1918 VLP vaccine was formulated with or without an oligonucleotide containing two CpG motifs and administered in two doses 2 wk apart via the intranasal route. The antibody titers in mice immunized with VLP vaccines were higher than in mice vaccinated with an inactivated swine virus (H1N1) control, when CHO cells expressing 1918 HA were used as antigen. The opposite result was obtained when disrupted swine virus was the antigen for the ELISA test. Vaccine efficacy was evaluated by challenging immunized mice with the 1918 antigenically related influenza virus A/swine/Iowa/15/30 (H1N1) and measuring viral titers in the upper and lower respiratory tract. Mice immunized with VLP vaccine plus CpG demonstrated significantly lower viral titers in the nose and lungs than did the control on days 2 and 4 postchallenge and completely cleared the virus by day 6. Furthermore, they did not show symptoms of disease although there was a minor decrease in body weight. Mice vaccinated with VLP alone also demonstrated significantly lower viral titers in the nose and lungs than did the placebo group as well as the inactivated virus group on days 4 and 6 postchallenge. These results suggest that it is feasible to make a safe and immunogenic vaccine to protect against the extremely virulent 1918 virus, using a novel and safe cell-based technology.

INTRODUCTION

In the last century, three significant influenza pandemics occurred, in 1918, 1957, and 1968 (1–3). Of these, the 1918 pandemic was the most devastating and widespread of all (4). It is estimated that 50 million people died worldwide because of infection with the virus, which demonstrated extreme virulence, high transmissibility, and the ability to predominantly affect young healthy adults (5–8). This is an unusual demographic for influenza infections, which typically infect young children and the elderly (9). This epidemic is a dire example of the devastation that pandemic influenza can bring about, particularly when it harnesses high virulence with high human transmissibility.

Because of the possibility of reemergence of the 1918 virus, it is of extreme importance that a prophylactic vaccine be developed that can effectively protect humans from infection (10). Interest in understanding the determinants of virulence and transmissibility, particularly

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stimulated by the emergence of the potentially pandemic H5N1 avian virus, have led to the reconstruction of the 1918 virus (11), which in experimental animals has demonstrated extreme virulence (8,12,13). The existence of this reconstructed virus further justifies the creation of a safe and efficacious vaccine to protect humans against this scourge. However, it will be technically difficult, because of the safety level required, as well as potentially dangerous to prepare vaccine against such a virulent virus using the conventional fertile egg production method. Therefore, newer and safer technologies will be required (14).

We have developed an innovative approach for creating influenza vaccines based on the formation of virus-like particles (VLPs) (15,16). These are structures resembling the influenza virus morphologically but unable to cause infection because of their lack of influenza genetic material. VLP vaccines do not require a chemical inactivation step that may potentially alter the structure of the antigenic epitopes of the surface glycoproteins. Animal studies with VLP vaccines have demonstrated that they are able to provide protection against lethal virus challenge when administered via intramuscular, intranasal (16,17), as well as subcutaneous routes (18).

In this study, we present data on the immunogenicity and protective efficacy of a 1918 VLP vaccine created with the HA and NA genes of the 1918 virus. The VLP vaccine was formulated with and without an oligodeoxynucleotide (ODN) as adjuvant and administered via the intranasal route in a prime–boost regimen. The antibody responses elicited by the vaccine as well as the protective efficacy against a virus challenge were evaluated.

MATERIALS AND METHODS

Generation of transfer vector

The HA gene of the influenza virus A/South Carolina/1/1918 (H1N1) recovered, reconstructed, and sequenced by Taubenberger's laboratory (19,20) was *de novo* synthesized with modifications by BlueHeron Biotechnology (Bothell, WA). The nucleotide sequence of the HA gene was obtained from GenBank (accession number AF117241) and the following changes were introduced to the synthesized gene: (*1*) the 3′ terminus, which encodes the transmembrane domain and cytoplasmic tail, was replaced with the analogous domains of HA3 of influenza A/Udorn/72 (H3N1); (*2*) the *Not*I and *Kpn*I restriction sites as well as the T7 polymerase promoter sequence were added at the 5′ terminus of the gene, whereas *Avr*II and *Not*I sites were added to the 3′ terminus.

The nucleotide sequence of the A/Brevig_Mission/1/1918 (H1N1) neuraminidase (NA) gene, also recovered, reconstructed, and sequenced by Taubenberger's laboratory (21), was obtained from GenBank (accession number AF250356). This gene was also *de novo* synthesized by BlueHeron Biotechnology with the following changes: (*1*) the 5′ terminal regions encoding the NH_2-terminal cytoplasmic tail and transmembrane anchoring domain were replaced with those of NA1 of influenza A/Udorn/72 (H3N1); (2) *SmaI* and *AscI* restriction sites were added to the 5′ end of the gene whereas *Fse*I, *Sma*I, and *Not*I sites were added at the 5′ terminus.

The NA gene was subcloned via the *Sma*I site into an intermediate shuttle vector (15), which already carried the M1 gene. In this intermediate construct, the NA gene was positioned under the transcriptional control of the baculovirus p10 promoter and the M1 gene was under the transcriptional control of the polyhedrin promoter. These two genes and respective promoters were cut out as a single fragment by digesting the shuttle vector with *PmeI* and *Sac*I restriction enzymes. The M1-NA insert was then subcloned into the *Pme*I/*Sac*I sites of the baculovirus transfer vector ($PAcAB_4$)-M2 (the M2 gene was already cloned into this plasmid) (15). Subsequently, the 1918 HA gene was subcloned into pAcAB4-M2-M1-NA via the *Not*I site. Restriction enzyme analysis was used to select the plasmids that carried the HA gene in the correct orientation with respect the polyhedrin promoter. The selected pAcAB4-M2-1918HA-1918NA-M1 plasmid was amplified, purified with an endotoxin-free plasmid purification kit from Qiagen (Valencia, CA), and sequenced to verify the gene and promoter nucleotide sequences as well as the gene orientations. In this final construct, the M1 and 1918HA genes were under the control of the polyhedrin promoter and in opposite direction whereas M2 and 1918NA were also in opposite direction to each other and under the control of the p10 promoter (Fig. 1A).

Generation of baculovirus recombinant virus that produces 1918 virus-like particles

To create a baculovirus recombinant, Sf9 insect cells were seeded at a density of $\sim 2 \times 10^6$ onto 60-mm dishes and subsequently transfected with a mixture of ~ 2 μg of the 1918-transfer vector and 0.5 μg of linearized BaculoGold Bright baculovirus DNA (BD Biosciences Pharmingen, San Diego, CA). This linear baculovirus DNA carries within its genome the green fluorescent protein (GFP) gene, and therefore the infectious virus generated by homologous recombination within the insect cells expresses GFP in addition to the influenza proteins required for VLP assembly. GFP serves as a marker for recognizing and selecting virus recombinants as well as for accurate determination of viral titers and multiplicities of infection. GFP-producing baculovirus recombinants were

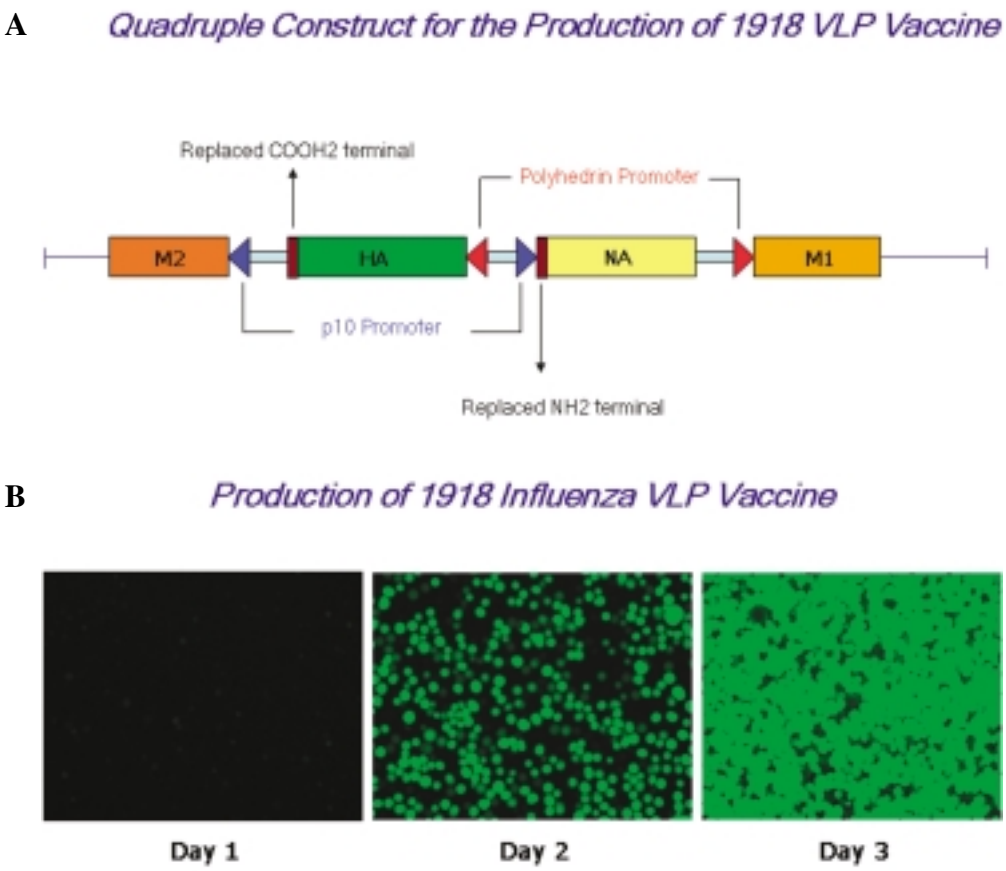

FIG. 1. (**A**) Quadruple construct for the production of 1918 VLP vaccine. Diagram of the position and orientation of the four influenza genes in the quadruple baculovirus recombinant. Formation of 1918 VLPs is accomplished by simultaneous expression of HA and NA of the 1918 influenza A virus together with the M1 and M2 proteins derived from influenza virus A/Udorn/73. The transmembrane domains and cytoplasmic tails of the 1918 HA and NA were replaced by those of the influenza A/Udorn virus glycoproteins (NH2 terminus in NA and COOH terminus in HA). In this construct, the HA and M1 genes are in opposite orientation to each other and under the transcriptional control of the baculovirus polyhedrin promoter, whereas M2 and NA are under the transcriptional control of the p10 promoter and in opposite orientation to each other. (**B**) Production of 1918 influenza VLP vaccine. Production of 1918 VLP vaccine by infected Sf9 cells was monitored by following the expression of the green fluorescent protein (GFP) on days 1, 2, and 3 postinfection. The quadruple baculovirus recombinant expresses GFP in infected cells in addition to the four influenza proteins required for VLP assembly; therefore GFP expression served as an indicator of infection and VLP production. On day 2 postinfection most of the cells were expressing GFP, which increased by day 3; this suggest that most of the cells were producing VLP by day 3.

selected, expanded, and analyzed by polymerase chain reaction (PCR) and Western blot to verify the presence of the four genes and their expression, respectively. The selected recombinant virus was amplified and subsequently titrated in Sf9 cells by using as readout the microscopic detection of GFP in the highest of triplicate end-point dilutions.

Production and purification of 1918 VLP vaccine

Sf9 cells were grown in shaker flasks with serum-free medium at 28°C. For vaccine production, cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 1, in one-tenth the final culture volume. Viral absorption to cells was allowed for 1 h, at which point fresh medium was added to bring the culture to its final volume. Progression of the infection was monitored by taking cell samples and observing, under a microscope, the expression of GFP (see Fig. 1B). Ninety-six hours postinfection, the culture supernatant, which contained the vaccine, was separated from the cells by low-speed centrifugation (2000 × *g* for 15 min at 4°C). The vaccine particles were then pelleted by centrifugation of the supernatant at 200,000 × *g* for 90 min. Depending on the number of cells initially infected, the vaccine pellet was resuspended in 0.5 or 1 mL of 1× phosphate-buffered saline (PBS), homogenized by brief sonication, and then loaded on top of an iodixanol (Op-

tiprep; Nycomed, Roskilde, Denmark) gradient (density of 1.08 to 1.32 g/mL). The gradient was spun at 200,000 × *g* for 3 h and the vaccine particles under these conditions formed a band within the top one-third of the gradient (15,16), from where they were collected. Vaccine particles were dialyzed in 1× PBS and this preparation was used as VLP vaccine alone. Purified vaccine material was analyzed by Western blot to confirm the presence of specific proteins and to determine the HA content as previously performed (16).

Western blot analysis of purified VLP vaccine

The protein content of the 1918 VLP vaccine was evaluated with antibodies to the 1918 HA and M1 proteins. A mouse monoclonal anti-1918 HA antibody (generously provided by P. Palese, Mount Sinai School of Medicine, New York, NY) was used as primary antibody to detect the 1918 HA. Rabbit anti-mouse horseradish peroxidase-conjugated IgG (Bio-Rad, Hercules, CA) was used as secondary antibody. Similarly, a mouse polyclonal anti-M1 antibody (AbD; Serotec, Raleigh, NC) was used to detect the M1 protein, which was derived from influenza A/Udorn/72. The M1 and M2 proteins share nine amino acids at their NH_2 termini and a band of the size expected for the M2 protein was observed with high levels of VLP and M1 antibody. We have not analyzed for the presence of the NA protein in the purified VLP vaccine by immunoblot.

Neuraminidase assay

The presence of neuraminidase (NA) was evaluated by detecting its enzymatic activity (sialidase), which cleaves the terminal sialic acid residues from glycoproteins (22). In this assay, purified VLPs were incubated with fetuin as substrate for 16 h at 37°C. The amount of free sialic acid released from the substrate by the enzymatic activity of the NA was detected with thiobarbituric acid, which produces a pink color in proportion to the amount of free sialic acid in the assay. A reaction with PBS and fetuin was carried out as control and used as a blank for the spectrophotometric readings. Influenza A/swine virus was tested as positive control. Color intensity was measured spectrophotometrically at a wavelength of 549 nm. NA enzymatic activity was expressed as optical density (OD) at 549 nm.

VLP vaccine hemagglutination assay

The ability of the 1918 VLP vaccine to agglutinate red blood cells (RBCs) was evaluated in a standard hemagglutination assay using cells from two different species, chicken and turkey. Briefly, 2-fold serial dilutions of the purified VLP vaccine were carried out with 1× PBS in V-shaped 96-well plates. An equal volume of a 0.5% solution of RBCs in 1× PBS was added to the wells and the plate was incubated at 4°C for 1 h. After this time, the appearance of an RBC precipitate (RBC button) indicated a lack of hemagglutination. Hemagglutination titers were expressed as the inverse of the highest dilution of the vaccine able to agglutinate RBCs.

Generation of CHO cells that express 1918 HA proteins

As an alternative source of antigen, a CHO cell line that expresses the 1918 HA protein was created by cloning the HA gene (see above) into the plasmid pcDNA 3.1/v5-His (Invitrogen, Carlsbad, CA) by ligation into the *Xba*I/*Kpn*I restriction sites, which were added to the ends of the synthesized HA genes by PCR. Purified recombinant plasmid DNA was transfected into CHO cells with Lipofectamine 2000 reagent (Invitrogen). Subsequently, transfected cells were cultured in serum-free medium containing neomycin at a concentration of 2 mg/mL as selecting agent (a kill curve with normal CHO showed that neomycin at 1 mg/mL kills all the cells in 7 d). Cells were subcultured under selective pressure conditions for 28 d and then tested for HA 1918 expression.

Antibody response elicited by 1918 VLP vaccination

The antibody response elicited by VLP vaccination was evaluated by ELISA, using 96-well plates (Immulon II; Thermo Lab Systems, Franklin, MA) coated with either detergent-disrupted purified influenza virus A/Swine/Iowa/15/30 (H1N1) or CHO cells expressing the 1918 HA protein as antigens. Virus-coated plates received 50 ng of total viral protein per well whereas the CHO cell-based ELISA plates were seeded with an equivalent number of cells and incubated until they reached confluency, at which time they were fixed with a 1:1 mixture of methanol–acetone for 10 min at room temperature. Both ELISA plates were blocked for 1 h at room temperature with PBS solution, pH 7.2, containing 1% bovine serum albumin (BSA), 2% goat serum, 2% nonfat milk, and 0.05% Tween 20 and subsequently washed three times with PBS containing 0.05% Tween 20 (PBST). Serial dilutions of individual serum samples were applied to either of the plates and incubated for 1 h at room temperature, followed by three washes with the PBST solution. Subsequently, the plates were incubated for 1 h at room temperature with 100 μL of a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody, diluted 1:1000 in PBS plus 1% BSA, 2% goat serum, and 0.05% Tween 20 and this was followed by another set of three washes with PBST.

Finally the plates were incubated with 100 μL of TMB

solution (Pierce Biotechnology, Rockford, IL) and monitored for color development. The color reaction was stopped by adding 100 μL of 0.1 M HCl. The absorbance was determined at 450 nm with a Thermo Multiskan EX plate reader. Absorbance titers were determined as the highest serum dilution that had an optical density twice that given by the preimmunized control serum.

The 1918 HA-CHO cell-based ELISA was optimized by using CHO cells that expressed the 1918 HA, parental CHO cells that did not express HA, an anti-1918 HA mouse monoclonal antibody as positive control serum (see above), and mouse preimmunization serum as negative control. Using the two cell lines and antibodies, a standard curve was established that verified the validity of the assay (data not shown).

Expansion and titration of influenza challenge virus

The influenza A Swine/Iowa/15/30 (H1N1) virus (VR-333; American Type Culture Collection [ATCC], Manassas, VA), antigenically related to and most contemporary with the 1918 influenza virus, was used as surrogate challenge. This virus was grown in 10-d-old embryonated chicken eggs (specific pathogen free [SPF]; Charles River Laboratories, Wilmington, MA) for 48 h postinfection at 37°C. The infected allantoic fluid was harvested and clarified by low-speed centrifugation and viral titer was determined by the plaque assay in MDCK cells. Aliquots of the virus were stored at −80°C until use.

Vaccination regimen and sample collection

The immunogenicity and protective efficacy of the 1918 VLP vaccine was tested in 7- to 8-wk-old, female BALB/c mice (Charles River Laboratories). Mice were housed in the Department of Comparative Medicine (New York Medical College, New York, NY) and the study was carried out according to institutional IACUC-approved protocols.

Four groups of 12 mice each received vaccine, placebo, and control treatment. The two vaccine groups received (via the intranasal route) two doses, 2 wk apart, of VLP vaccine (1 μg of HA content per dose) alone or admixed with 10 μg per dose of an oligonucleotide (20-mer ODN). This ODN contained two CpG motifs located in the middle of the molecule and spaced by two bases (CGXXCG) and flanked by complementary sequences able to form a stem–loop structure. The placebo and control groups also received via the intranasal route two doses of either PBS or formalin-inactivated influenza A/Swine/Iowa/15/30 (H1N1) (~1 μg of HA content), respectively (16).

Two weeks after the primary and booster immunizations, mice were anesthetized with ketamine–xylazine (70 and 6 mg/kg body weight, respectively) and blood samples were collected via retro-orbital bleeding. Eighteen days after the booster immunization, mice in all groups (anesthetized as described above) were challenged via the intranasal route with 1×10^6 plaque-forming units (PFU)/20 μL of influenza virus A/Swine/Iowa/15/30 (H1N1), a surrogate for the extinct 1918 influenza virus. The challenge dose was delivered as small drops (10 μL per nostril), using a Pipetman with an ultraslim capillary sequencing tip. Mice were monitored daily for weight loss and clinical signs and severity of infection. On days 2, 4, 6, and 8 postchallenge, four mice from each group were sacrificed and their nasal passages and trachea/lungs were harvested and placed in 1.5 mL of SPG (0.22 M sucrose, 0.01 M potassium phosphate, and 0.005 M potassium glutamate in PBS, pH 7.2). Tissues were homogenized for 1.5 min with an Omni International TH homogenizer equipped with a saw-tooth generator. Samples were centrifuged at $2500 \times g$ for 10 min to pellet cell debris and clarified supernatant was stored at −80°C until viral titrations were performed.

Determination of viral titers in the upper and lower respiratory tract by NP ELISA

Viral load in the nasal passages and trachea/lung tissues was determined in a cell-based ELISA using an anti-NP antibody that recognizes the nucleoprotein (NP) of influenza A viruses. This assay is sensitive and highly specific allowing for the detection of low levels of NP protein expressed in infected cells. MDCK cells were seeded at a concentration of 5×10^4 cells per well in 96-well tissue culture plates and incubated overnight at 37°C in 5% CO_2. Cell monolayers were washed with PBS and infected with 100 μL of 10-fold serial dilutions of tissue homogenates in PBS. Lung/trachea and nasal tissue samples were assayed in septaplicate on the same 96-well plate. Infected plates were incubated at room temperature for 1 h and then the inoculum was removed and replaced with 100 μL of minimal essential medium (MEM) containing penicillin (50 U/mL), streptomycin (50 mg/mL), and TPCK-treated trypsin (1 μg/mL); (Worthington Biochemical, Lakewood, NJ). Subsequently, plates were incubated at 37°C in 5% CO_2 for an additional 40 h, at which time the plates were spun down at $2800 \times g$ for 12 min and then fixed for 10 min at room temperature with 100 μL of an acetone–methanol (1:1) mixture.

Plates were then washed six times with buffer (PBS with 0.05% Tween 20), blocked for 1 h at room temperature with 150 μL of blocking solution (5% nonfat milk, 1% BSA [Pierce Biotechnology], 2% normal goat serum [Vector Laboratories, Burlingame, CA], and 0.05% Tween 20 in PBS), and washed again three times with wash buffer. Subsequently, 100 μL of a mouse mono-

clonal anti-NP primary antibody (influenza A H1N1 clone IVF8 [Biodesign International, Saco, ME] diluted 1:3000 in 1% BSA, 2% normal goat serum, and 0.05% Tween 20 in PBS) was added and then plates were incubated for 1 h at room temperature. Primary antibody was not added to rows 6 and 12 so that background signals could be subtracted during viral titer calculation. Plates were again washed three times with wash buffer and then 100 μL of a secondary goat anti-mouse antibody conjugated with HRP (1:2500 dilution in 1% BSA, 2% normal goat serum, and 0.05% Tween 20 in PBS) (Bio-Rad) was added and incubated for 1 h at room temperature. Again, plates were washed six times with wash buffer and 100 μL of Ultra-TMB (Pierce Biotechnology) was added and rocked at room temperature until color development. The reaction was stopped by adding 100 μL of a 0.2 M HCl acid solution when the desired color intensity was achieved. Absorbance in each plate was measured at 450 nm with a Thermo Multiskan EX plate reader. Viral titers were expressed as $TCID_{50}$ and were calculated according to the Reed–Muench method (23).

Statistical analysis

Statistical analyses were performed using the two-tailed Student *t* test for unpaired samples with unequal variance. *p* Values (*p*) less than 0.05 were considered statistically significant.

RESULTS

Production and analysis of 1918 influenza virus-like particle vaccine

The 1918 VLP vaccine was produced in Sf9 cells after infection with the 1918 baculovirus recombinant (MOI of 1), which carries the HA (A/South Carolina/1/1918) and NA (A/Brevig_Mission/1/1918) genes of the 1918 pandemic influenza virus (H1N1) (19,20,24) and the M1 and M2 genes derived from influenza A/Udon/72 (H3N2) (Fig. 1A). To maintain optimal interactions between the surface glycoproteins and the matrix protein, the sequence of the HA gene encoding the COOH-terminal 30 amino acids comprising the cytoplasmic tail and transmembrane domains, was replaced during synthesis with those of HA3 of the influenza A/Udorn virus (15). Similarly, the sequence encoding the NH2-terminal 26 amino acids of the NA gene, comprising the cytoplasmic tail and transmembrane domains, was also replaced with the analogous sequence of NA of the influenza A/Udorn virus (Fig. 1A).

Infected Sf9 cells were cultured in suspension in shaker flasks (120 rpm) and the efficiency and progression of infection were monitored by tracking the expression of green fluorescent protein (GFP). Fluorescence microscopic examination of infected cells was performed 20, 40, and 60 h postinfection to determine the percentage of cells expressing GFP and the intensity of the GFP signal. Because the GFP gene is carried within the genome of the recombinant virus that expresses the VLP-forming proteins, its expression denotes production of the 1918 VLP vaccine by the infected cells (Fig. 1B).

The 1918 VPL vaccine was purified from the culture supernatant as described above and the presence of the HA and M1 proteins in the final vaccine preparation was evaluated by Western blot. This test demonstrated that the 1918 HA protein was indeed present in the purified 1918 VLP vaccine fractions (Fig. 2A, panel A). When an antibody to the M1 protein was used in Western blot, it was demonstrated that M1 protein was present in the same two VLP vaccine fractions as the HA protein (Fig. 2A, panel B). The M2 protein was detected when higher concentrations of purified material were loaded in the gel (data not shown). Expression and incorporation of the NA protein were evaluated in purified VLPs by a neuraminidase assay (Fig. 2C), which showed that NA was indeed assembled onto the VLP as previously shown by immunofluorescence and immunogold-labeled electron microscopy (15).

An intrinsic property of all influenza viruses is the capacity to agglutinate red blood cells (RBCs) of different species. Therefore, it was important to determine whether the purified 1918 VLPs, which morphologically resemble the influenza virus, were able to agglutinate RBCs. The agglutination assay was performed with RBCs of two different species, chicken and turkey. The RBCs of both of these species express on their surface a mixture of HA receptor with sialic acid linked to galactose by either an α-2,3 (SAα-2,3) or an α-2,6 linkage (SAα-2,6) (25,26). It was quite surprising to find that the 1918 VLPs were functional in hemagglutination with turkey RBCs but were unable to perform this function, even at lower dilutions, with chicken RBCs (Fig. 2B). This appears to be a peculiarity of the 1918 HA because VLPs bearing surface antigens derived from other viruses, such as HA7 and HA5, were able to perform this function with RBCs of both species (data not shown).

Immune response elicited by intranasal administration of 1918 VLP vaccine

The magnitude of the serum antibody response elicited by two doses of the 1918 VLP vaccine administered by the intranasal route and formulated in either PBS alone or with PBS plus 10 μg per dose of an ODN containing two CpG motifs was determined by ELISA utilizing sucrose gradient-purified and detergent-disrupted influenza virus A/Swine/Iowa/15/30 (H1N1) or CHO cells expressing the 1918 HA protein as antigens. Similar assays were used to measure the serum antibody response elicited by

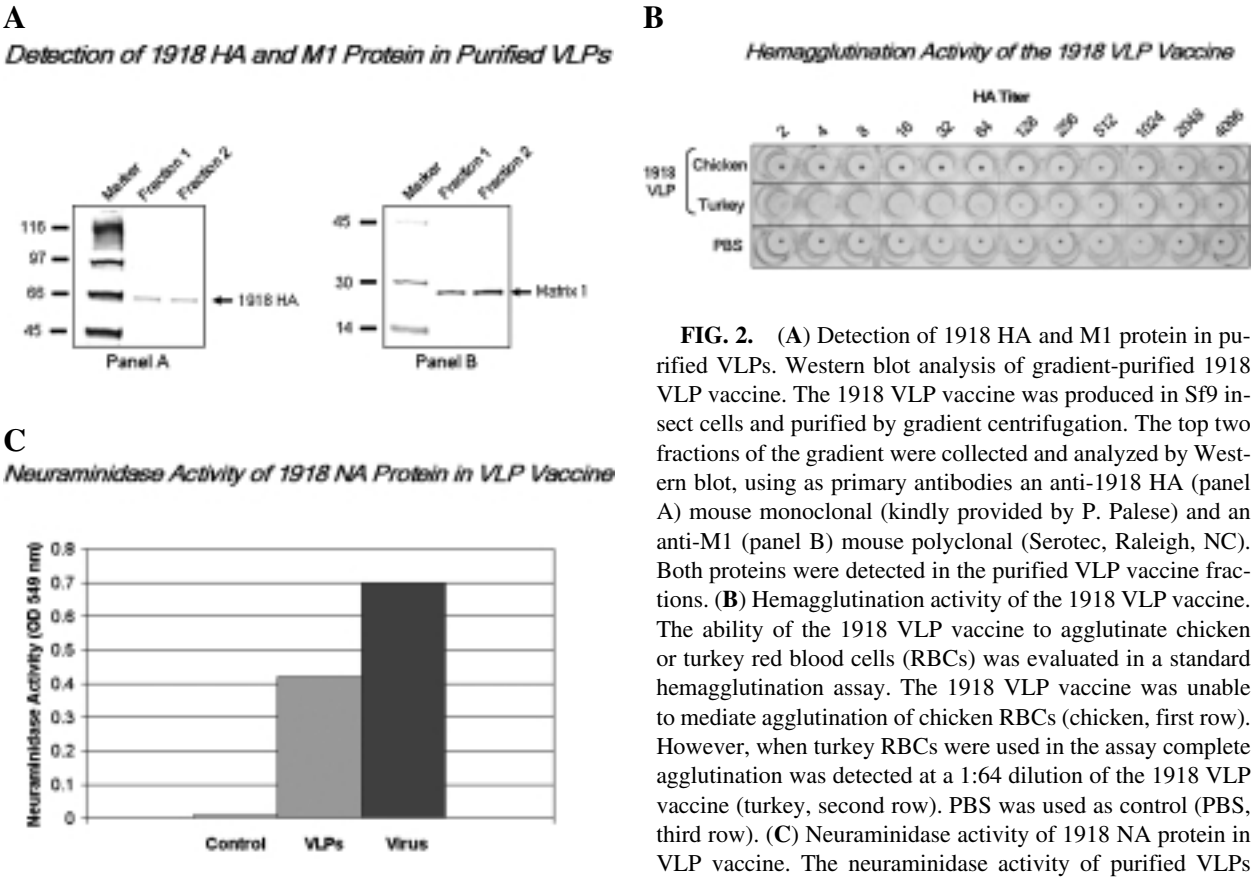

FIG. 2. (**A**) Detection of 1918 HA and M1 protein in purified VLPs. Western blot analysis of gradient-purified 1918 VLP vaccine. The 1918 VLP vaccine was produced in Sf9 insect cells and purified by gradient centrifugation. The top two fractions of the gradient were collected and analyzed by Western blot, using as primary antibodies an anti-1918 HA (panel A) mouse monoclonal (kindly provided by P. Palese) and an anti-M1 (panel B) mouse polyclonal (Serotec, Raleigh, NC). Both proteins were detected in the purified VLP vaccine fractions. (**B**) Hemagglutination activity of the 1918 VLP vaccine. The ability of the 1918 VLP vaccine to agglutinate chicken or turkey red blood cells (RBCs) was evaluated in a standard hemagglutination assay. The 1918 VLP vaccine was unable to mediate agglutination of chicken RBCs (chicken, first row). However, when turkey RBCs were used in the assay complete agglutination was detected at a 1:64 dilution of the 1918 VLP vaccine (turkey, second row). PBS was used as control (PBS, third row). (**C**) Neuraminidase activity of 1918 NA protein in VLP vaccine. The neuraminidase activity of purified VLPs was assayed in an enzymatic reaction with fetuin as substrate. A half-dilution of purified VLP was tested in this assay. Influenza A/swine virus was used as positive control and a complete reaction without enzyme served as the negative control. Neuraminidase activity is expressed as the optical density (OD) at a wavelength of 549 nm.

two intranasal immunizations with inactivated swine influenza virus and placebo control. Mice vaccinated with the 1918 VLP vaccine with or without CpG as well as mice immunized with the inactivated virus demonstrated an antibody response when disrupted virus was used as antigen (Fig. 3). However, the magnitude of the antibody response in mice immunized with inactivated virus was almost three times higher than the response elicited by either formulation of the 1918 VLP vaccine. Two important aspects of the viral antigen ELISA may account for the difference between the antibody responses elicited by the 1918 VLP vaccine and the inactivated virus. First, there are antigenic differences between the 1918 HA, which is part of the VLP vaccine, and the HA of the swine virus, which is the coating antigen for the virus ELISA (see Fig. 4). Second, the inactivated virus vaccine delivers a larger number of virus-specific proteins/antigens (e.g., nucleoprotein [NP]) than the number of antigens present in the 1918 VLP vaccine. The antibodies these additional antigens elicit would enhance the overall immune response in the ELISA.

The level of serum antibody elicited by either the 1918 VLP vaccine alone or admixed with CpG was similar (Fig. 3). Thus, adding CpG–ODN to the 1918 VLP vaccine did not enhance the serum antibody response. This outcome might be expected because CpG–ODN mediates immune modulation through interaction with the innate immunity TL9 receptor, which in turn triggers the release of cytokines (such as interferon [IFN]-γ, IFN-α, interleukin [IL]-6, and IL-10) activating B cells and the cellular arm of the immune system (27).

When the ELISA was carried out with CHO cells expressing the 1918 HA as antigen, serum IgG titers were also detected in mice immunized with either of the 1918 VLP vaccine formulations or inactivated virus. With this approach, however, the antibody levels measured in both of the 1918 VLP vaccine groups were higher than the level measured with the inactivated virus (Fig. 3). This variation is expected because, as presented above, there are antigenic differences between the HAs of the 1918 and Swine viruses, which could account for the antibody level difference (Fig. 3).

When CHO cells expressing the 1918 HA were used as antigen in the ELISA, as also found with the disrupted virus ELISA, there was not a significant difference in the levels of serum IgG between mice immunized with the

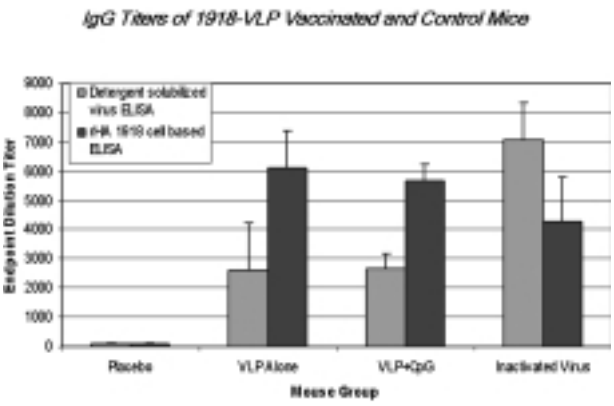

FIG. 3. IgG titers of 1918-VLP vaccinated and control mice. Serum antibody responses to 1918 virus-like particles (VLPs). The humoral immune response elicited by two intranasal immunizations, 2 wk apart, with 1918 VLP vaccine alone and plus CpG, inactivated challenge virus, or placebo control was evaluated by ELISA in serum samples collected 3 d before challenge with the influenza virus A/Swine/Iowa/15/30 (H1N1). Two alternative antigens, detergent-disrupted virus or CHO cells expressing a 1918 rHA, were used as antigens to coat the ELISA plates. The data shown represent the average of all the individual measurements.

1918 VLP vaccine alone or adjuvanted with CpG–ODN. However, the CHO cell ELISA measured higher antibody levels for both 1918 VLP vaccine formulations than the virus-disrupted ELISA for the same groups. The differences in the levels of serum antibody measured with the two ELISAs are likely to be a reflection of the antigenic differences between the HAs of the 1918 and swine virus (Fig. 4). The antibody response elicited by a single VLP immunization stimulated a low level of antibody production (data not shown).

Protective efficacy afforded by 1918 VLP vaccine

To evaluate the level of protection afforded by two doses of the 1918 VLP vaccine, formulated in PBS alone or in PBS plus CpG–ODN, inactivated virus control, and placebo inoculations, administered via the intranasal route, mice in all groups were challenged 17 d after the second immunization with 1×10^6 PFU of influenza A/Swine/Iowa/15/30 (H1N1) virus. This influenza virus was selected as a surrogate challenge because it is the most contemporary and antigenically related to the 1918 virus currently available, other than the reconstructed 1918 virus (28). Preliminary experiments in mice showed that the swine virus at the dose used as challenge causes severe influenza illness with typical clinical signs of the disease such as progressive inactivity beginning around day 3 postinfection, ruffled fur, labored breathing, a tendency to huddle, and reduced or absent water and food intake leading to severe weight loss. All these parameters were subsequently monitored during the protective efficacy study with the 1918 VLP vaccine.

Vaccinated and control mice were slightly anesthetized, as described above, before receiving the virus challenge (1×10^6 PFU), which was contained in a volume of 20 μL and administered by intranasal instillation of very small droplets delivered by ultraslim sequence tips (10 μL per nostril). After virus challenge, three different measurements were used to assess vaccine protective efficacy: body weight measurement, clinical signs of illness, and viral titers in nasal and trachea/lung tissues. Clinical signs of influenza illness were scored as follows: (+) no clinical sign of disease, although body weight measurement may indicate slight body mass losses; (++) ruffled fur, inactivity, tendency to huddle; (+++) hunched back, pronounced ruffled fur, severe inactivity; (++++) labored breathing (high frequency and abdominal panting), severely hunched back, ruffled fur, complete inactivity (no response to stimulation), and severe weight loss.

Group average of daily weight measurements showed that mice immunized with the 1918 VLP vaccine plus CpG experienced a slight decrease in body weight between days 4 and 8 postchallenge, without clinical signs of influenza infection (score, +; Fig. 5); however, the weight did not drop lower than their starting weights on day 0. The mice in the 1918 VLP vaccine-alone group experienced a more pronounced reduction of body weight that continued until day 6, at which point the trend reversed and mice began to gain weight. Mice in this group,

FIG. 4. Sequence alignment of antigenic sites of the 1918 and swine virus HA. Amino acid differences between swine and 1918 HAs. Four antigenic sites have been mapped on the HA1 globular portion of the H1 subtype of HA (Ca, Cb, Sa, and Sb) (30,31). Amino acid changes at these sites drive antigenic drift and immunoevasion. Alignment of these regions between the swine/15/30 and South Carolina/1/1918 HAs demonstrates that 5 (1–5) of the 22 amino acid differences on HA1 mapped to the four antigenic sites. One change (o) is adjacent to the Sb site and may influence the antibody interaction with this site.

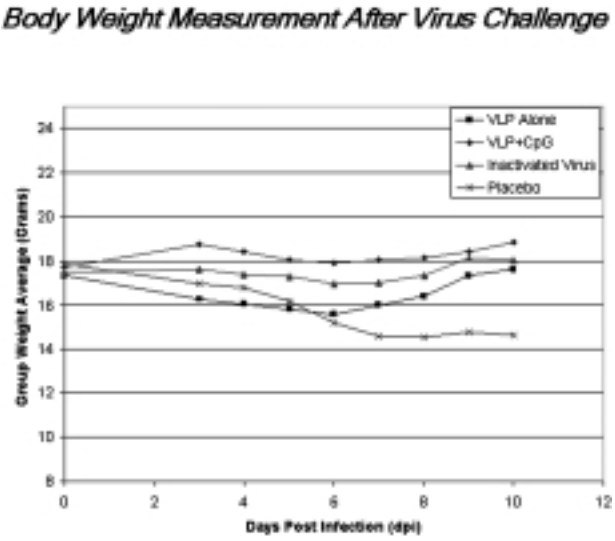

FIG. 5. Body weight measurement after virus challenge. The body weight of each mouse was monitored daily after challenge and data are presented as group weight average in grams versus days postinfection. The total number of animals per group was reduced by four after days 2, 4, 6, and 8 when mice were sacrificed for viral load determination. After day 10, the remaining mice in the VLP vaccine and inactivated virus groups were completely normal whereas the remaining four mice in the placebo group continued a slow recovery until day 15, when the experiment was concluded.

however, showed only minor clinical signs of illness (score, ++), quite opposite to the inactivated virus control group, which showed more severe clinical signs of disease (score, between ++ and +++) with a less pronounced body weight loss tendency until day 6, when these mice also began gaining weight (Fig. 5). The placebo control group showed a pronounced weight loss that persisted beyond day 6 and stabilized around day 8 without clear recovery on day 15, when the experiment was terminated. Symptoms were quite severe in this group (score, ++++) and one mouse died on day 5 postchallenge. Two, 4, 6, and 8 d postchallenge, four animals per group were killed and viral titers in the nasal tissue as well as in the trachea/lungs were determined in a cell-based assay (see Materials and Methods). Hence, the score of clinical signs and group average of body weight reported after each time point were those collected from the remaining animals in each group.

Mice immunized with the 1918 VLP vaccine alone or with the 1918 VLP plus CpG had significantly lower viral titers in the nasal tissue on days 2 ($p < 0.0005$), 4 ($p < 0.05$), and 6 ($p < 0.005$) postchallenge as compared with the placebo group (Fig. 6A). On day 6 postchallenge, the 1918 VLP plus CpG-vaccinated mice showed complete clearance of the swine virus whereas the VLP-alone vaccine group still showed virus in the nasal tissue (Fig. 6A). Mice immunized with the inactivated virus also had a significantly lower titer than the placebo control group on days 2 ($p < 0.005$) and 4 ($p < 0.05$) postchallenge; however, these titers were slightly higher on average than the viral load detected in the nose of mice immunized with either of the two 1918 VLP vaccines (Fig. 6A). Virus was not detected in the nose of any of the groups on day 8 postchallenge.

The level of protection of the lower respiratory tract provided by either of the two 1918 VLP vaccine formulations, the inactivated virus, or the placebo treatment was also evaluated by assessing viral loads in the trachea/lung tissues on days 2, 4, 6, and 8 postchallenge. This showed that mice vaccinated with the 1918 VLP vaccine alone, or with 1918 VLP plus CpG, had significantly lower viral titers in the trachea/lung tissues, on days 2 ($p < 0.05$), 4 ($p < 0.05$), and 6 ($p < 0.005$) postchallenge than mice in the placebo group (Fig. 6B). Furthermore, mice immunized with the 1918 VLP vaccine plus CpG were able to completely clear the viral infection from the lower respiratory tract by day 6 postchallenge, whereas mice in the 1918 VLP vaccine-alone and inactivated virus groups still had virus at this time, although at significantly lower titers than the placebo control group. Viral titers in the trachea/lungs of mice immunized with the 1918 VLP vaccine alone were on day 4 significantly lower than those of mice immunized with the inactivated virus control; however, on days 2 and 6 they showed similar levels of virus that cleared by day 8 in both groups as well as in the placebo control.

Even though virus was detected in mice vaccinated either with the 1918 VLP alone, 1918 VLP plus CpG, or inactivated swine virus vaccine, on day 2 postchallenge there was a significant difference in viral titers by about a 1- or 2-log difference in the trachea/lung and nasal tissues, respectively, as compared with the placebo group. The data also indicated that the viral load was higher in the lower respiratory tract than in the upper tract, but complete virus clearance occurred on day 8 postchallenge in both upper and lower tracts of all the groups.

DISCUSSION

Here, we present data on the production, properties, immunogenicity, and efficacy of a virus-like particle (VLP) vaccine designed to protect against infection with the 1918 influenza virus. VLPs are structures that morphologically resemble an influenza virus, but are devoid of the genetic material required for viral replication and infection. The use of VLP technology allows for the safe creation of vaccines against extremely dangerous pathogens such as the 1918 influenza virus. We have previously demonstrated

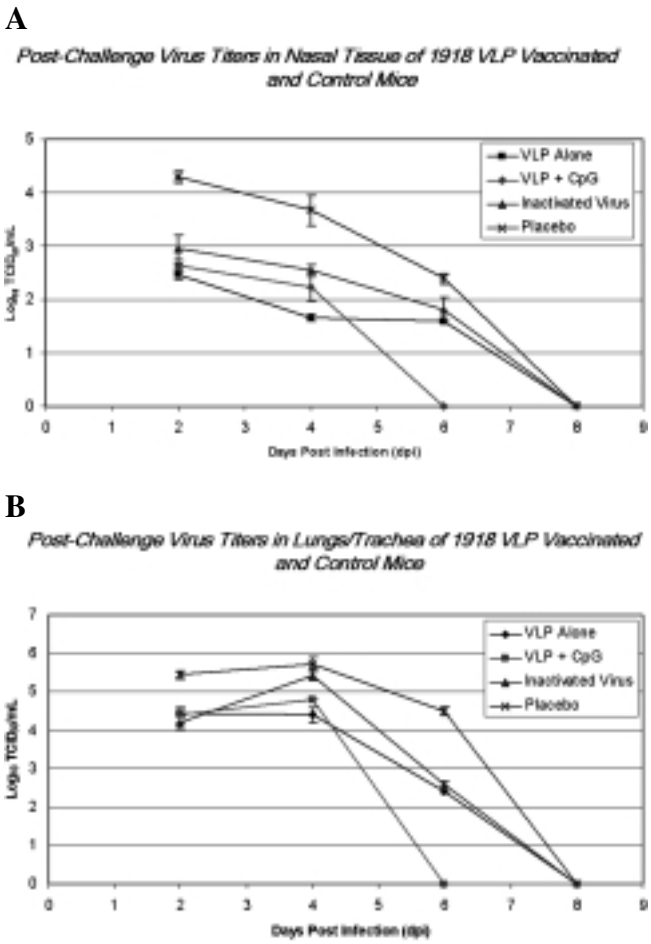

FIG. 6. **A:** Post-challenge virus titers in nasal tissue of 1918 VLP vaccinated and control mice. (**A** and **B**) **B:** Post-challenge virus titers in lung/trachea of 1918 VLP vaccinated and control mice. Viral titers in nasal tissue (**A**) and trachea/lung tissue (**B**) of VLP-vaccinated and control mice were measured four times after virus challenge. VLP-vaccinated and control mice were challenged via the intranasal route with 1×10^6 PFU of the influenza A/Swine/Iowa/15/30 (H1N1) virus. On days 2, 4, 6, and 8 postchallenge, four animals per group were killed, trachea/lung tissues were harvested, and viral titers were determined by an MDCK cell-based ELISA. Viral titers ($\log_{10}$ $TCID_{50}$/mL) were plotted versus days postinfection (dpi). Each time point represents the average titer of four mice and the vertical lines indicate the standard deviation values.

the feasibility of the formation and release of influenza VLPs from the cell surface (15) and also that these structures are able to stimulate an immune response able to protect against a lethal influenza virus challenge (16). Here we have created VLP structures that carry the HA and NA surface glycoprotein of the 1918 influenza virus. These VLPs were purified from the culture supernatant of Sf9 insect cells infected with a quadruple baculovirus recombinant that expresses the four influenza structural proteins needed to form the VLP structure.

To generate this construct, the HA and NA genes of the 1918 virus were synthesized *de novo* with changes in the sequences that encode the cytoplasmic tails and transmembrane domains, which were entirely replaced with the same domains of HA and NA of the influenza virus A/Udorn/72 (H3N2). These changes were introduced to optimize their interaction with the matrix protein M1, which is also derived from the same influenza virus A/Udorn/72 (H3N1).

Purified VLPs were functional in the agglutination of RBCs, a property of all influenza viruses; however, this activity was demonstrated with turkey RBCs and not with chicken RBCs, which are typically used for this type of assay. There are significant differences in the glycosylation patterns between insect and mammalian cells that can account for this hemagglutination distinction of the 1918 VLPs; however, we have not seen this discrimination with other HA subtypes that have been incorporated onto the surface of the VLPs. This difference may reflect intrinsic properties of the 1918 HA-produced insect cells and its interaction with surface receptor molecules on the red blood cells. Studies on the 1918 HA (A/South Caroline/1/18) receptor specificity have shown that this HA preferentially binds chicken red blood cells with recep-

tors bearing sialic acid attached to galactose in an α-2,6 linkage (SAα-2,6Gal) (28), which is predominant in human tracheal epithelium. In addition, substitution of two amino acids can alter the HA specificity from an α-2,6 to an α-2,3 (SAα-2,3Gal) receptor preference, abrogating viral transmission in a ferret model (29). We have not modified the primary sequence of H1 where the receptor-binding pocket resides, suggesting that other posttranslational modifications such as glycosylation may influence the HA–receptor binding interaction, which may also be important in viral transmission or virulence.

In this study, we used a homologous swine virus as a challenge in immunized mice because previous studies have shown that mice vaccinated via the intramuscular route with 10 μg of inactivated swine virus were protected against a lethal dose of a 1918 HA/NA/M/NP/NS:WSN recombinant virus (13). This previous work has also shown that challenge virus was undetected in brain and lung tissue on day 5 postchallenge; however, it is not clear whether mice vaccinated with the swine virus showed any clinical signs of influenza disease or weight loss. In our study, the level of anti-HA antibody elicited by the 1918 VLP vaccine administered via the intranasal route appears to confer significant protection against the swine virus challenge, even though there is not complete homology between the vaccine and viral challenge HAs. Amino acid alignment of the HA1 portion of these two H1 subtype HAs showed that there are 22 amino acid differences between these two molecules, 5 of which are located in the antigenic sites previously identified for H1 subtypes (30,31). One additional change is adjacent to the antigenic site Sb and could potentially affect the antigenic properties of this site. The significance of these changes in our study resides in the fact that one amino acid mutation in each of these sites may significantly reduce or even preclude virus neutralization (32). Despite these differences, VLP vaccination conferred significant protection against the swine virus.

Only serum anti-HA antibody responses were measured in this study; however, it is reasonable to speculate that intranasal immunization with VLPs may have stimulated a more cross-protective local IgA response that enhanced control of virus replication at the mucosal surface. Several studies have shown that mucosal immunization elicits broader cross-protective (heterosubtypic) responses than parenteral vaccination (33,34). Both VLP vaccines have shown on average a statistically significant lower viral titer in both the upper and lower respiratory tracts than that found in the placebo group. And although, there was not a significant difference in the serum antibody titers, the VLP plus CpG vaccine conferred complete protection against disease and cleared the virus from the respiratory tract faster than VLP alone or the swine virus vaccine. The enhanced protection provided by the VLP plus CpG vaccine suggests that the CpG–ODN may have either stimulated a specific cell-mediated response that hastened virus clearance in the airways or augmented the levels of cross-reacting anti-HA IgA, improving cross-protection against the swine virus challenge, which is an antigenic drift from the 1918 virus. Although the VLP vaccine was purified away from the baculovirus, we cannot completely rule out the possible presence of contaminant baculovirus material, which could enhance the immunogenicity of the VLP vaccine. Studies aimed at the characterization of the mucosal immune response as well as at understanding how to enhance VLP vaccine potency are planned.

The vaccine created and evaluated in this study serves as the basis for advancing the production and further characterization of an effective VLP vaccine to protect humans against the 1918 influenza virus. VLP vaccine production can be attained in a cell-based system without generating infectious material, thus eliminating manufacturing safety concerns. Furthermore, the intranasal route of immunization offers a noninvasive vaccine delivery approach that not only stimulates systemic and local immune responses but could also neutralize influenza virus infection at the mucosal port of entry. The VLP vaccine for this study was produced at laboratory scale; however, the system is suitable for scale-up and large production.

Considering the devastation of the 1918 pandemic, it would be prudent to pursue the development of a safe and efficacious vaccine that could be available to halt a potential outbreak and prevent a potential pandemic in the event that the 1918 virus or an antigenically related strain reappears.

ACKNOWLEDGMENTS

The authors thank Dr. George Martin for reviewing this manuscript. This work was supported by NIAID/NIH SBIR grant 1-R43-AI063830-01 to J.M.G.

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