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Brief Report

Virus-Like Particle (VLP) Vaccine Conferred Complete Protection against a Lethal Influenza Virus Challenge

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ABSTRACT

We have previously demonstrated the formation and release of influenza virus-like particles (VLPs) from the surface of Sf9 cells infected with either a quadruple baculovirus recombinant that simultaneously expresses the influenza structural proteins hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1) and M2, or a combination of single recombinants that include the M1 protein. In this work, we present data on the immunogenicity and protective efficacy afforded by VLPs (formed by M1 and HA) following immunization of mice. VLP vaccine (~1 μg HA) were formulated with or without IL-12 as adjuvant and administered twice, at two weeks intervals, by either intranasal instillation or intramuscular injection. All VLP-vaccinated and influenza-immunized control mice demonstrated high antibody titers to the HA protein; however, intranasal instillation of VLPs elicited antibody titers that were higher than those induced by either intramuscular inoculation of VLPs or intranasal inoculation with two sub-lethal doses of the challenge influenza virus (control group). Antibody responses were enhanced when VLP vaccine was formulated with IL12 as adjuvant. All mice were challenged with 5 LD50 of a mouse-adapted influenza A/Hong Kong/68 (H3N2) virus. Intramuscular administration of VLP vaccine formulated with or without IL-12 afforded 100% protection against a lethal influenza virus challenge. Similarly, intranasal instillation of VLP vaccine alone protected 100% of the mice, whereas VLP formulated with IL-12 protected 90% of the vaccinated mice. Not only do these results suggest a novel approach to the development of VLP vaccines for diverse influenza virus strains, but also the creation of multivalent vaccines by decoration of the surface of the VLPs with antigens from other pathogens.

INTRODUCTION

EMERGING AND RE-EMERGING viral pathogens with great capacity to cause serious public health emergencies as well as significant economic disruption (5,7,12,13,20,25) pose a clear challenge to our ability to generate innovative, safe and efficacious prophylactic vaccines. Influenza A viruses which are amongst this category of pathogen, are able to maintain or increase their epidemic or pandemic disease potential through mutation

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of antigenic determinants within their surface glycoproteins (antigenic drift) and by reassortment and exchange of entire gene segments (antigenic shift) between different virus strains, some of which may not have previously circulated in the human population (17,26). The resulting rapid evolution of influenza A allows the virus to evade the host immune response and necessitate periodic updating of vaccine formulations to include new viral antigens (3,11).

The respiratory mucosal surface is the natural port of entry and the primary replication site of the influenza virus in humans as well as other mammalian species; hence it seems appropriate to develop new vaccines that are aimed at using the mucosal surface as the immunization route. Many studies using live attenuated (recently licensed) or inactivated vaccine formulations have investigated the type and level of immune response as well as the level of protection provided by mucosal immunization (2,6,16,18,21). Live attenuated vaccine has demonstrated a significant level of protection and is currently available (1); however, this type of vaccine approach is not a suitable strategy for immunization against emergent viruses derived from avian or other reservoir species. Therefore, novel approaches to vaccine production such as the one described in this work merit serious consideration because they offer a solution for the rapid and safe production of influenza vaccine to protect against newly emerging virus strains.

Animal model studies with inactivated and subunit influenza vaccines administered by the nasal mucosal route have shown induction of protective immune responses (8,9,22). Furthermore, it appears that inactivated influenza vaccines can induce a primary T ($CD8^+$) cell response which is both broad and balanced, including many of the epitopes recognized by T ($CD8^+$) cell induced by infectious virus (4). Hence, these studies have clearly shown that mucosal immunization with subunit vaccines can not only provide protection against infection but can also elicit a balanced humoral and cell-mediated immune response.

Vaccine composed of virus-like particles (VLP) have been generated for a variety of viruses belonging to diverse families and have demonstrated great potential as vaccines for the prevention of infectious disease (10,19,24). We have recently demonstrated the assembly and release from the cell surface of an enveloped virus-like particle that can accommodate different surface spikes indicating the potential for the creation of vaccines against multiple pathogens (14).

The production of non-infectious influenza VLP vaccines that are able to induce a comprehensive immune response involving the activation of both B and T cell population would likely be a safe and effective approach to prophylaxis. This technology can address the problem of rapid influenza vaccine production for both newly emerging antigenic variants as well as for those viruses with pandemic potential (H5N1, H7N7, and H9N2) (15,25).

In this work, we present the first data on immunogenicity and protective efficacy afforded by a virus-like particle (VLP) vaccine in a murine lethal challenge model. VLP vaccine was formulated in phosphate buffer saline (PBS) alone or admixed with recombinant murine IL 12 as adjuvant, and delivered via either intranasal (IN) or intramuscular (IM) routes. We evaluated the serum antibody responses elicited by VLP immunization as well as protective efficacy by challenging vaccinated mice with a lethal dose of a mouse adapted influenza virus. We discuss these results and present new directions for this novel and promising approach to vaccine development and production.

MATERIALS AND METHODS

Generation of individual M1 and HA baculovirus recombinants. Genomic RNA from gradient purified influenza A virus Udorn/72 (H3N2) was purified using an RNA extraction procedure (Qiagen, Valencia, CA). cDNA clones of segments 4 and 7 which encode HA and M1 protein respectively were generated by RT-PCR using specific primers annealing to the 3′ and 5′ termini of each gene. The sequence of the M1 and HA genes 3′ and 5′ terminus specific primers were as follows: M1-3′ terminus 5 ′AGCAAAAGCAGGTAG 3′, M1-5′ terminus 5′AGTAGAAACAAGGTA 3′, HA-3′ terminus 5′ AGCAAAAGCAGGGGATAATTCTA 3′ and HA-5′ terminus 5′ AGTAGAAACAAGGGTGTTTTTAA 3′, respectively.

The M1 gene was cloned into pGem (Promega, Madison, WI) in the T7 orientation (pGT-M1) and two of the donor splicing sites at the 5′ end of the gene, one of which serves as a donor site to produce mRNA encoding the M2 protein, were mutated using a Quick Change kit (Stratagene, La Jolla, CA) and two set of primers as follows: M1 splice 1F 5′ AGCAAAAGCA***C***GTAGATATTG 3′, M1 splice 1R 5′ CAATATCTAC***G***TGCTTTTGCT 3′ and M1 splice 2F 5′ GACCGAGGTCGAAAC***C***TATGTTCTCTCTATC 3′ and M1 splice 2R 5′ GATAGAGAGAACATA***G***GTTTCGACCTCGGTC 3′, respectively (14). This generated the plasmid pGT-M1 splice. To subclone the M1 gene, pGT-M1 splice was digested with SacII and SalI sequentially. SacI linkers (New England Biolabs) were ligated onto the insert which was then digested with SacI/SalI (New England Biolabs Beverly, MA) and subsequently gel purified using gel extraction kit (Qiagen, Valencia, CA). The insert was ligated into a SacI/SalI digested pBlueBac4.5 baculovirus

transfer vector (PharMigen, San Diego, CA), and competent DH5a *E. coli* cells (Invitrogen, Carlsbad, CA) were transformed with the ligation mix.

The HA gene was initially cloned into pGemT (Promega) in the T7 orientation and subsequently subcloned into the baculovirus transfer vector pBlueBac 4.5 as follows: the pGT-HA clone was digested with SacII and blunt-ended with T4 DNA Polymerase. The DNA was then redigested with SalI to release the HA insert which was then gel purified. The HA insert was ligated into NheI (blunt)/SalI-digested pBlueBac 4.5, and JM109 *E. coli* competent cells (Stratagene) were transformed with the ligation mix.

The sequence integrity of the HA and M1 genes inserted into the pBlueBac 4.5 transfer vectors were verified by dye termination sequencing reactions with specific primers and an automated ABI 377 DNA sequencer. Subsequently, Sf9 insect cells were transfected with 5 μg of each pBlueBac clones and 10 μg of Bac & Blue DNA (Invitrogen) by using a liposome-mediated method. Cells were incubated for 5 days, and the virus harvested from the supernatant was subjected to three rounds of plaque purification. Single blue plaques were grown and amplified in SF9 cells and protein expression was evaluated by Western blots (14) using anti-HA (mouse monoclonal, Clone 12CA5; Roche Molecular Biochemical, Indianapolis, IN) or anti-M1 antibodies (goat polyclonal; Biodesign, Saco, ME)

Formation and purification of influenza virus-like particles. Influenza virus–like particles VLP carrying the hemagglutinin (HA) as the sole surface antigen were attained by co-infection of Sf9 insect cells with the M1 and HA single baculovirus recombinants. These influenza structural proteins are sufficient for VLP formation, as has been demonstrated in our previous work (14). Sf9 cells were seeded at a density of 4.5×10^7 per flask and allowed to settle at room temperature for 30 min. Subsequently, the Sf9 insect cells were co-infected with the HA and M1 baculovirus recombinants at an MOI of 5 and infection allowed to proceed for 72 h at 28°C, at which time culture supernantant containing the VLPs was harvested and clarified by low-speed centrifugation (2,000$\times g$ for 20 min at 4°C). VLPs were pelleted by centrifugation (200,000$\times g$ for 60 min at 4°C), resuspended in 200 μL of phosphate buffer saline ($1 \times$ PBS) and homogenized by a brief sonication and then loaded on top of an iodixanol (Optiprep, Nycomed) gradient (density of 1.08 to 1.32 g/mL). The gradient was spun at 200,000$\times g$ for 3 h, and top fractions containing the VLPs were harvested and dialyzed overnight against PBS. The protein content of the purified material was evaluated by comassie blue staining of SDS-PAGE as well as Western blot using a combination of anti HA and M1 antibodies. This material constituted the basic VLP vaccine.

SDS-PAGE and Western blot analysis. The protein content and identity of the VLP vaccine was evaluated by a sodium dodecyl sulfate (SDS)–4–20% polyacrylamide gel and Western blot (14). Blots were blocked with a solution of Tris-buffered saline containing 5% non-fat milk and 0.1% Tween 20, and subsequently probed with a mixture of anti-M1 and anti-HA monoclonal antibodies. The presence of the influenza proteins M1 and HA were detected with AP-conjugated anti-mouse secondary antibody. The amount of HA protein present in the VLP vaccine was estimated by densitometry of coomassie blue stained SDS-PAGE gels (23).

Vaccine formulation and immunization schedule. The influenza VLP vaccine composed of the influenza virus structural proteins HA and M1 was formulated as a suspension in PBS alone or admixed with recombinant murine IL12 (produced and purified at Wyeth) as adjuvant. VLP vaccines were tested in female BALB/c mice (Charles River Laboratories, Wilmington, MA) aged 6–8 weeks old. Vaccine and control groups consisted of 10 mice each or as otherwise specified. VLP vaccines were administered by intranasal instillation (10 μL per nostril) or by intramuscular injection (50 μL volume) of vaccine containing a total amount of $\sim$1 μg of HA protein per dose. Placebo control mice received PBS inoculations of the same volume and via the same routes as the vaccine groups. Influenza immunized control mice received one-third of an LD50 of the influenza A/Hong Kong/68 (H3N2) challenging virus by intranasal instillation (10 μL per nostril). All mice–vaccine, placebo, or influenza immunized–received two doses of vaccine 2 weeks apart (Table 1). During the inoculation procedure, mice were lightly anesthetized with a mixture of Ketamine and Xylazine at the dose of 70 and 6 mg/kg b.w. respectively. Each group of mice was separately housed in insulator-lid cages.

Evaluation of the serum immune response. The level of antibodies elicited by VLP vaccine and controls were evaluated by ELISA. Blood samples were collected from each mouse by retro-orbital bleeding (anesthetized as described above) 3 days prior to the initiation of the immunization schedule (pre-immunization samples) and 2 weeks after the second immunization. ELISA plates were coated with sucrose-gradient purified influenza virus A/Udorn/72 (H3N2) 100 μL/well (20 ng total protein concentration). Plates were incubated overnight at 4°C and subsequently blocked with PBS containing 5% milk and 5% BSA. Dilutions of the mouse sera were applied in triplicate (100 μL per well) and incubated at room temperature for 2 h. After three consecutive washes with PBS containing 0.01% of Tween 20, a secondary goat anti-mouse antibody conjugated with horseradish peroxidase (Sigma, St. Louis, MO) was added and incubated for 1 h at room temperature. After four consecutive washes

TABLE 1. IMMUNIZATION REGIMEN AND VLP VACCINE FORMULATION

Groups	*No. of mice*	*Route*	*Two doses*	*Volume*	*Adjuvant*
Placebo (PBS) control[a]	10	IN	1 × PBS	50 μL	—
Flu-immunized control[b]	10	IN	1/3LD50[b]	30 μL	—
VLP vaccine (group 1)	10	IM	~1 μg HA	50 μL	—
VLP vaccine (group 2)	10	IM	~1 μg HA	50 μL	1 μg IL-12[c]
VLP vaccine (group 3)	10	IN	~1 μg HA	20 μL	—
VLP vaccine (group 4)	10	IN	~1 μg HA	20 μL	1 μg IL-12[c]

[a]1 × phosphate buffer saline.
[b]Immunization with influenza A/Hong Kong/68 (H3N2) challenging virus.
[c]Recombinant murine IL12.

with the PBS–Tween 20 solution, a single TMB substrate solution (BIO-RAD, Hercules, CA) was added to each well, and the plates were incubated at room temperature for color development. The reaction was stopped with 0.18 M H_2S0_4, and absorbance was determined at 450 nm.

Determination of vaccine efficacy. To evaluate the protective efficacy of the VLP vaccine, all vaccinated mice including control animals were challenged 17 days after the booster immunization with 5 LD50 of a mouse adapted influenza A/Hong Kong/68 (H3N2) virus (~60 PFU as determined by plaque assay in MDCK cells). Prior to receiving the virus challenge, mice were lightly anesthetized as described above. The 5 LD50 of the challenge virus constituted a total volume of 20ul and was administered by intranasal instillation of tiny droplets delivered by ultra-slim sequencing gel loading tips (10 μL per nostril). All mice were observed twice daily for 15 days, at which time vaccine efficacy was assessed by determining the number of mice that survived the virus challenge.

Measuring of body weight as an indicator of protection. Daily measurements of body weight and monitoring of clinical signs of influenza illness were used as additional indicators of vaccine protection. The weight of each mouse was measured on day one after challenge and daily for 15 days. Changes in body weight, together with the general clinical appearance of the mice were used as additional indicators of the level of protection afforded by vaccine treatment.

RESULTS

Formation and composition of VLP vaccine. Formation of two component VLPs was accomplished by infecting Sf9 insect cells with two individual baculovirus recombinants that express either the M1 or HA proteins. These influenza virus structural proteins are sufficient to

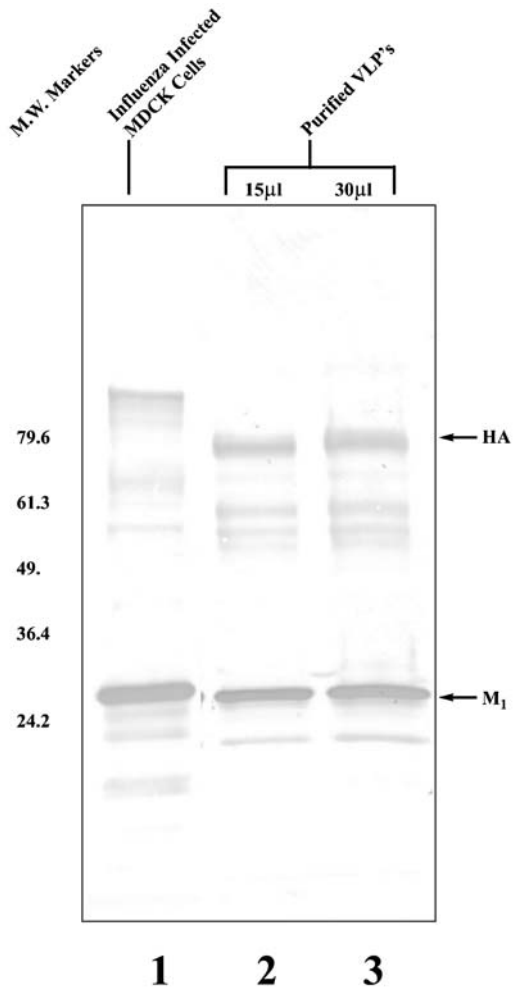

FIG. 1. The protein content of gradient purified VLPs were evaluated by western blot using a mixture of anti HA and M1 monoclonal antibodies. Lane 1, influenza infected MDCK cells. Lane 2 and 3 show two different amounts of purified VLPs. The VLP HA migrates faster than the HA synthesized in influenza infected MDCK cells, presumably reflecting glycosylation differences present in insect cells.

drive formation and release of VLPs from the cell surface, as has been previously described (14). VLPs were purified from the culture supernatant as described above and the presence of HA and M1 proteins in the final vaccine preparation were evaluated by western blot. This analysis demonstrated that indeed the HA and M1 influenza proteins were present in the purified VLP vaccine (Fig. 1, lanes 2 and 3). The amounts of HA and M1 proteins contained in the vaccine preparation were estimated by densitometry of Coomassie blue–stained SDS-PAGE (data not shown).

Immune response elicited by intranasal and intramuscular VLP vaccine immunization. The level of serum antibody elicited by two doses of VLP vaccine administered by either the intranasal (IN) or intramuscular (IM) route, formulated in either PBS alone or in combination with recombinant murine IL-12 as adjuvant (Table 1) was determined by ELISA utilizing gradient purified influenza A/Udorn/72 (H3N2) virus as antigen. All mice immunized with VLP vaccine by either route, with or without IL-12 as adjuvant, demonstrated high serum antibody titers (Fig. 2). Intranasal administration of VLP vaccine induced on average a stronger antibody response than two intranasal inoculations of a sub-lethal dose of the challenge virus (influenza A/Hong Kong/68 H3N2). In addition, the immune response was enhanced when murine IL-12 was used as adjuvant (Fig. 2A). Administration of VLP vaccine by the IM route elicited lower antibody titers than did intranasal VLP or influenza virus immunization (control group; Fig. 2B). The VLP vaccine formulated with IL-12 induced higher antibody titers than VLP vaccine alone (Fig. 2B). The antibody response to M1 protein has not yet been evaluated.

Protective efficacy afforded by VLP vaccination. To assess vaccine efficacy, all VLP vaccinated mice, placebo (PBS) and influenza virus immunized (influenza A/Hong Kong/68) controls were challenged 17 days after the second immunization with 5 LD50 of a mouse-adapted influenza virus A/Hong Kong/68 (H3N2) by intranasal instillation. All experimental groups were observed for 15 days, at which time vaccine efficacy was assessed by determining the number of mice that survived the lethal challenge. In addition, daily measurement of body weight was used as an indicator of protection and disease progression in all the groups. One hundred percent of mice that received two intramuscular injections of VLP vaccine (with or without IL-12) survived the challenge (Fig. 3). In addition, they were able to maintain their weight without any clinical signs of influenza disease (Fig. 4). Furthermore, intranasal immunization with VLP vaccine in PBS also protected 100% of the mice, whereas VLP vaccine with IL-12 protected 90% of the mice. Almost all of the mice in these two groups were able to maintain or even increase body weight (Fig. 4), with the excep-

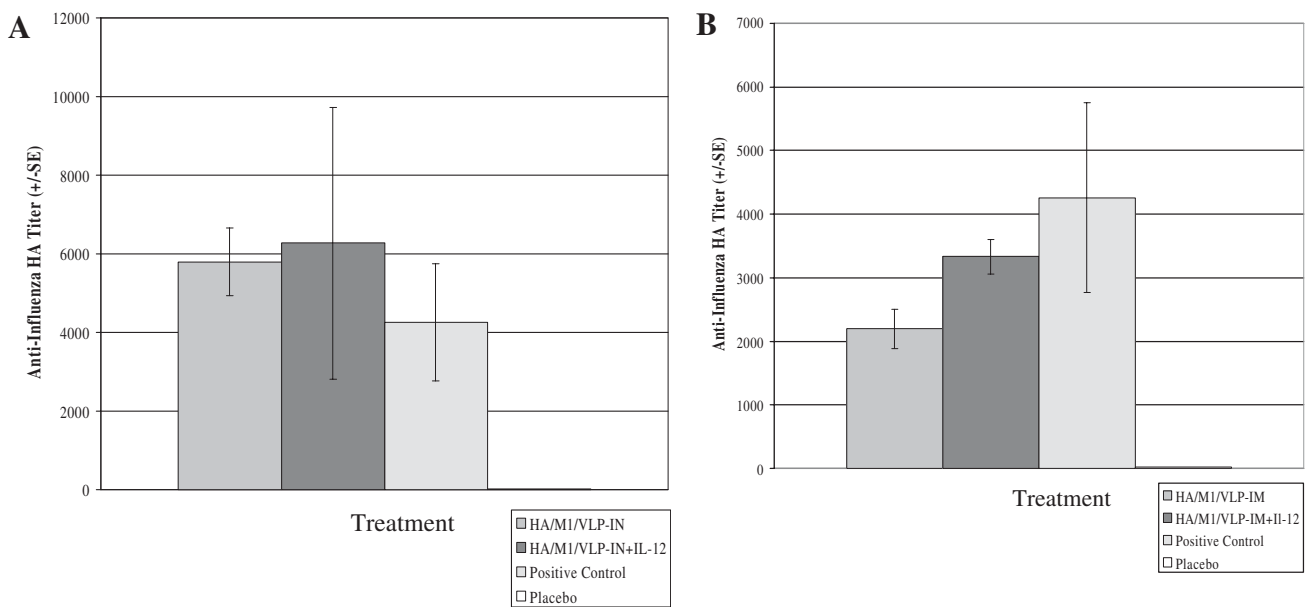

FIG. 2. Serum antibody responses to influenza virus-like particles (VLPs). Mice were vaccinated twice (2 weeks apart) with 1 μg of HA-containing VLPs formulated with or without IL12 as adjuvant by intranasal (IN) (**A**) or intramuscular (IM) (**B**) routes. Control and placebo groups received two sub-lethal doses of the challenge virus or PBS respectively. Total serum anti-influenza antibodies were measured by ELISA 2 weeks after the second vaccination and prior to challenge with a lethal dose of a mouse-adapted influenza virus. Results are depicted as the average of the all the individual measurements.

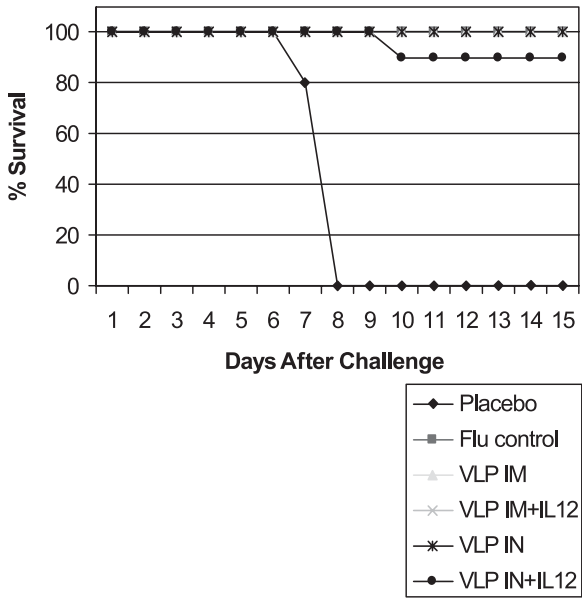

FIG. 3. Control and VLP immunized mice were challenged with 5LD50 of a mouse-adapted influenza A/Hong Kong/68 (H3N2) virus. All mice immunized intramuscularly with VLP vaccine formulated with or without IL-12 survived the virus challenge. Also, intranasal instillation of VLP alone conferred complete protection, whereas VLP+IL12 protected 90% of the vaccinated mice. All control mice died between days 7 and 8.

tion of one mouse in the IN+IL12 group that lost weight for two consecutive days and died. This mouse was not included in the group weight average, but it is indicated as a dead animal. This mouse demonstrated antibody titers as high as the other animals in the group, suggesting that its death may be unrelated to the challenge (data not shown). All mice in the influenza-immunized control group (vaccinated with two sub-lethal doses of the challenge virus) survived, whereas 100% of the mice in the placebo group died between day 7 and 8 after challenge.

DISCUSSION

In this work, we present data on the immunogenicity and protective efficacy afforded by a two-component (HA, M1) virus-like particle (VLP) vaccine against a lethal influenza virus challenge in a murine model. VLP vaccine was generated by co-infection of Sf9 cells with two individual baculovirus recombinants carrying the influenza structural genes encoding either the HA surface glycoprotein or the matrix protein M1. We have previously demonstrated (Latham and Galarza, 2001) that formation of VLPs containing only HA, HA and NA, HA, NA and M2, and even heterologous surface spike glycoproteins can be accomplished by concomitant expression of the protein/s with matrix proteins (M1). We decided to initiate our VLP vaccine studies using a two-component particle, the simplest of the VLP structures, to assess the protective immune response elicited by this type of vaccine. The two-component VLPs were able to stimulate an immune response that fully protected vaccinated mice against a lethal influenza virus challenge, suggesting that the HA spikes displayed neutralizing epitopes, and therefore presumably a structural conformation that is analogous to wild-type HA spikes.

Immunization of mice with two IM injections of VLP vaccine elicited a serum antibody response that was able to afford 100% protection against a highly pathogenic mouse-adapted influenza A/Hong Kong/68 (H3N2) virus challenge. However, mice immunized by this route and dosage were not completely protected, because some animals experienced a small weight loss indicating minor virus replication, which did not affect normal behavior of the animals or translate into any sign of clinical disease.

Two intranasal administrations of VLP vaccine alone also afforded 100% protection against the lethal virus challenge, whereas VLP with IL-12 had demonstrated a 90% protection efficacy. All mice immunized with VLP vaccine by the IN as well as by the IM routes demonstrated high antibody titers to the HA protein as measured by ELISA. Utilization of murine IL-12 as adjuvant appeared to slightly enhance the magnitude of the antibody

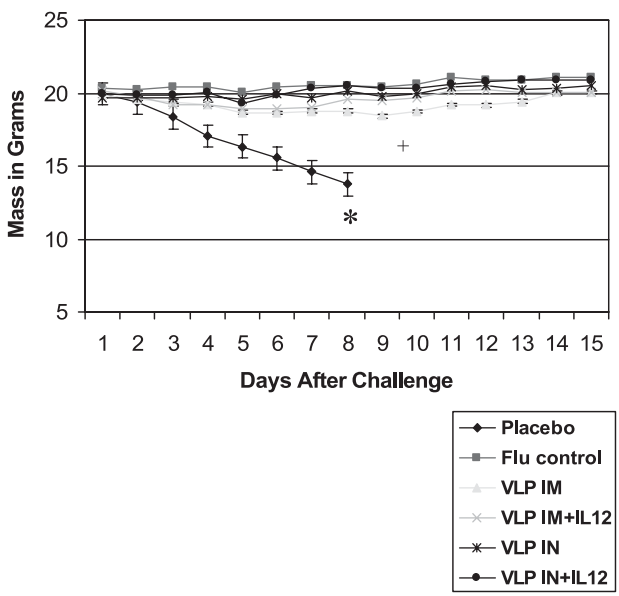

FIG. 4. Body weight was monitored for all the groups, and weight average was plotted versus the days after virus challenge. All mice in the placebo control group (*) died between days 7 and 8. Also, one mouse in the VLP IN+IL-12 group (+) died at day 9.

response induced by VLP vaccine administered via either IN or IM route. The level of protection afforded by VLP vaccine delivered by intranasal immunization underlined the effectiveness of these particles in triggering a response that not only protects from death but also from clinical disease. However, we cannot completely rule out virus replication in the lungs because we have not determined in these experiments virus titers in the upper and lower respiratory system.

Previous studies (9) have shown the inability of purified HA vaccines administered by intranasal route to afford protection, even after two doses of vaccine containing 10 μg of rHA protein. It is therefore quite significant that a two-component VLP vaccine, which carries a much lower HA content (1 μg per dose) than the study mentioned above (9), was able to induce complete protection. It seems reasonable to assume that the native conformation of the HA molecules on the surface of the VLPs as well as the particulate nature of the vaccine will facilitate interaction with cell surface receptors and initiate processes that lead to a strong local and systemic immune response. In this work, we have not yet characterized either the IgG subclasses or assessed cell-mediated immunity or measured local IgA production; however the level of protection afforded by intranasal vaccination permit us to infer a significant contribution of local mucosal immunity in preventing virus infection.

The protective efficacy afforded by VLP vaccination clearly demonstrates the potential of this approach to generate influenza virus vaccines of different HA compositions. Furthermore, the fact that these VLPs do not carry influenza virus genetic material make them an attractive approach for the generation of prophylactic vaccines against influenza viruses such as H5N1, H9N2, or H7N7, which pose a pandemic threat because they have not circulated in the human population. Formation of VLPs containing surface antigens of other pathogens may create opportunities to design mono- and multivalent vaccines for a wide range of disease targets.

The safety and efficacy of VLP vaccine together with the simplicity of delivery make this new vaccine technology a promising approach to address serious public health issues. It may allow us to produce quickly and safely not only vaccines against influenza viruses but also vaccines against other emerging pathogens by accommodating key antigen on the surface of the VLP (pseudotyping).

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