

Evaluation of influenza virus-like particles and Novasome adjuvant as candidate vaccine for avian influenza

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Abstract

The development of safe and effective vaccines for avian influenza viruses is a priority for pandemic preparedness. Adjuvants improve the efficacy of vaccines and may allow antigen sparing during a pandemic. We have previously shown that influenza virus-like particles (VLPs) comprised of HA, NA, and M1 proteins represent a candidate vaccine for avian influenza H9N2 virus [Pushko P, Tumpey TM, Fang Bu, Knell J, Robinson R, Smith G. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. *Vaccine* 2005;23(50):5751–9]. In this study, an H9N2 VLP vaccine and recombinant HA (rH9) vaccine were evaluated in three animal models. The H9N2 VLP vaccine protected mice and ferrets from challenge with A/Hong Kong/1073/99 (H9N2) virus. Novasome adjuvant improved immunogenicity and protection. Positive effect of the adjuvant was also detected using the rH9 vaccine. The results have implications for the development of safe and effective vaccines for avian influenza viruses with pandemic potential.

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1. Introduction

Development of effective vaccines for avian influenza is a priority in preparedness for influenza pandemic [2–4]. Since 1997, direct avian-to-human transmission of pathogenic avian influenza viruses have been recorded and caused fatal human disease [5]. The H5N1 subtype of avian influenza virus is widespread in domestic poultry and is considered the most likely culprit for the next pandemic [6]. Furthermore, more recent H5N1 viruses isolated from humans exhibit increased virulence in mammals [7]. However, avian influenza A H9N2 viruses are also widespread among domestic poultry and have been isolated from children after

influenza-like disease in Hong Kong [8]. Antigenic and phylogenetic analyses have shown an increasing genetic and biologic diversity of H9N2 viruses that also suggests their potential role as pandemic influenza threats [9].

The development of vaccines from pathogenic avian influenza viruses including H9N2 is difficult because of safety as well as technical reasons. We have recently shown that recombinant virus-like particles (VLPs) comprised of the hemagglutinin (HA), neuraminidase (NA), and matrix (M1) proteins of H9N2 influenza virus protect mice from influenza virus challenge [1]. We have also developed non-phospholipid liposome nanoparticles, Novasomes, which showed promising characteristics as vaccine adjuvant [10]. Both VLPs and liposome nanoparticles containing vaccine-relevant antigens represent effective immunogens, because they are capable of presenting antigens in

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native conformation in the context of highly immunogenic oligomeric complexes [11,12].

In this study, we evaluated H9N2 VLPs in rats, mice, and ferrets as candidate influenza vaccines. We have also assessed the potential of Novasome or alum adjuvants to enhance immunogenicity and protection efficacy of H9N2 VLPs as well as of recombinant rH9 protein vaccines. Vaccinated mice and ferrets were challenged with influenza A/Hong Kong/1073/99 (H9N2) virus, and the effects of vaccines and adjuvants on the immunogenicity and protection against virus challenge were evaluated.

2. Materials and methods

2.1. Cells, viruses, and constructs

Spodoptera frugiperda Sf9 insect cells (ATCC CRL-1711) were maintained as suspension cultures in HyQ-SFX insect serum free medium (HyClone, Logan, UT) at 28 °C. A Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA) was used with pFastBac1 transfer vectors in *E. coli* DH10Bac cells for the generation of recombinant baculovirus vectors expressing influenza genes.

Influenza A/Hong Kong/1073/99 (H9N2) virus (Influenza Branch, CDC, Atlanta, GA) was used as source of HA, NA, and M1 genes. Viral RNAs were extracted under BSL3 containment. The resulting H9N2 HA, NA, and M1 cDNA genes of 1.7, 1.4, and 0.7 kb in length, respectively, were initially cloned into a pCR2.1-TOPO vector, then transferred into pFastBac1 downstream of the AcMNPV polyhedrin promoter, and finally combined within the single transfer vector pNAHAM derived from pFastBac1 (Fig. 1a), as described in detail previously [1].

For generation of the pH9 transfer vector for expression of rH9 protein, primers 5'-AGGATCCatgGAAACAATA-TCACTAATAAC-3' and 5'-AGGTACCTTAgtggtggtggtggtggtgTATACAAATGTTGCATCTGC-3' were used (ATG and codons corresponding to histidine (His) tag shown in

small case). The rH9 gene was generated using PCR. The resulting rH9 gene encoding the entire rH9 protein with six His residues at the carboxy terminus was cloned as a *Bam*HI-*Kpn*I DNA fragment (1.7 kb) downstream of the polyhedrin promoter within pFastBac1 vector. This resulted in transfer plasmid pH9 (Fig. 1b).

Recombinant baculoviruses were generated by site-specific homologous recombination following transformation of pNAHAM or pH9 transfer plasmids containing influenza genes into *E. coli* DH10Bac competent cells, which contained the AcMNPV baculovirus genome (Invitrogen). The recombinant bacmid DNA was extracted from *E. coli* cells and transfected into the Sf9 cells using CellFectin (Invitrogen). The recombinant baculoviruses were recovered, plaque-purified, amplified, and the titers of recombinant baculovirus stocks were determined by agarose plaque assay using Sf9 cells.

2.2. Purification of influenza antigens

For purification of influenza antigens, Sf9 cells were infected at a multiplicity of infection (MOI) of 3 for 72 h at a cell density of 2×10^6 cells/ml with recombinant baculoviruses encoding either H9N2 HA, NA, and M1 for VLPs or just HA for rH9 antigen production (Fig. 1).

H9N2 influenza VLPs were purified from Sf9 cultures using 20–60% sucrose density gradients [1]. For purification of rH9 protein, Sf9 cells were harvested and lysed for 1 h at 25 °C in buffer containing 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 1.5% Triton X100. Ni-NTA Superflow column (Qiagen, Valencia, CA) was equilibrated with buffer A containing 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 10 mM imidazole. Sf9 cell lysate containing rH9 protein was loaded onto the column, the column was washed with buffer A, and the rH9 protein was eluted with buffer B containing 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 300 mM imidazole.

Influenza proteins were characterized by SDS-PAGE using 4–12% gradient polyacrylamide gels (Invitrogen) and Coomassie staining as well as by Western blot using rabbit antiserum R#55 (obtained from A. Klimov, CDC) specific for influenza A/Hong Kong/1073/99 (H9N2) virus (CDC, Atlanta, GA). Secondary antibodies were alkaline phosphatase conjugated goat anti-rabbit IgG (H+L) (Kirkegaard and Perry, Gaithersburg, MD). Total protein in rH9 and VLP preparations was determined by BCA protein assay (Pierce, Rockford, IL) followed by calculating the HA content from scanned Coomassie stained SDS gels. Additionally, for determining the HA content in VLPs, single radial immunodiffusion assay was carried out according to [13] using antiserum for influenza A/Hong Kong/1073/99 (H9N2) virus and purified rH9 protein as a standard.

Negative staining electron microscopy of H9N2 VLP preparations was carried out essentially as described previously [1].

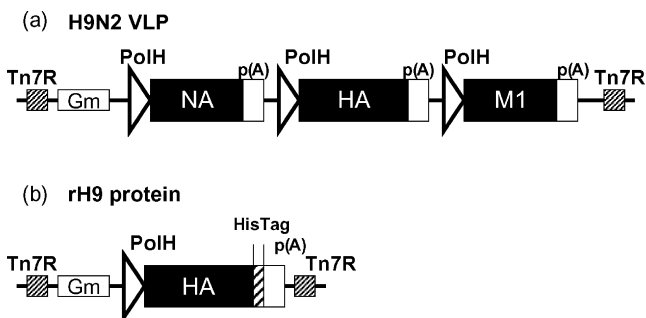


Fig. 1. (a) Baculovirus construct for expression of influenza H9N2 VLPs; (b) baculovirus construct for expression of recombinant influenza H9 (rH9) protein. Indicated are the polyhedrin promoter (Polh), polyadenylation signal, Tn7 regions, gentamicin resistance gene (Gm), and influenza A/Hong Kong/1073/99 (H9N2) genes. HA: hemagglutinin; NA: neuraminidase; M1: matrix protein.

2.3. Formulation of H9N2 antigens with adjuvants

Purified H9N2 VLPs or rH9 protein was formulated with the Novasome B52CCPCE nonphospholipid adjuvant comprised of polyoxyethylene-2-cetyl ether (Brij 52), cholesterol, cetylpyridinium chloride, and vitamin E. Briefly, the antigen was intermixed with pre-made B52CCPCE Novasomes at a ratio 1:1 using a tuberculin syringe fitted with an 18 gauge needle. In order to ensure maximal absorption of antigens on the surface of adjuvant nanoparticles, the suspension was fully homogenized by passing the suspension back and forth between two tuberculin syringes fifty times as described previously [10].

Aluminum hydroxide gel (alum) was prepared using Alhydrogel (Superfos Biosectors, Kvistgaard, Denmark). A 2% alum suspension was mixed with an equal volume of vaccine in PBS for 6 h at 4 °C before immunization.

2.4. Vaccination and challenge

Influenza H9N2 VLP and rH9 antigens (with and without adjuvant) were resuspended in PBS, pH 7.2. Sprague Dawley (SD) female rats, six animals per group, were vaccinated intramuscularly (i.m.) into left hind leg on days 0 and 21 with inoculations of H9N2 VLPs without adjuvant containing 0.12, 0.6, 3, or 15 µg of HA. Additional groups of animals were vaccinated with the doses of 0.12 and 0.6 µg formulated with either Novasome or alum adjuvants.

Male ferrets *Mustela putorius furo* (Fitch strain), 6–12 months of age, six animals per group, were vaccinated i.m. (left hind leg) with H9N2 VLPs containing 0.15, 1.5, or 15 µg of HA on days 0 and 21. Prior to vaccinations, animals were confirmed by HAI assay to be seronegative for circulating influenza A (H1N1 and H3N2) and influenza B viruses. Because of space constraints for ferrets in the BSL3+ lab, we did not include adjuvants in this experiment. PBS was used as a negative control inoculation.

Female BALB/c mice, 13 animals per group, were inoculated i.m. into left hind leg on days 0 and 28 with 10 µg of H9N2 VLPs containing 3.2 µg of HA, or with 10 µg of purified rH9 antigen. Both VLP and rH9 antigens were administered with or without Novasome adjuvant.

Following vaccinations, serum was collected and tested by hemagglutination inhibition (HAI), enzyme-linked immunosorbent assay (ELISA), or virus neutralization (VN) tests for the presence of influenza-specific humoral responses. HAI assays were performed with 0.5% turkey erythrocytes using standard methods [14]. ELISA was carried out as previously described [15]. ELISA plates were coated with a purified baculovirus-expressed recombinant HK/1073 HA protein (1 µg/ml; Protein Sciences Corp., Meriden, CT).

Titers of VN antibody were determined essentially as described elsewhere [16,17] and are shown as the reciprocal of the highest dilution of serum that neutralized 100 plaque-

forming units or 100 50% tissue culture infectious doses (TCID₅₀) of virus in Madin–Darby canine kidney (MDCK) cell cultures.

For challenge, vaccinated ferrets and mice were transferred into a BSL3+ containment facility and challenged intranasally (i.n.) with 10⁶ 50% egg infectious doses, (EID₅₀) of influenza A/Hong Kong/1073/99 virus [15]. Nasal wash samples were taken from ferrets on days 3, 5, and 7 postchallenge, and influenza virus was titrated in eggs to determine virus shedding in upper respiratory tissues [7]. In ferrets, we determined virus titers in the upper respiratory tissues. In mice, virus was determined in both upper and lower respiratory tissues. Five mice were sacrificed on day 3 and another four on day 5 postchallenge. Tissues were collected, snap frozen at –70 °C and later thawed, homogenized in 1 ml of cold PBS and pelleted by centrifugation. Clarified homogenates were titrated in eggs for virus infectivity from initial dilutions of 1:10. The limit of virus detection was 10^{1.2} EID₅₀/ml. The remaining mice were observed and weighed daily for 14 days for morbidity.

3. Results

3.1. Expression of influenza VLPs in Sf9 cells

In order to generate H9N2 VLPs in Sf9 cells, the HA, NA, and M1 genes were derived from influenza A/Hong Kong/1073/99 (H9N2) virus and introduced into recombinant baculovirus bNAHAM, each gene within its own expression cassette, which included a polyhedrin promoter and transcription termination sequences (Fig. 1a).

Influenza VLPs were purified from culture media by sucrose gradient centrifugation. Presence of HA, NA, and M1 in VLPs were confirmed by SDS-PAGE and Western blot, as well as by the hemagglutination and neuraminidase functional assays, as described previously [1]. Electron microscopic examination of negatively-stained samples revealed the presence of H9N2 VLPs with a diameter of approximately 80–120 nm, which showed surface spikes, characteristic of influenza HA protein on virions. VLPs were associated frequently as groups (Fig. 2a).

3.2. Expression of rH9 protein

Recombinant baculovirus for expression of rH9 protein in Sf9 cells contained a single expression cassette comprised of the polyhedrin promoter, the rH9 gene, and transcription termination and polyadenylation signals (Fig. 1b). The rH9 protein containing six His residues at the carboxy terminus was purified using Ni-NTA affinity chromatography and used in animal studies described below. As expected, purified rH9 protein derived from Sf9 cells represented HA₀ protein with a molecular weight of approximately 62–65 kDa (Fig. 2b, lane 1).

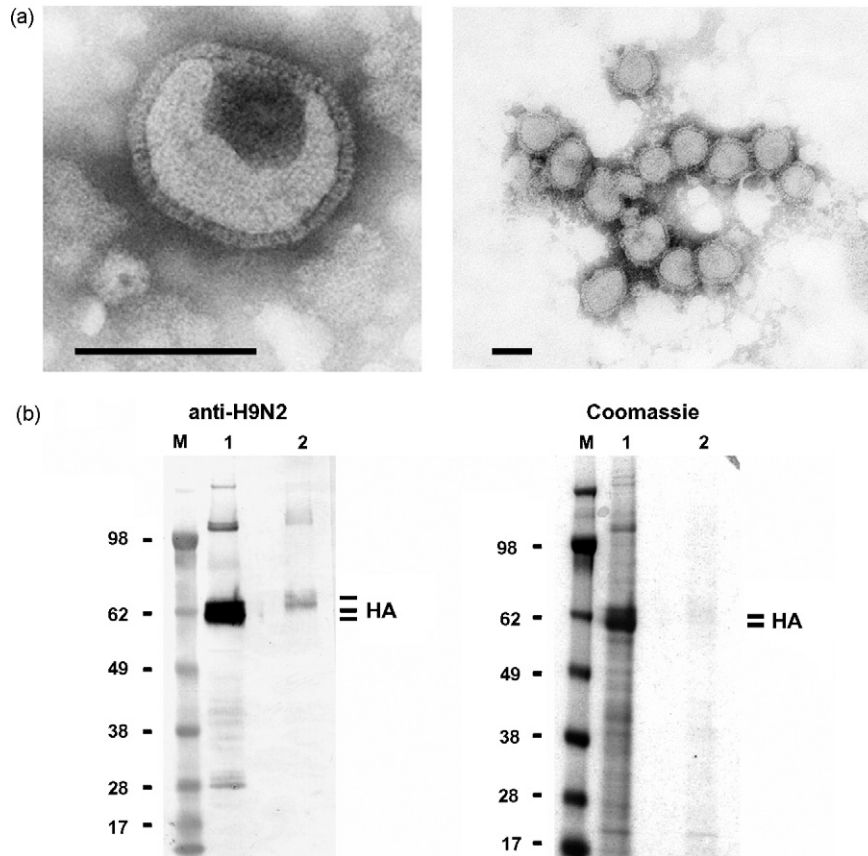


Fig. 2. (a) Negative staining electron microscopy of influenza H9N2 VLPs comprised of the HA, NA, and M1 proteins. Bars represent 100 nm; (b) Western blot and Coomassie staining of rH9 protein before formulation with Novasomes (lane 1), and after formulation with Novasomes and removal of Novasome-rH9 complexes by centrifugation (lane 2). Western blot was carried out using rabbit antiserum for influenza A/Hong Kong/1073/99 (H9N2) virus (CDC, Atlanta, GA). Position of rH9 (HA) is indicated.

3.3. Formulation of H9N2 antigens with Novasome adjuvant

For vaccinations, VLPs as well as rH9 protein were formulated with, as well as without Novasome adjuvant nanoparticles. Without adjuvant, the rH9 protein migrated at the expected molecular weight of approximately 62 kDa (Fig. 2, lane 1). Additional minor bands of ~120 kDa and ~200 kDa were also detected, which likely represented dimers and trimers of the rH9 protein. The efficacy of incorporation of rH9 protein into the Novasome nanoparticles was monitored by removing adjuvant-rH9 complexes using centrifugation and determining the amount of soluble rH9 protein in the remaining liquid phase. Only residual quantities of H9 were detected in the liquid phase showing high efficiency of incorporation of rH9 protein into Novasome adjuvant (Fig. 2b, lane 2). The residual rH9 had higher apparent molecular weight in SDS gels as compared to the purified rH9 protein (Fig. 2b, lane 2) suggesting that the adjuvant may affect protein mobility, possibly by adhering to the protein or changing protein conformation.

3.4. Immunogenicity of VLPs in rats

Initially, the immunogenicity of H9N2 VLP vaccine, dose response, and the effects of adjuvant were studied in SD rats (Fig. 3). As expected, no influenza-specific serum antibody was detected in animals before vaccinations or in animals inoculated with PBS control only. After a single administration of 0.6, 3.0, or 15 μ g of VLPs all animals developed HAI titers that exceeded titer of 1:240, and the titers increased after booster inoculations. Two vaccinations with 0.12 μ g were required to achieve similar HAI titers (Fig. 3a). Vaccination with 0.12 μ g of VLP vaccine with Novasome adjuvant resulted in increased HAI antibody titers as compared to vaccination with 0.12 μ g of VLPs without Novasome adjuvant (Fig. 3b). Although statistically not significant ($p > 0.1$), an increase of GMT in animals indicated that coadministration of vaccine with Novasome adjuvant may have a beneficial effect. This effect was confirmed in BALB/c mice, as shown below. In contrast, formulation of the VLP vaccine with alum did not enhance the HAI response to homologous HK/1073 virus.

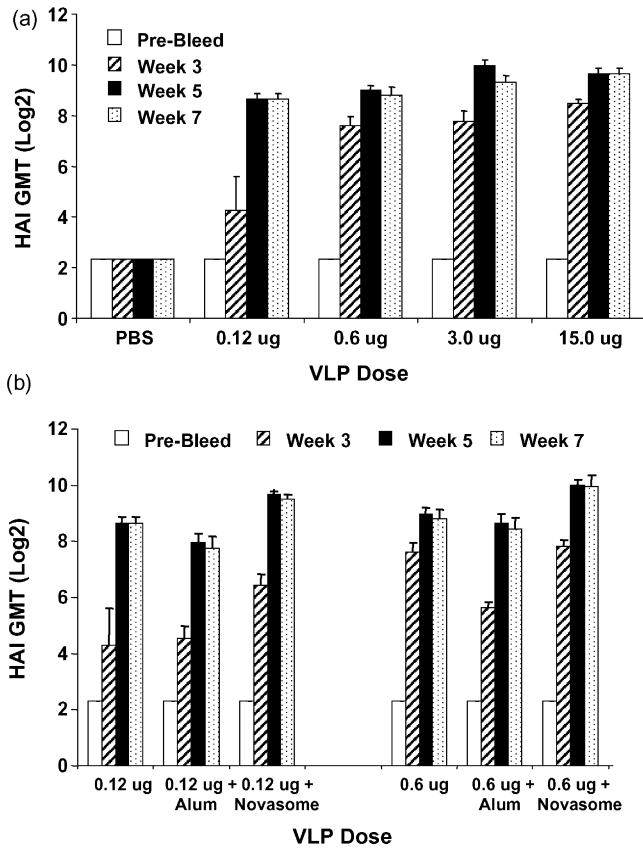


Fig. 3. (a) Induction of serum HAI antibody in Sprague Dawley rats vaccinated with escalating doses of influenza H9N2 VLPs without adjuvant; (b) the effects of alum or Novasome adjuvants on serum HAI antibody response. Animals were vaccinated i.m. on days 0 and 21 with VLPs with or without adjuvant as indicated. Detection limit was 2.0 log₂. Bars indicate standard deviation. GMT: geometric mean titer.

3.5. Immunogenicity and protection with VLPs in ferrets

Ferrets are considered to be the most suitable animal model for preclinical evaluation of human influenza vaccines prior to clinical trials. Ferrets received primary and booster vaccinations with 0.15, 1.5, or 15 µg of HA in H9N2 VLPs without Novasome adjuvant. Control animals received PBS only. As little as 0.15 µg of VLPs induced detectable HAI antibody responses that increased 2- to 9-fold with the 15 µg dose (Fig. 4). Following vaccinations, animals that received vaccinations with 1.5 or 15 µg of VLPs were challenged with A/Hong Kong/1073/99 (H9N2) virus, and replication of the challenge virus was determined by monitoring virus shedding in nasal washes post challenge. Infectious virus was detected in nasal washes of all animals on day 3 postchallenge. However, on day 5, only low titers of virus were detected in the animals vaccinated with 1.5 or 15 µg of VLPs. In these groups, virus replication was undetectable on day 7, whereas control animals that received PBS showed over 3 log₁₀ EID₅₀/ml of replicating influenza virus (Fig. 4).

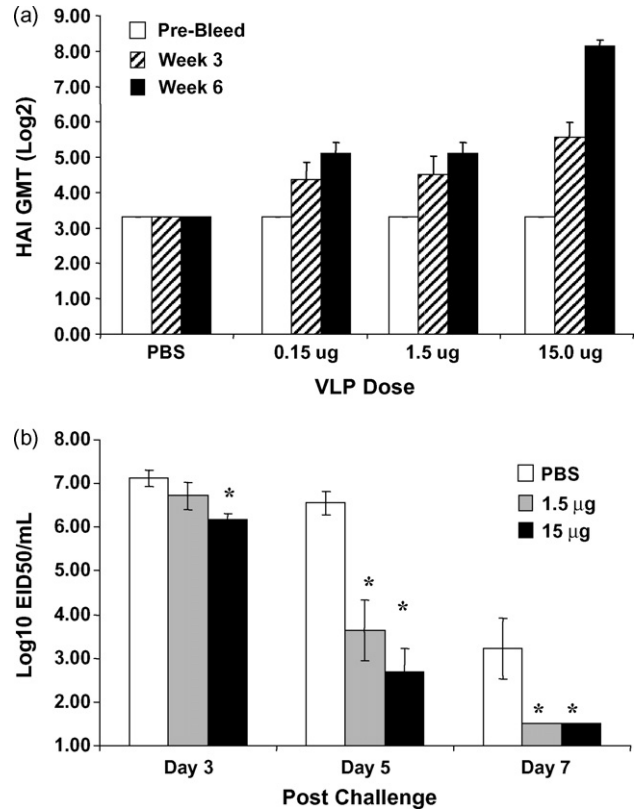


Fig. 4. (a) Induction of serum HAI antibody in ferrets (Fitch strain) vaccinated with escalating doses of influenza H9N2 VLPs. Bars indicate standard deviation. GMT: geometric mean titer; (b) protection of VLP-vaccinated ferrets against challenge with influenza A/Hong Kong/1073/99 (H9N2) virus. Shown are the titers of replicating virus in nose tissues of VLP-vaccinated (filled bars) and non-vaccinated (open bars) ferrets. Animals were challenged i.n. with 106 EID₅₀ of influenza A/Hong Kong/1073/99 (H9N2) virus. Detection limit was 1.2 log₁₀ EID₅₀/ml of virus. Also shown are standard deviation values. An asterisk indicates the vaccinated group was significantly ($p < 0.05$) different from the non-vaccinated control group by using analysis of variance (ANOVA).

3.6. Immunogenicity and protection in mice

Protective capacity of VLPs and the effects of Novasome adjuvant were further evaluated in BALB/c mice. Animals were vaccinated twice i.m. with 10 µg doses of influenza VLPs with or without adjuvant. According to densitometric analysis of SDS gels, 10 µg dose of VLPs contained 3.23 µg of HA, with the remaining influenza proteins represented by NA and M1 proteins (data not shown). In order to evaluate the effects of the adjuvant on antigens, we also included vaccinations with 10 µg of a purified baculovirus-expressed recombinant HK/1073 rH9 protein with or without adjuvant.

Following a single inoculation with VLP or rH9 antigens, serum antibody was detected by ELISA (data not shown) as well as HAI in all animals except the controls (Fig. 5). After booster inoculations, the HAI titers increased. Novasome adjuvant increased immunogenicity of both antigens. The highest HAI titers were detected in the groups vaccinated

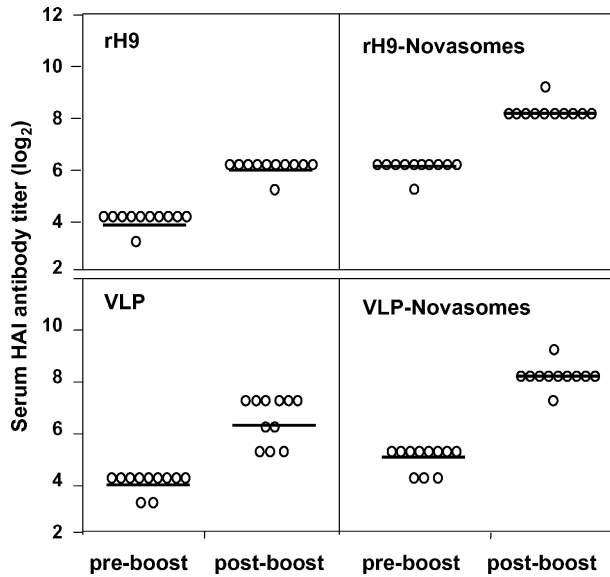


Fig. 5. Induction of HAI serum antibody in individual BALB/c mice vaccinated with influenza H9N2 VLPs or rH9. BALB/c mice received vaccine inoculations with or without Novasome adjuvant or PBS as negative control on days 0 and 28. HAI titers were determined on day 14 after primary inoculation (pre-boost) as well as on day 42 after booster inoculation (post-boost).

with either rH9 or VLP antigen in the presence of Novasomes (Fig. 5).

Influenza VN antibodies were detected in the sera from all four experimental groups after primary vaccinations (Table 1). High levels of VN titers were observed after a

Table 1
Virus-neutralizing (VN) responses in BALB/c mice^a

Vaccine group	No. of doses ^b	Titer, pooled serum ^c	Titer, individual sera, log ₁₀ GMT + S.D.
PBS	2	10	1.14 ± 0.44
VLP	1	390	2.59 ± 0.05
VLP	2	549	2.72 ± 0.05 ^d
VLP + Novasomes	1	265	2.48 ± 0.03
VLP + Novasomes	2	1338	3.11 ± 0.05 ^{d,e}
rH9	1	294	2.44 ± 0.06
rH9	2	378	2.61 ± 0.05 ^d
rH9 + Novasomes	1	639	2.82 ± 0.03
rH9 + Novasomes	2	4358	3.63 ± 0.02 ^{d,e}

^a Influenza A/Hong Kong/1073/99 (H9N2) virus (100 TCID₅₀) was used for VN titer determination. All sera were diluted 1:10 prior VN assay. As positive control, we used reference serum from ferret infected with A/Hong Kong/1073/99 (H9N2), which had VN titer of 365 in this assay.

^b BALB/c mice received vaccine inoculations or PBS as negative control on days 0 and 28. VN titers were determined on day 14 after primary inoculation (1 dose) as well as on day 42 after booster inoculation (2 doses).

^c A titer of 10 indicates that the serum was negative for neutralizing antibodies. All vaccinated groups developed neutralizing antibody response that was significantly ($p < 0.001$) different from the negative control group (PBS) by Student's *t*-test. GMT: geometric mean titer; S.D.: standard deviation.

^d Significant statistical difference between one dose of vaccine and two doses of vaccine, $p < 0.001$, by Student's *t*-test.

^e Significant statistical difference between two doses of vaccine without Novasome adjuvant, and two doses of vaccine with Novasome adjuvant, $p < 0.001$, by Student's *t*-test.

single inoculation in the rH9-Novasome group, as well as in the VLP group. This suggests that a single dose of VLPs or rH9-Novasomes may provide effective vaccination. Interestingly, inoculations with VLPs in the presence of adjuvant resulted in the decrease of VN titers. However, the lowest VN titers after a single inoculation were observed in the animals inoculated with rH9 without adjuvant, whereas the highest VN titers were found in the animals that received rH9 with adjuvant. After booster vaccinations, the VN titers in all experimental groups increased significantly ($p \leq 0.001$). Further, booster inoculations with Novasome-containing vaccines resulted in statistically significant final VN antibody titer increase ($p < 0.001$) as compared to vaccinations without Novasomes (Table 1).

Following i.n. challenge with A/Hong Kong/1073/99 virus, we determined virus replication and morbidity (measured by weight loss) in the challenged animals. Average weight loss of up to 17% were observed in non-vaccinated controls as well as in the animals vaccinated with rH9 without adjuvant (Fig. 6a). However, animals vaccinated with VLPs with or without Novasomes showed only approximately 10% weight loss demonstrating protective effect of VLPs. Protection was also observed in the animals, which received rH9 protein with adjuvant. Protection correlated with the VN titers (Table 1). Active replication of influenza virus was detected in lung and upper respiratory tissues of control animals at days 3 and 5 postchallenge (Fig. 6b). In contrast, a significant ($p < 0.05$) reduction of virus titers in the lung and nose tissues were demonstrated among all vaccinated animals at day 3 postchallenge, as compared to non-vaccinated controls. Among vaccinated groups, the lowest protection was detected in animals vaccinated with rH9 protein without adjuvant, which correlated with weight loss data. No virus was detected on day 5 postchallenge in nose tissues of all vaccinated animals.

4. Discussion

The development of vaccines for avian influenza by conventional methods is complicated both technically and by the need for high containment facilities. Strategies adopted to overcome these issues include the use of baculovirus-expressed recombinant influenza proteins, particularly, influenza HA, a surface glycoprotein important for elicitation of influenza virus-neutralizing antibody response. In animal models, protection was achieved with recombinant H5 and H7 HA [18]. However, baculovirus-expressed recombinant HA from H5 avian influenza virus was found to be poorly immunogenic in people [19]. Thus, improved vaccines are needed for avian influenza.

Recently, influenza VLPs emerged as promising candidates for influenza vaccines [1,20]. In this study, we have demonstrated that H9N2 VLPs are immunogenic and protective in animal models. The i.m. administration was chosen to best parallel the conventional route of influenza

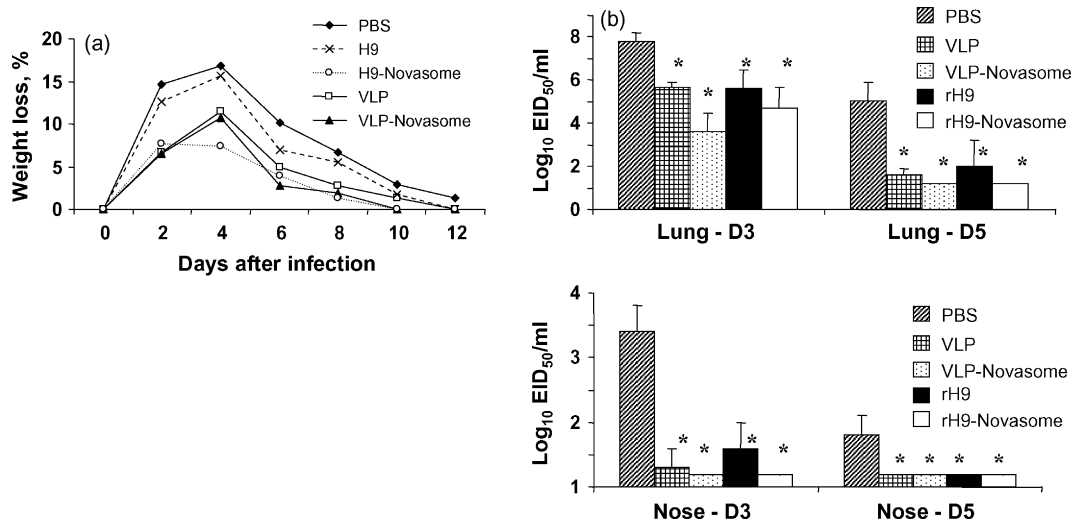


Fig. 6. Protective response in BALB/c mice vaccinated with H9N2 VLPs or rH9. Antigens were administered with and without Novasome adjuvant as indicated. (a) Weight loss in vaccinated vs. non-vaccinated mice (8 mice/group). (b) Titers of replicating influenza A/Hong Kong/1073/99 virus in lungs and nose tissues of vaccinated vs. non-vaccinated mice (5 mice/group). Detection limit was $1.2 \log_{10}$ EID₅₀/ml of virus. Also shown are standard deviation values. An asterisk indicates the vaccinated group was significantly ($p < 0.05$) different from the unvaccinated-control group by using analysis of variance (ANOVA).

vaccination for humans. We have also tested rH9 protein as vaccine antigen and Novasome nanoparticles as vaccine adjuvant.

The direct comparison between the VLP- and rH9-based vaccines is hampered due to distinct differences in antigen composition and presentation, as well as other factors. Without adjuvant, the VN titers were consistently higher in VLP-vaccinated mice. An enhancing effect of Novasomes on immunogenicity and protection was detected in SD rats as well as in BALB/c mice with either VLP or rH9 vaccine. The use of adjuvants for augmenting immune response is well-documented (reviewed in [11,21]). Most adjuvants function by attracting macrophages to the antigen, providing an antigen depot, and presenting the antigen to regional lymph nodes. Novasomes represent liposome nanoparticles made of nonphospholipid amphiphiles, which may also act as carriers for the antigen [10]. In Novasomes, 2–10 peripheral lipid bilayers encapsulate vaccine-relevant antigen in aqueous volume, which is interspersed between the bilayers and may also be encapsulated in the amorphous central cavity. Such paucilamellar structure ensures high load capacity and stability of the antigen.

The data suggest that the HA protein has higher immunogenicity when placed in the context of VLPs or in the context of Novasomes. Previous observations have shown that assembly of virus proteins into VLPs augments their immunogenicity due to presentation of epitopes in the native spatial conformation and highly repetitive fashion to the immune system (reviewed in [12]). We hypothesize that paucilamellar structure of Novasome nanoparticles may mimic the VLP structure. Interestingly, alum adjuvant, the only adjuvant currently licensed for use in humans in the U.S., did not increase the antibody response to the VLPs in our experi-

ments. This is in line with previous observation that alum fails to adsorb influenza virus HA and thus may be a poor adjuvant for this antigen [22]. However, more research is needed to determine long-term safety and efficacy of Novasomes *in vivo* following parenteral administration, as well as the mechanism of action of Novasomes.

In VLP-based influenza vaccines, in addition to HA, the NA and M1 proteins within VLPs may have also contributed to the protection observed with VLPs. Previously, baculovirus-expressed NA was immunogenic and protective in mice [23]. Epitope vaccine derived from influenza M1 also provided detectable level of protection in mice [24]. Highly conserved M1 protein, which is present in the VLP vaccine, may provide broader coverage including heterotypic protection against related strains of influenza [25].

Taken together, the data suggest that H9N2 VLPs represent promising vaccine for H9N2 influenza virus. Further, Novasome adjuvant improves immunogenicity and protection of both influenza VLP- and rH9-based baculovirus-generated recombinant vaccines. Therefore, the use of Novasome adjuvant deserves further study as potential vaccine component or antigen sparing agent. The latter may be important when many vaccine doses are needed within a short period of time, such as in the case of an influenza viruses with pandemic potential, such as H5N1 viruses.

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