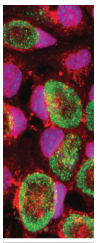

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RESEARCH ARTICLE

An H1-H3 chimeric influenza virosome confers complete protection against lethal challenge with PR8 (H1N1) and X47 (H3N2) viruses in mice

Asghar Abdoli[1], Hoorieh Soleimanjahi[1], Masoumeh Tavassoti Kheiri[2], Abbas Jamali[2], Vahideh Mazaheri[2] & Meghdad Abdollahpour Alitappeh[3]

1 Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2 Influenza Research Lab, Pasteur Institute of Iran, Tehran, Iran
3 Department of Immunology, Pasteur Institute of Iran, Tehran, Iran

This article reports some interesting results obtained immunizing mice against influenza using chimeric virosomes, furthering the advancement of virosome technology. Virosomes could become attractive system to vaccinate using multiple epitopes, providing broad protection against pathogens that have multiple serotypes or are highly mutagenic.

Keywords
influenza virus; protection; vaccination; virosome.

Correspondence
Hoorieh Soleimanjahi, Virology Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Tel.: +(98) 21 82883561
fax: +(98) 21 82883581
e-mail: soleim_h@modares.ac.ir

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Abstract

Annual health threats and economic damages caused by influenza virus are still a main concern of the World Health Organization and other health departments all over the world. An influenza virosome is a highly efficient immunomodulating carrier mimicking the natural antigen presentation pathway and has shown an excellent tolerability profile due to its biocompatibility and purity. The major purpose of this study was to construct a new chimeric virosome influenza vaccine containing hemagglutinin (HA) and neuraminidase (NA) proteins derived from the A/PR/8/1934 (H1N1) (PR8) and A/X/47 (H3N2) (X47) viruses, and to evaluate its efficacy as a vaccine candidate in mice. A single intramuscular vaccination with the chimeric virosomes provided complete protection against lethal challenge with the PR8 and X47 viruses. The chimeric virosomes induced high IgG antibody responses as well as hemagglutination inhibition (HAI) titers. HAI titers following the chimeric virosome vaccination were at the same level as the whole inactivated influenza vaccine. Mice immunized with the chimeric virosomes displayed considerably less weight loss and exhibited significantly reduced viral load in their lungs compared with the controls. The chimeric virosomes can be used as an innovative vaccine formulation to confer protection against a broad range of influenza viruses.

Introduction

Virosomes are *in vitro*-assembled virus-like particles from purified components, lacking a viral genome and internal proteins of the native virus. Influenza virosomes combine the immunological benefits of a virus-like particle with the technical advantages of a tightly controllable composition and the *in vitro* particle assembly process (Moser *et al.*, 2011). In contrast to liposomes, virosomes contain functional envelope glycoproteins of influenza virus, hemagglutinin (HA) and neuraminidase (NA), inserted into the phospholipid bilayer. These viral proteins not only confer structural stability and homogeneity to virosomal formulations but also significantly contribute to the immunological properties of virosomes, which are clearly distinct from other liposomal and proteoliposomal carrier systems (Wilschut, 2009). The fully functional fusion activity of virosomes using hemagglutinin (HA) protein enables receptor-mediated uptake and natural intracellular processing of the antigen, stimulating both arms of the immune system, the humoral and cellular immune responses (Huckriede *et al.*, 2005). Antibodies directed against the HA are most important for protection against influenza illness. In the mouse model, even low levels of pre-existing immunity (immunological memory) against influenza substantially enhance the antibody response against unrelated antigens delivered in the context

of an influenza virosome (Zurbriggen & Glück, 1999; Moser *et al.*, 2007). Beyond classical vaccine approaches based on antigen delivery, influenza virosomes have also been applied as local and systemic delivery vehicles for gene therapy as well as siRNA and drug delivery (Bungener *et al.*, 2002, 2005; Felnerova *et al.*, 2004; de Jonge *et al.*, 2005; Huckriede *et al.*, 2007).

There are several types of influenza vaccines, such as live attenuated influenza virus (LAIV), whole inactivated virus (WIV), split, subunit and virosomal vaccines (Jefferson *et al.*, 2005; Hagenaars *et al.*, 2008). The most common adverse events associated with an LAIV vaccine are nasal congestion, headache, myalgia and fever. LAIV may very rarely revert to its virulent form and cause disease (Gruber *et al.*, 1996). A WIV vaccine is superior to split and subunit vaccines in naïve human populations (Samdal *et al.*, 2005; Quan *et al.*, 2010). However, the WIV vaccine was withdrawn from the market because side effects such as fever and headache were observed more frequently than split and subunit vaccines (Gross *et al.*, 1977). A subunit vaccine does not contain the structure of original virus, and split vaccines consist of a mixture of solubilized membrane proteins and viral internal components. One of the major disadvantages of subunit and split vaccines is the fact that they generally require larger doses of vaccines, and/or strong adjuvants will be required, often inducing tissue reactions. Since virosomes (100–300 nm) have hemagglutinin (HA) and neuraminidase (NA) glycoproteins in vesicles resembling the structure of the wild-type virus, they mimic the natural antigen presentation route of the influenza virus and do not require any adjuvant (Hagenaars *et al.*, 2008). The virosomal influenza vaccine is the first influenza vaccine licensed for all age groups due to its biocompatibility and purity. Inflexal® V (Crucell, Switzerland) contains a mixture of three monovalent virosome pools, each of which is made from the specific hemagglutinin and neuraminidase glycoproteins of one influenza strain. Since 1997, Inflexal® V has been used widely in several European countries and has presented good results in terms of tolerability and safety in the elderly, the immunocompromised and children. Furthermore, it has been demonstrated that intramuscular administration of virosomes enhances hemagglutination-inhibition (HI) titers in human, similar to those induced by conventional whole virus or subunit vaccines (Kanra *et al.*, 2004; Herzog *et al.*, 2009).

In addition, influenza virosome has been successfully applied commercially as an adjuvant, like alum salts and oil emulsions (MF59, AS03 and AF03). Since 1994, influenza virosomes as an adjuvant have been efficiently used in the development of Epaxal® for vaccination of Hepatitis A virus (Glück *et al.*, 1992; D'Acremont *et al.*, 2006; Bovier, 2008). The influenza virosomes as a vaccine carrier and adjuvant system have been recently used in the formulation of vaccines against human diseases such as malaria, leishmania, cancer and infectious agents such as HCV and HIV (Liu *et al.*, 2006; Tanzi *et al.*, 2006; Thompson *et al.*, 2008; Wiedermann *et al.*, 2010; Jamali *et al.*, 2012).

Almeida *et al.* (1975) were the first group to report the construction of lipid vesicles comprising viral spike proteins derived from the influenza virus. In 1987, Stegmann *et al.* (1987) described a new procedure to construct influenza virosomes by reconstitution of virus-like particles only containing viral membrane phospholipids and glycoproteins. Whereas virosomes have been prepared from a variety of enveloped viruses, influenza virosomes remain the only ones applied in clinical stage vaccine candidates and in licensed products (Moser *et al.*, 2013). Influenza virosomes were used to deliver the DNA vaccine to induce protective immune responses against both homologous and heterologous challenges (Kheiri *et al.*, 2012).

In this study, H1-H3 chimeric and monomeric virosomal influenza vaccines were constructed by means of a dialyzable short-chain phospholipid 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine (DCPC) as a solubilizing agent, and their efficiency as a vaccine in mice was compared with the inactivated influenza vaccine and monomeric influenza virosomes.

Materials and methods

Cell line and virus strains

Madin–Darby canine kidney (MDCK) cell line (ATCC CCL_{-34}) was obtained from the National Cell Bank of Iran, Pasteur Institute of Iran. Influenza virus X47 (a re-assortant between the HA and NA of A/Victoria/3/75 H3N2 and internal genes of A/Puerto Rico/8/34 H1N1) and A/PR8/34 (H1N1) were kindly provided by Xavier Saelens (University of Ghent, Ghent, Belgium) and Anke Hueckride (University of Groningen, Groningen, Netherlands), respectively.

Microcarrier cell culture

Cytodex 1 microcarriers were used to scale-up a cell-based influenza virus production process to construct virosomes. The Cytodex 1 microcarriers (Sigma-Aldrich, Sweden) were sterilized by autoclaving at 120 °C for 15 min. Microcarriers were dispensed at 2 g L^{-1} in a siliconized spinner flask (Cellspin Integra Biosciences). MDCK cells were added at a density of 2×10^5 cells mL^{-1} to a spinner flask, and then fresh complete DMEM was added. The spinner flasks were placed on a stirrer in a 37 °C, 5% CO_2 incubator and agitated at 55 r.p.m. for 4 h (1 min with 20-min intervals). Cell attachment to the microcarriers was observed under a microscope. Subsequently, an appropriate amount of complete medium was added to 70% of full volume of the spinner flask stirred while incubating at 37 °C in an atmosphere of 5% CO_2 (Genzel *et al.*, 2004; Bock *et al.*, 2011; Abdoli *et al.*, 2013b).

Virus propagation and purification

To propagate the viruses, the cell culture growth medium was replaced with serum-free DMEM containing antibiotics (100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin), with 2 μg mL^{-1} of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). The cells were infected with 0.01 multiplicity of infection (moi) in spinner

flasks and harvested after 72 h (Abdoli *et al.*, 2013a). Discontinuous sucrose gradient ultracentrifugation (10%/60% w/v) was applied for 1.5 h (100 000 ***g*** at 4 °C in a Optima XL-90 Beckman rotor Ti-90) for purification of the virus. The purified viruses were harvested from the boundary of the two sucrose layers (10%/60% w/v), and dialyzed against Hank's buffer solution (HBS) overnight at 4 °C to eliminate the residual sucrose. Subsequently, the dialyzed influenza viruses were sedimented by ultracentrifugation (100 000 ***g*** for 1 h at 4 °C), and 5 mg of each purified virus pellet was resuspended in 375 μL of HBS (Noori *et al.*, 2012).

Virosome construction

The monovalent inactivated whole-virus pools were separately ultracentrifuged. The pellet was then solubilized with the 200 mM detergent 1,2-dicaproyl-sn-glycero-3-phosphocholine (DCPC) in HBS. Subsequently, the solutions were incubated on ice for 30 min and mixed. Then, viral ribonucleoproteins (RNPs) were removed by ultracentrifugation (85 000 ***g*** for 30 min at 4 °C). After removing the RNP, the supernatant containing the viral glycoproteins and lipids was harvested and dialyzed to remove DCPC to prepare crude virosomes. To separate non-incorporated and non-chimeric virosomes from the reconstituted chimeric virosomes, the crude virosomes were purified by discontinuous sucrose gradient (10%/60% w/v sucrose in HBS) and centrifuged for 1.5 h (100 000 ***g*** at 4 °C). The purified chimeric virosomes were harvested from the boundary of the two sucrose layers and dialyzed against HBS overnight at 4 °C to remove the sucrose and then filter-sterilized (de Jonge *et al.*, 2006). The protein content of the virosomes was analyzed by a micro Lowry protein assay (Lowry *et al.*, 1951).

SDS-PAGE and Western blotting

The purified H3N2, H1N1, and chimeric virosomes were loaded on a 12% polyacrylamide gel to perform SDS-PAGE and transferred onto the nitrocellulose membrane (0.45 μm, S&S Bioscience GmbH, Whatman group, Dassel, Germany) using a semidry electro-transfer apparatus (Applex, 016932). The blotting was carried out in transfer buffer (pH 8) containing 10% methanol, 24 mM Tris (Sigma-Aldrich), and 194 mM glycine (Sigma-Aldrich), at 10 V for 30 min. To block nonspecific binding, the membrane was incubated in blocking solution containing 2.5% bovine serum albumin (Roche, Mannheim, Germany) in phosphate-buffered saline at room temperature (RT) for 1.5 h. The blotted HA protein reacted with mouse primary monoclonal antibody (Abcam) directed against HA of H3N2 and H1N1 viruses. An anti-mouse IgG antibody (Sinobiological, China) conjugated with HRP was applied as a secondary antibody. The TMB substrate solution (Sigma-Aldrich) was added to visualize the reacted protein bands (Mallick *et al.*, 2011; Yousefi *et al.*, 2012).

Hemagglutination assay (HA)

To verify whether the reconstituted virosomes are able to bind to sialic acid receptors, HA assay was performed. The purified constructed virosomes were diluted in serial twofold dilutions with phosphate-buffered saline (PBS). Chicken red blood (0.5%) cell suspension was added to each dilution in the U-shaped microtiter plates. Following gentle agitation, the plates were left undisturbed for 30 min at RT. The last dilution showing complete hemagglutination was considered the end point, and its dilution factor expressed as hemagglutination units (HAU) per test volume (Kistner *et al.*, 1998).

Determination of particle size

The average particle sizes of the virosomes were determined by dynamic light scattering by means of photon correlation spectroscopy. The measurements were performed at 25 °C with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK) equipped with a helium-neon laser and a scattering angle of 173 °C (Ghanbari Safari & Hosseinkhani, 2013).

Sandwich ELISA

Sandwich ELISA was performed to confirm the presence of both HAs (HA3 and HA1) of two different influenza subtypes on the surface of a virosome particle. Anti-H3-specific monoclonal antibody, as a capture antibody (Abcam), was coated onto 96-well ELISA plates. After overnight incubation at 4 °C, the plates were washed three times. Next, the coated plates were blocked with 5% skim milk in PBS at 37 °C for 1 h and then washed three times with PBS buffer containing 0.05% Tween-20 (PBST). Then, chimeric virosome samples were added and incubated overnight at 4 °C with mixing, followed by washing the blocked plates with PBS-Tween. Subsequently, the plates were incubated with a 1 : 5000 dilution of mouse IgG anti-HA1 monoclonal antibodies (Sinobiological, China) overnight at 4 °C with agitation. The plates were then incubated with the 1 : 5000 secondary antibody conjugated with horseradish-peroxidase (Sinobiological, China) at 37 °C for 1.5 h with shaking. After washing the plate, color was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) chromagen at 37 °C for 15 min with mixing. The reaction was then stopped with an equal volume of 2 N H_2SO_4, and optical density (OD) was measured at 450 nm. The ELISA cut-off values were determined using the mean absorbance of negative control plus three times the standard deviation (Kumar *et al.*, 2010; Hashemi *et al.*, 2012).

Mice and immunization protocols

Female BALB/c mice 6–8 weeks old were obtained from the animal facilities of Pasteur Institute of Iran (Karaj, Iran). Mice were housed for 1 week before the experiment, given free access to food and water, and maintained in a light/dark cycle with lights on from 6:00 to 18:00 hours. All mouse experiments were performed in agreement with Institutional Animal Care and Research Advisory Committee of Pasteur Institute of Iran. Mice were divided into eight groups of 10 mice. All mice were injected intramuscularly with a single dose (100 μL) of each vaccine. Groups 1–8 were injected

with 5 μg of PR8 virosomes, 5 μg of X47 virosomes, 10 μg of chimeric virosomes, 10 μg of co-adminstered PR8 and X47 virosomes, 5 μg of inactivated PR8 viruses, 5 μg of inactivated X47 viruses, 10 μg of co-adminstered inactivated X47 and PR8 viruses, and 100 μL of PBS as a negative control, respectively.

Animal challenge

Three weeks after the immunization, BALB/c mice were anesthetized with a cocktail of 10% ketamine (100 mg kg^{-1}) and 2% xylazine (10 mg kg^{-1}), and 10 mice per group were challenged intranasally with five mouse infectious doses of 50% (MID50) of X47 and/or PR8 viruses. Mortality and weight loss were monitored for 14 days after challenge (Jamali *et al.*, 2010).

Quantitation of viral loads in lungs

Six days after challenge, four mice from each group were sacrificed by cervical dislocation for titration of residual viruses in the lungs. The lungs of the mice were aseptically removed, rinsed in sterile PBS, and homogenized in 2 mL ice-cold PBS buffer containing 100 IU mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. Lung homogenates were clarified by centrifugation at 1159 ***g*** for 10 min to pellet cell debris, and the supernatants were then stored at −20 °C until the assay was performed. Semi-confluent MDCK cells in 96-well plates were infected with serial log10 dilutions of clarified lung samples in DMEM containing TPCK-treated trypsin. After 72 h of incubation at 37 °C in a 5% CO_2 atmosphere, the culture supernatants were tested for the presence of influenza virus HA activity by mixing 50 μL of supernatant with 50 μL of a 0.5% suspension of chicken RBCs. The viral titer in each lung specimen was calculated by the Karber method and expressed as the 50% cell culture infective dose ($CCID_{50}$). The viral titers were represented by the mean 6 SD of the viral titer per mL in the lung homogenates prepared from five mice in each group (Jamali *et al.*, 2010).

HI assay

To measure anti-HA Ab titers by the HI assay, sera were heat-inactivated by incubation at 56 °C for 30 min. To reduce non-specific hemagglutination inhibition, 225 μL of a 25% kaolin suspension was added to heat-inactivated sera. The mixture was vortexed and incubated at RT for 20 min. After centrifugation at 6500 ***g*** for 2 min, 50 μL of the supernatant was harvested and serially diluted (two-fold) in PBS in U-bottom 96-well plates (Greiner, Germany). Four HA units of X47 or PR8 viruses were separately added to each sample and incubated at RT for 1 h, followed by addition of 0.5% chicken red blood cells and incubation at RT for 30 min. The HI titer for each sample was reported as the reciprocal of the highest serum dilution that completely inhibited hemagglutination (Kheiri *et al.*, 2012).

ELISA

To determine pre-challenge IgG titers, five mice per group were bled via the retro-orbital vein 21 days after the immunization. The blood samples were left to clot at RT for 1 h followed by at least 5 h at 4 °C for contraction of clots. Sera from mice were collected by centrifugation at 290 ***g*** for 10 min, stored at −20 °C until analysis and then tested individually. The titers of total IgG in each mouse serum directed against H1 and H3 chimeric virosomes were measured by ELISA. Maxisorp ELISA plates (Nunc, Denmark) were coated with X47 and PR8 viruses in Tris-NaCl resuspension buffer (pH 8), and the plates were incubated for 1 h at RT and then overnight at 4 °C. The plates were blocked with 5% skim milk in PBS at 37 °C for 1 h, washed twice with PBS buffer containing 0.05% Tween-20 (PBST), and then incubated with twofold serially diluted sera in PBS (100 μL per well) at 37 °C for 2 h. Next, all plates were incubated with 100 μL of a 1 : 5000 dilution of the horseradish peroxidase-conjugated goat polyclonal anti-mouse-IgG secondary antibody (Abcam) for 30 min, and washed three times. After washing, color was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) chromogen (100 μL per well) at 37 °C for 15 min with mixing. The reaction was then stopped with an equal volume of 2 N H_2SO_4, and optical density was then measured at 450 nm ($OD_{450\ nm}$). All serum samples from individual mice were assessed in triplicate (Lee *et al.*, 1993;, Zandi *et al.*, 2007; Anvar *et al.*, 2013) .

Statistical analysis

Antibody titers, body weight changes and residual virus titers in the lungs were compared among different groups of mice by Student's *t*-test and one-way ANOVA followed by Tukey's and Dunnett's post-tests. Survival data were analyzed by the Kaplan–Meier method and log-rank test. Results at the $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ levels were considered statistically significant. GRAPHPAD PRISM 5 software (La Jolla, CA) was used for all statistical analyses.

Results

Purified virosome preparation, SDS-PAGE and Western blot analysis

Influenza virion buoyant density in sucrose is 1.19–1.21 g mL^{-1}; therefore, 10% and 60% sucrose step-gradient concentrations were used to purify virosomes. Purified virosomes were loaded on a polyacrylamide gel for electrophoresis. Two viral glycoproteins, hemagglutinin and neuraminidase, were inserted into the virosome membrane. HA is the major envelope glycoprotein of influenza virus, composed of two separate molecules, termed HA1 (50 kDa) and HA2 (27 kDa), joined by a disulfide bond (Weis *et al.*, 1988; Skehel & Wiley, 2000). The complete HA molecule is composed of three subunits each with a molecular weight of approximately 75–80 kDa and 20% carbohydrate to give a

total molecular weight of approximately 225 kDa (Daniels *et al.*, 1985). NA is composed of four identical subunits, each with a molecular weight of approximately 55–60 kDa (Shaw *et al.*, 2008). Figure 1 shows the efficient removal of major nuclear proteins while sparing the major surface glycoproteins (HA and NA). The constructed X47, chimeric, and PR8 virosomes were separated on a 12% polyacrylamide gel, and the desired bands were revealed with Western blot analysis using anti-H1 and -H3 monoclonal antibodies (Fig. 1).

HA assay and size distribution of particle diameters

The result of the HA assay revealed that reconstituted virosomes are able to bind and agglutinate the red blood cells via their surface glycoproteins. The HA units of whole viral particles before and after the construction of virosomes were 655 360 HA mL^{-1} and 163 840 HA mL^{-1}, respectively. The great majority of chimeric virosomes showed a Z-average of about 148 nm, and a moderate particle size distribution (PDI) of 0.282 (Fig. 2).

Sandwich ELISA

The antigen to be measured by sandwich ELISA must contain at least two antigenic sites capable of binding to the antibody, since at least two antibodies act in sandwich ELISA. The cut-off point of the ELISA test in this study was an absorbance value of 0.6, and the absorbance value for chimeric virosomes were 1.8. This confirmed the simultaneous presence of two different HA1-HA3 proteins on the surface of a virosome particle (Fig. 3).

HI assay

Serum samples were collected 21 days after vaccination, and tested for the HI assay. Vaccine-induced antibody titers were observed in all vaccinated groups. As shown in Fig. 4, no statistically significant differences were observed among vaccinated groups. Moreover, no cross-reactive HI antibody response to the heterologous PR8 virus was detected in the sera of mice vaccinated with monomeric X47 virosomes or inactivated H3N2 viruses, or vice versa. HI antibody responses against both strains of influenza virus were detected in the sera of mice injected with chimeric virosomes and co-adminstered inactivated viruses.

Lethal dose challenge

To determine the potency of protective efficacies, mice were challenged with two mouse-adapted subtypes of influenza A virus, PR8 and X47. As shown in Fig. 5, the chimeric virosomes vaccinated group exhibited a significantly higher

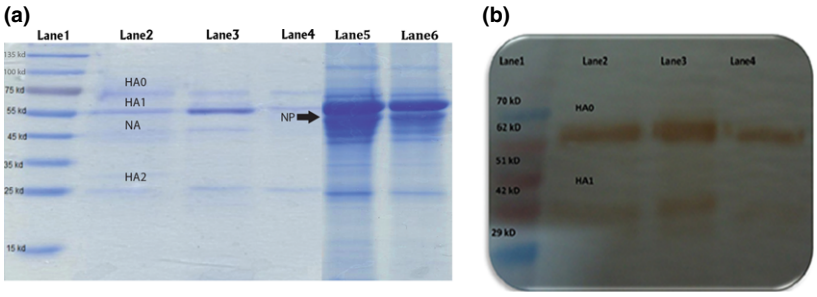

Fig. 1 SDS-PAGE and Western Blot analysis of purified influenza virosomes. The constructed H3N2, chimeric and H1N1 virosomes (10 μg of each virosome) were loaded on a 12% polyacrylamide gel and stained with Coomassie blue (a). HA is seen in SDS-PAGE in three forms: uncleaved (HA0 = 75 kDa) and cleaved (HA1 = 50 kDa and HA2 = 27 kDa); only one form of NA (approximately 55–60 kDa) can be observed in SDS-PAGE gels. Anti-influenza virus H1 and H3 monoclonal antibodies were used for Western blot analysis (b). Lane 1: protein marker. Lane 2: monomeric H3N2 virosome. Lane 3: chimeric virosome. Lane 4: monomeric H1N1 virosome. Lanes 5 and 6: wild-type viruses (control), demonstrating the nuclear proteins [NP, shown by a vertical arrow in (a)] that were effectively removed from virosome structures.

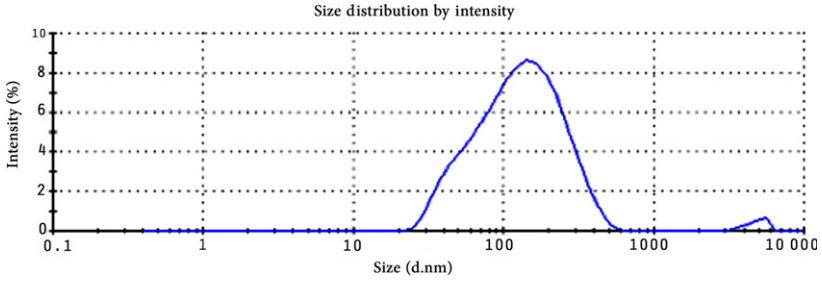

Fig. 2 The size distribution profile of chimeric virosomes measured by zetasizer.

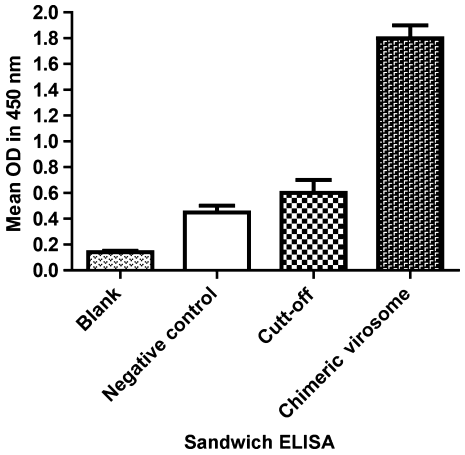

Fig. 3 Sandwich ELISA. A double-antibody sandwich ELISA was employed for detection of H1 and H3 on the surface of the chimeric virosome using anti-H1 and -H3 antibodies. Cut-off values were estimated at mean absorbance of negative controls plus three times the standard deviation.

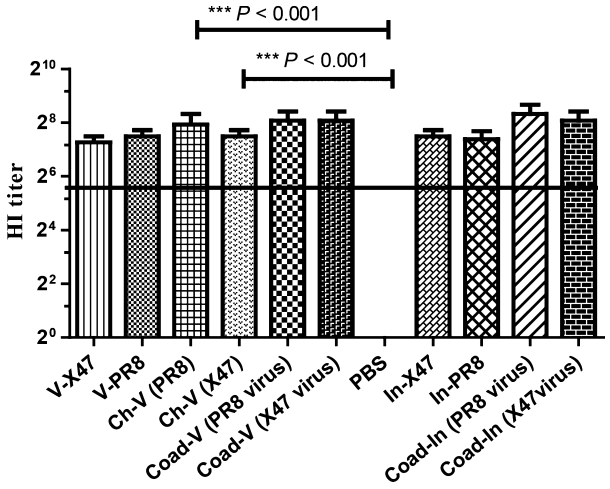

Fig. 4 Hemaggutination inhibition (HI) titers. Mice were bled 21 days after vaccination and serum samples were tested for the HI assay. The viruses within parentheses indicate the virus for which the HI assay were done. The horizontal line represents the 1 : 40 dilution. Groups of mice were immunized with V-X47 [monomeric X47(H3N2) virosomes], V-PR8 [monomeric PR8 (H1N1) virosomes], Ch-V (chimeric virosomes), Coad-V (co-administered PR8 and X47 monomeric virosomes), In-H1N1 (inactivated PR8 viruses), In-X47 (inactivated X47 viruses), Coad-In (co-administered inactivated PR8 and X47 viruses), and PBS as a negative control.

survival rate ($P < 0.001$) in comparison with monomeric inactivated viruses, monomeric virosomes and non-vaccinated control group when challenged with both viruses. Chimeric virosomes protected mice against both lethal virus infections, whereas 80 and 100% mice injected with monomeric-inactivated viruses and monomeric virosomes succumbed to lethal challenge with heterologous viruses,

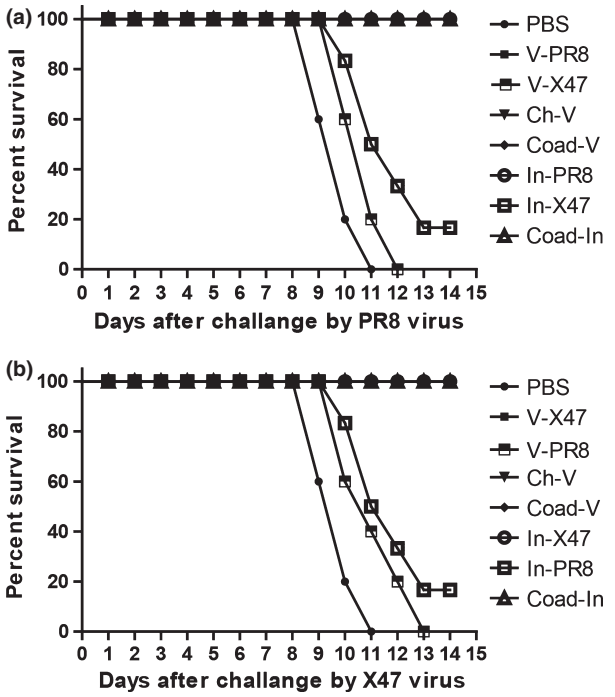

Fig. 5 Chimeric virosome vaccine efficacy in mice following lethal challenge with PR8 (a) or X47 (b) viruses. At 21 days after the vaccine injection, mice were challenged with five lethal doses (LD50) of PR8 and X47 viruses. Mice were monitored daily for 14 days post challange. Survival rates following the challenge were calculated based on percent survival within each experimental group ($n = 6$ mice per experimental group; $^{***}P < 0.001$). Mice immunised with chimeric virosomes showed significat improved survival rates compared with those immunized with monomeric inactivated viruses and virosomes ($^{***}P < 0.001$) when challenged with both viruses. Groups of mice were immunized with V-X47 [monomeric X47(H3N2) virosomes], V-PR8 [monomeric PR8(H1N1) virosomes], Ch-V (chimeric virosomes), Coad-V (co-administered PR8 and X47 monomeric virosomes), In-H1N1 (inactivated PR8 viruses), In-X47 (inactivated X47 viruses), Coad-In (co-administered inactivated PR8 and X47 viruses) or PBS as a negative control.

respectively. All control mice showed excessive weight loss and succumbed to lethal challenge with both viruses.

Prevention of weight loss in mice with chimeric influenza virosome after challenge

The mice were monitored daily for weight loss for 2 weeks after challenge with X47 and PR8 viruses. As shown in Fig. 6, mouse groups injected with chimeric virosomes and co-adminstered inactivated viruses experienced only a small and transient weight loss after challenge with homologous and heterologous viruses compared with those injected with monomeric virosomes, inactivated viruses or PBS ($P < 0.001$). The least weight loss occurred in those receiving co-adminstered inactivated viruses (5%) and chimeric virosomes (9%), with recovery to the initial level within 12 days. Mice immunized with monomeric virosomes

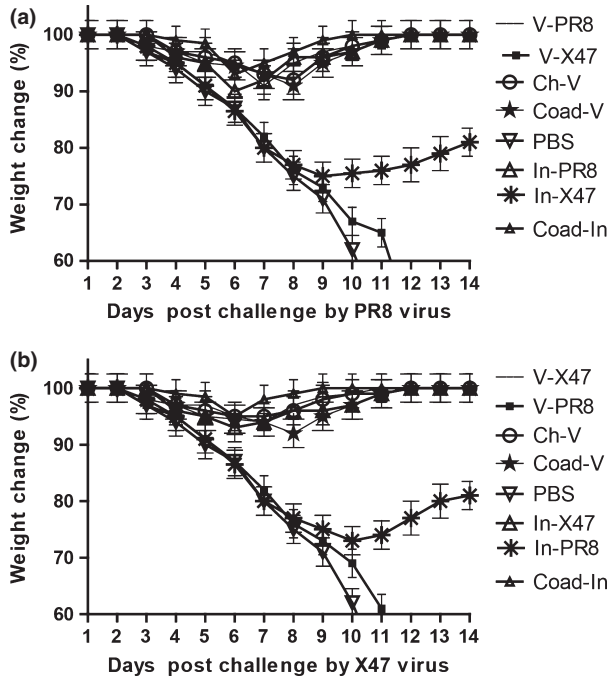

Fig. 6 The mice weight loss after virus challenge. The immunized mice were challenged by 5 LD50 of mouse-adapted PR8 (a) and X47 (b) viruses 21 days post immunization. Mice were monitored for changes in body weight daily for 14 days. Average weights in each treatment group were measured for the duration of the study, and the percent original body weight was calculated based on the average starting weight for each group on day 0. Groups of mice were immunized with V-X47 [monomeric X47(H3N2) virosomes], V-PR8 [monomeric PR8(H1N1) virosomes], Ch-V (chimeric virosomes), Coad-V (co-administered PR8 and X47 monomeric virosomes), In-H1N1 (inactivated PR8 viruses), In-X47 (inactivated X47 viruses), Coad-In (co-administered inactivated PR8 and X47 viruses) or PBS as a negative control.

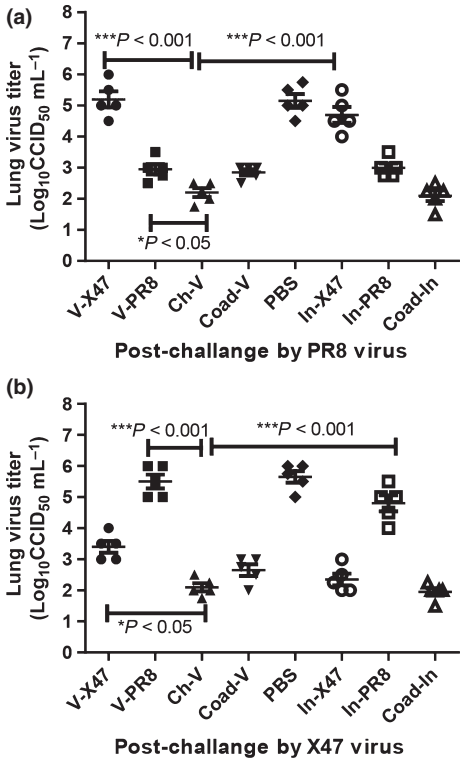

Fig. 7 Influenza A viral titers in the mice lungs. Five days post-challenge by PR8 and X47 viruses, four mice in each group were sacrificed and residual virus titers were measured in the lungs as $CCID_{50}$ per mL of each lung homogenate. Values were the means ± SE of the means for three experiments. Groups of mice were immunized by V-X47 [monomeric X47(H3N2) virosomes], V-PR8 [monomeric PR8(H1N1) virosomes], Ch-V (chimeric virosomes), Coad-V (co-administered PR8 and X47 monomeric virosomes), In-H1N1 (inactivated PR8 viruses), In-X47 (inactivated X47 viruses), Coad-In (co-administered inactivated PR8 and X47 viruses) or PBS as a negative control.

and inactivated viruses showed severe weight loss when challenged with hetrologus viruses. Mice injected with PBS exhibited excessive weight loss (40%) within 7–10 days post challenge. Only one mouse from each group immunized with inactivated viruses survived and they only recovered 90% of body weight at the end of the 14-day follow-up.

Viral clearance from the lungs of immunized mice after challenge

To evaluate the effect of chimeric virosome formulation on the virus clearance rate in the lungs, the immunized mice were challenged intranasally with five LD_{50} of PR8 and X47 influenza viruses. As shown in Fig. 7a, influenza virus titers after PR8 challenge were significantly lower in mice immunized with chimeric virosomes compared with those injected with inactivated X47 viruses, monomeric X47 virosomes, PBS ($P < 0.001$) or monomeric PR8 virosomes ($P < 0.01$). After challenge with both viruses, mice injected with chimeric virosomes but not those injected with monomeric virosomes or inactivated viruses showed a significant reduction in lung virus titers compared with PBS-treated controls ($P < 0.001$). Furthermore, a higher level of viral clearance was observed in mice immunized with chimeric virosomes and co-adminstered inactivated viruses with no significant difference (Fig. 7a and b).

Serum IgG titers

To assess the ability of chimeric virosomes to mount serum IgG production, sera were collected at 3 weeks post immunization, and an ELISA assay was applied to detect the IgG titer in addition to the HI titer. Mice immunized with chimeric virosomes showed significantly higher specific IgG antibody responses against both X47 and PR8 viruses than those induced by monomeric virosomes (Fig. 8). In contrast, animals receiving monomeric virosomes could not induce anti-heterologous virus strain antibodies. Furthermore, chimeric virosomes enhanced antibody production to the same level as inactivated viruses. In the control groups (PBS-treated groups), no specific IgG antibodies were found ($P < 0.001$).

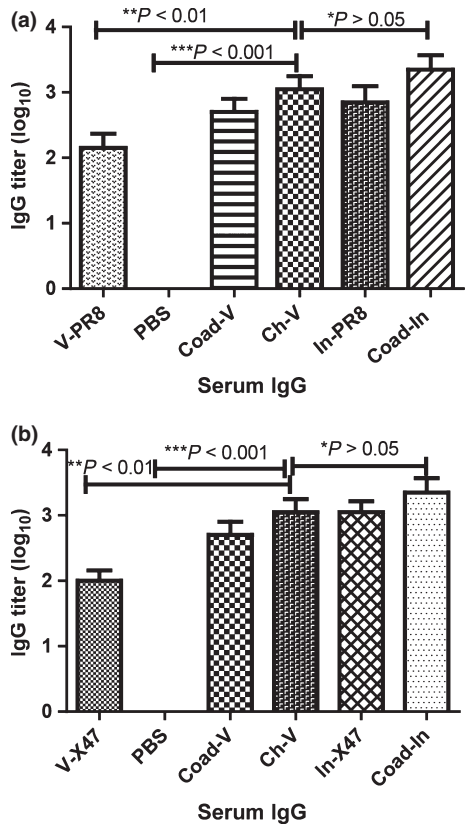

Fig. 8 Antibody responses in BALB/c mice. (a) Measurements of anti-influenza chimeric virosome IgG antibodies by ELISA using PR8-coated microplates. Results are stated as antibody endpoint titer, where the OD value was threefold higher than the background value obtained with a 1 : 50 dilution of pre-immune serum from BALB/c mice. Data represent the mean ± SD of five mice. Statistically significant differences were signified by $*P < 0.05$, $**P < 0.01$, and $**P < 0.001$. Results at levels $P > 0.05$ were not considered statistically significant. (b) Measurements of anti-influenza chimeric virosome IgG antibodies by ELISA using X-47-coated microplates. Groups of mice were immunized by V-X47 [monomeric X47(H3N2) virosomes], V-PR8 [monomeric PR8 (H1N1) virosomes], Ch-V (chimeric virosomes), Coad-V (co-administered PR8 and X47 monomeric virosomes), In-H1N1 (inactivated PR8 viruses), In-X47 (inactivated X47 viruses), Coad-In (co-administered inactivated PR8 and X47 viruses) or PBS as a negative control.

Discussion

Designing novel vaccine candidates that closely mimic specific pathogens without being pathogenic themselves remains a key challenge for influenza vaccine development (Kapoor *et al.*, 2013). Virosomes are tightly controllable virus-like particles suitable for vaccine formulation (Moser & Amacker, 2013).

In the present study, we have constructed a chimeric virosome directly from the PR8 and X47 envelope viruses by a reconstitution method using short-chain phospholipid 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DCPC), and its efficiency as a vaccine candidate was evaluated in mice. de Jonge *et al.* (2006) demonstrated that the use of a dialyzable DCPC as a viral membrane solubilizer offers several benefits over the traditional method of virosome preparation involving viral envelope solubilization with either Triton X-100 or C12E8. It allows more efficient removal of detergent molecules from the virosomal membranes by dialysis, which favors membrane stability without antigenic changes and can preserve the ability to agglutinate chicken red blood cells (Stegmann *et al.*, 2010; Kheiri *et al.*, 2012; Shafique *et al.*, 2013).

SDS-PAGE analysis demonstrated that chimeric virosomes contained both HA and NA protein bands and were devoid of nucleocapsid complexes of the virus. The average diameters of chimeric virosomes was about 148 nm, which is consistent with observations made in earlier experiments on monomeric virosomes constructed by de Jonge *et al.* (2006).The sandwich ELISA analysis confirmed that HA of PR8 and X47 viruses are simultaneously present on the surface of a chimeric particle. We hypothesize that the coexistence of HA3 and HA1 ligands on the surface of chimeric virosomes might enhance the uptake of particles by attaching them to both α-2, 6- and α-2, 3-linked sialic acid receptors. This may lead to the presentation of more antigens by major histocompatibility complex class I and II (MHC-I and MHC-II) proteins, resulting in activation of B cells, dendritic cells and T cells, as observed during viral infection (Lamb & Krug, 2001; Sharma & Yasir, 2010). As suggested by Rezaei *et al.* (2013), chimeric influenza VLPs play an helpful role in antigen-sparing effects. We demonstrated that the chimeric virosomes are highly immunogenic and elicit a strong immune response against both viruses. Collectively, these results are consistent with those reported by Rezaei *et al.* (2013), showing that influenza virus-like particles containing two different subtypes of hemagglutinin (H1-H3) proteins are able to protect against both the lethal challenges of influenza viruses.

Three weeks after a single intramuscular vaccination with the chimeric virosomes, the HI titers were at the same level as inactivated influenza virus in BALB/c mice. Similarly, virosome particles containing HA of PR8 and X47 viruses elicited higher IgG titers against both viruses compared with monomeric virosomes. Four weeks after vaccination, the mean serum HI and IgG titers of a group of mice receiving chimeric virosomes correlated with a high level of protection, conferring a 100% protection against challenge with five LD_{50} of mouse-adapted PR8 or X47 viruses. They indicated only a transient body weight loss, whereas the animals receiving monomeric virosomes showed excessive weight loss and were not protected against heterologous virus infection. In addition, lower virus titers were detected in the lungs of mice receiving chimeric virosomes in comparison with monomeric virosomes. In a subset of subjects with low pre-vaccination titers, De Bruijn *et al.* (2004) showed that 76–99% of subjects attained protective hemagglutination inhibiting (HI) antibody titers after vaccination with the virosomal influenza vaccine. They also investigated the safety and immunogenicity of virosomal vaccines in individuals 61 years and older as compared with standard inactivated influenza vaccines. The vaccines had similar immunogenicity results, whereas the tolerability

profile of the virosome vaccine was better than that of inactivated subunit vaccine (De Bruijn *et al.*, 2006). The result of the present study is in contrast with the result of Hagenaars *et al.* (2008). Those authors reported the lowest HI and IgG titer levels for influenza virosomal vaccine compared with whole inactivated, subunit and split influenza vaccines.

From a validation and quality assurance point of view, the costs of establishment, formulation, quality, safety, efficacy and speed of vaccine production are the major problems hindering the development of new vaccines. In the chimeric virosome, all steps of the vaccine preparation, including ultracentrifugation, sterilized filtration, determination of virosome size as well as detergent and endotoxin assays, last a shorter time than three monovalent virosome vaccines (Mischler & Metcalfe, 2002). Therefore, the chimeric virosome strategy seems to be a reliable method for commercialization.

The virosomal vaccine has shown an excellent tolerability profile due to its biocompatibility and good immunogenicity in both healthy and immunocompromised elderly, adults and children (Herzog *et al.*, 2009).

Amacker *et al.* (2005) developed a chimeric liposome based on the virosome [chimeric immunopotentiating reconstituted influenza virosome (CIRIV)] as a carrier system for the delivery of HCV core peptides into cells. However, the composition and method of virosome development by Amacker *et al.* is completely different from the method used in this study.

In conclusion, the present study investigated whether a vaccination platform based on chimeric influenza virosomes in BALB/c mice provides a highly effective protection against lethal dose challenge of both PR8 and X47 viruses. It was shown that the chimeric virosome vaccination prevents weight loss in mice, which show resistance to five lethal $dose_{50}$ challenges by both homologous and heterologous viruses. Administration of chimeric virosomes significantly enhances the viral clearance from the lungs of the challenged mice. The chimeric virosomes elicit robust anti X47 and anti PR8-IgG antibody responses. Chimeric virosomes are an attractive vaccine platform, possibly due to their particulate nature along with the presentation of functional glycoproteins in a native conformation without being pathogenic.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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