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Influenza virosome/DNA vaccine complex as a new formulation to induce intra-subtypic protection against influenza virus challenge

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ABSTRACT

Influenza virosome is one of the commercially available vaccines that have been used for a number of years. Like other influenza vaccines, the efficacy of the virosomal vaccine is significantly compromised when circulating viruses do not have a good match with vaccine strains due to antigenic drift or less frequent emergence of a pandemic virus. A major advantage of virosome over other influenza vaccine platforms is its intrinsic adjuvant activity and potential carrier capability which have been exploited in this study to broaden vaccine protectivity by incorporating a conserved component of influenza virus in seasonal vaccine formulation. Influenza nucleoprotein (NP)-encoding plasmid was adsorbed onto surface of influenza virosomes as a virosome/DNA vaccine complex. Mice were immunized with a single dose of the influenza virosome attached with the NP plasmid or NP plasmid alone where both influenza virosomes and NP gene were derived from influenza A virus H1N1 New/Caledonia strain. Analysis of the cellular immune responses showed that 5 μg (10-fold reduced dose) of the NP plasmid attached to the virosomes induced T cell responses equivalent to those elicited by 50 μg of NP plasmid alone as assessed by IFN-γ and granzyme B ELISPOT. Furthermore, the influenza virosome/NP plasmid complex protected mice against intra-subtypic challenge with the mouse adapted H1N1 PR8 virus, while mice immunized with the virosome alone did not survive. Results of hemagglutination inhibition test showed that the observed intra-subtypic cross-protection could not be attributed to neutralizing antibodies. These findings suggest that influenza virosomes could be equipped with an NP-encoding plasmid in a dose-sparing fashion to elicit anti-influenza cytotoxic immune responses and broaden the vaccine coverage against antigenic drift.

1. Introduction

Influenza virus causes serious morbidity and mortality in human populations (Nicholson et al., 2003; Palese, 2004; Stohr et al., 2006). During seasonal epidemics, 5–15% of the worldwide population is typically infected, resulting in 3–5 million cases of severe illness and up to 500,000 deaths per year (Stohr et al., 2006). Vaccination is the most effective means for the prevention of influenza virus infection (Rimmelzwaan and Osterhaus, 2001). Current licensed influenza vaccine strategies are based upon induction of neutralizing antibodies mainly against haemagglutinin and include whole-inactivated virus, split virus, subunit and virosomal vaccines (Geeraedts and Huckriede, 2011; Herzog et al., 2009). Virosomal influenza vaccines have been on the European market for over 11 years, and more than 41 million doses have been sold (Herzog et al., 2009). Virosome is a reconstituted envelope of the influenza virus devoid of viral genetic material that preserve the receptor-binding and membrane fusion activity of the viral haemagglutinin (Stegmann et al., 1987). Virosomal vaccines have been shown to induce antibody titers comparable to the whole inactivated or subunit vaccines. Moreover, they appear to induce a long-term sero-protection rate in the elderly (Zamparo and Little, 2011).

Efficacy of the virosomal influenza vaccine like other available influenza vaccine formulations is significantly compromised when circulating viruses do not have a good match with vaccine strains

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due to antigenic drift or inaccurate epidemiological predictions. Antigenic drift is the gradual evolution of viral strains due to frequent mutations (Both et al., 1983). Most of these mutations are 'neutral' as they do not affect the conformation of the proteins; however, some mutations cause changes to the viral proteins such that the binding of host neutralizing antibodies is compromised. Influenza virus strains that are not matched with the seasonal vaccine circulate on a regular basis and can have a significant impact on vaccine effectiveness (Carrat and Flahault, 2007). Indeed it has been suggested that approximately once every decade the mismatch between virus and vaccine is enough to reduce vaccine effectiveness by 70% (Carrat and Flahault, 2007). Consequently, infecting viruses can no longer be inhibited effectively by host antibodies raised against the previously circulating strains, allowing the virus to spread more rapidly among the population by escape selection from the adaptive immune response (Webby and Webster, 2001). To circumvent this problem, many studies have been conducted against more conserved influenza virus proteins. These vaccines have been shown to protect against multiple influenza A virus subtypes in animal models and are promising complements or alternatives for the currently used HA-based, strain-specific vaccines (Hillaire et al., 2011; Stanekova and Vareckova, 2011). DNA plasmids (pDNA) expressing conserved influenza proteins especially nucleoprotein (NP) elicit T-cell responses and provide cross-protection against lethal challenge in mice. However, the initial clinical results using naked pDNA immunization have not been promising in large animals and humans (Ulmer et al., 1993, 1998). A major drawback in DNA vaccines development is the need for relatively high doses of pDNA to elicit minimal responses (Liu, 2011). Hence, there has been an ongoing challenge to find new strategies to improve potency of pDNA immunogens.

Interestingly, influenza virosomes appear to be a flexible platform that is particularly suited to resolve these drawbacks of DNA vaccines (Daemen et al., 2005). Adjuvant property of influenza virosomes is related to strong capacity of virosomes to induce maturation of DCs and trigger the secretion of various cytokines such as TNF-α, IFN-γ and IL-12 (Gluck et al., 2004; Moser et al., 2003). In this study, we introduce an alternative vaccine formulation composed of influenza virosome/NP pDNA complex to broaden immunogenicity and protectivity of currently used seasonal flu vaccines.

2. Material and methods

2.1. Construction of NP-expressing plasmid

PcDNA3 plasmid encoding influenza virus NP gene was constructed and characterized as described before (Jamali et al., 2010). Briefly, NP gene was amplified by RT–PCR from influenza A/New Caledonia/20/99 H1N1 virus (hereafter called NC virus) and cloned into pcDNA3 plasmid. Expression of NP was analyzed by immunoflourescence staining. pcDNA3–NP was amplified in *Escherichia coli* DH5-α and purified using Endo-Free Mega kit (Qiagen, USA).

2.2. Formulation of influenza virosome/NP pDNA complex

Influenza virosomes with pDNA attached to the surface were prepared from NC virus as described by Schoen et al. (1999) and modified by de Jonge et al. (2007). Briefly, the influenza viral membrane was solubilized with detergent 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DCPC) followed by reconstitution after removal of DCPC by dialysis. Cationic lipid 1,2-dioleoyl-3-trimethyl-ammoniumpropane (DOTAP, Avanti Polar Lipids, Inc., USA) was added 34% relative to total lipid and 43% relative to viral lipid prior to detergent removal. For the binding of NP pDNA to the surface of the virosome, 5 μg of pDNA was incubated with 20 nmol of virosomal phospholipids at room temperature for 30 min. This molar ratio of NP pDNA:virosome corresponds to the saturation capacity of the virosome as described previously (Schoen et al., 1999).

2.3. NP pDNA transfection by influenza virosomes

Carrier potential of influenza virosomes was evaluated *in vitro* by transient transfection of NP pDNA into BHK-21 cells and subsequent immunofluorescence staining using specific antibodies. BHK-21 cells were transfected with 1 μg of the pcDNA3–NP or pcDNA3 empty vector loaded on 4 nmol of influenza virosomes. As positive control, 1 μg of NP pDNA was transfected by Lipofectamine 2000® (Invitrogen) according to manufacturer's instruction. Two days post-transfection, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS). Next, the cells were treated with Triton X-100 and then anti-NP monoclonal antibody (Ab) (Serotec, USA), followed by incubation with anti-mouse IgG–rhodamine conjugate (Invitrogen, USA).

2.4. Immunization schedule

Six to eight-week old female BALB/c mice 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 on a 12-h light/12-h dark cycle. All experiments were performed according to the Animal Care and Use Protocol of the Pasteur institute of Iran. Mice were divided into five groups and immunized once intradermally. Group I and II were inoculated with 5 and 50 μg of pDNA–NP, hereafter referred to as pNP-5 and pNP-50 respectively. Group III received pDNA/virosome complex in which 5 μg of pDNA–NP was attached to the surface of influenza virosomes (containing 15 μg HA) and group IV injected with virosomes without NP–pDNA, hereafter referred to as V-NP and V-only respectively. As negative control, mice were immunized with PBS.

2.5. Virus challenge

For challenge experiments, three weeks after immunization, the mice were slightly anesthetized with a mixture of Ketamine/Xylazine (1.98 and 0.198 mg per mouse, respectively) and challenged intranasally with 4 LD_{50} of influenza viruses in 50 μl PBS. The mouse-adapted strains used in this study included PR8 [A/Puerto Rico/8/34 (H1N1)] and NC. PR8 virus was a kind gift from Prof. Anke Hueckride, University of Groningen, Netherlands. NC virus was adapted to mice by sequential lung-to-lung passages of the virus in six to eight week-old BALB/c mice. Briefly, female 6-week-old BALB/c mice were inoculated intranasally under light anesthesia with 50 μl of NC virus. Lungs were harvested after 3–4 days; homogenized and 50 μl of the clarified homogenate was used as the inoculum for the next passage. After a total of 10 passages, filtered mouse lung homogenates served as the viral challenge stocks. Adaptation process was verified by rapid and widespread viral replication in the lungs and death of the unimmunized mice in 6–8 days. Mortality rate and weight changes were recorded regularly for 2 weeks after challenge.

2.6. Lung virus titration

The virus titers were measured in mice lungs 5 days after the challenge. Lungs were harvested from five mice out of each group and prepared as clarified homogenates. MDCK cells were inoculated with serial $\log_{10}$ dilution of the lung homogenates and the