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Incorporation of conserved nucleoprotein into influenza virus-like particles could provoke a broad protective immune response in BALB/c mice and chickens

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ABSTRACT

We engineered influenza A/goose/GD/1996 (H5N1) (clade 0) virus-like particles (VLPs) by coinfecting Sf9 cells with triple/quadruple recombinant baculovirus that expressed hemagglutinin (HA), neuraminidase (NA), and matrix 1 (M1) with or without nucleoprotein (NP). VLP3 (HA, NA, and M1) and VLP4 (HA, NA, M1, and NP) vaccines (containing 1 μg HA) with oil emulsion were administered to mice and chickens by intramuscular injection, and the immune responses were analyzed. The VLP-vaccinated mice demonstrated high antigen specific antibody titers and effective cellular immune responses. The mice and chickens vaccinated with VLP4 demonstrated more robust humoral and cellular immune responses than those vaccinated with VLP3. The VLP4 vaccine afforded 100% protection against a heterologous lethal influenza virus challenge (clade 2.3.4) whereas the VLP3 vaccine conferred 50% protection in chickens. These results implied that the incorporation of conserved NP protein into the VLPs could elicit a broad protective immune response in BALB/c mice and chickens. To the best of our knowledge, this study is the first report describing the immunological profile of the NP-containing VLPs vaccines in mice and chicken models, and the results demonstrate that the non-infectious, genome less VLPs, particularly those containing NP, represent a promising strategy for the development of a safe and effective vaccine to control pandemic influenza.

1. Introduction

Avian influenza (AI), causing morbidity and mortality in humans and domestic animals, remains an increasingly global threat to economic and social well-being (Horimoto and Kawaoka, 2001). In many developing countries, H5N1 viruses have caused a serious impact on the poultry industry. Since 1997, H5 subtype lethal AI viruses have been reported to transmit directly from avian to human and cause fatal human diseases (Webby and Webster, 2003), which increased the need to control AI in addition to economic considerations. Vaccination has been a cost-effective means to control influenza. The approved influenza vaccines are primarily detergent-solubilized virions inactivated chemically that cannot induce strong cytotoxic lymphocyte responses (Cox et al., 2004; Zhirnov et al., 2007). The progressive amino acid substitutions of hemagglutinin (HA) and neuraminidase (NA), known as antigenic drift and shift, may result in escape of previously acquired immunity. Thus, influenza vaccines must be updated annually to effectively prevent the epidemic viral strains. The attenuated influenza virus vaccine for seasonal influenza could cause the appearance of more aggressive revertants when a limited number of amino acids changes in vaccine strains. The emergence of pandemic influenza A strains remains a constant threat because of the reassortment of segmented viral genomes (Abdel-Ghafar et al., 2008; Fu et al., 2009). The production of both types of the licensed influenza vaccines described above primarily depend on fertilized chicken egg systems, which is time-consuming process in an influenza pandemic outbreak that could threaten global egg supply for the manufacture of vaccine. Because current influenza vaccines have some limitations (Demicheli et al., 2004), there is an urgent requirement to develop novel vaccines

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targeting conserved viral antigens to prevent the H5N1 influenza effectively.

As an alternative to conventional egg-based influenza vaccine, scientists developed influenza virus-like particles (VLPs) platform. VLPs, lacking the viral genome, were non-infectious and assembled themselves. They were shown to have a strong and broad immune response because the overall structure of VLPs accurately mimics that of virions and presents conformational epitopes of surface proteins that are easily recognized and processed by antigen presenting cells (Bessa et al., 2008; Haynes, 2009; Kang et al., 2009a; Noad and Roy, 2003; Rudolf et al., 2001). The production of recombinant influenza VLPs is not needed to use live infectious viruses, so it does not require exceptional biosafety containment or pose a threat to vaccine production workers. Recombinant VLPs can be quickly manufactured for an emergency influenza pandemic, and particularly VLPs have the advantage of the ability to easily differentiate vaccinated birds from infected ones. Influenza VLPs can be achieved in high yield in insect cells using the Bac-to-Bac baculovirus expression system; for example, 5–10 mg of purified VLPs can be obtained from 1 L of insect-cell culture that is similar in range to the amount of purified influenza virus produced in 1 L of egg allantoic fluid (Wen et al., 2009). The insect cells are cultured in suspension, so it is relatively easy to expand them to large bioreactors, and the manufacturing costs is competitive. Because of these advantages over the licensed influenza vaccines, VLPs have received significant attention in recent years.

In previous studies, VLPs (containing the HA, NA, and M1/M2 proteins) have been shown to be efficient inductors of an immune response in a mice/ferret model (Bright et al., 2007, 2008; Kang et al., 2009b; Mahmood et al., 2008; Pushko et al., 2005; Quan et al., 2007, 2008). The nucleoprotein (NP) of the influenza virus is highly conserved, and the $CD8^+$ T cells against the conserved epitopes of the NP could contribute to protection against morbidity and mortality from influenza (Braciale and Yap, 1978; Epstein et al., 2005; Gschoesser et al., 2002; Kang et al., 2009b; Zhirnov et al., 2007). The NP is associated with RNA segments and then packaged into virions. There are no studies on whether the NP can be packaged in VLPs without RNA segments.

If the NP is incorporated into virions, then the VLPs should have an enhanced immune response. To investigate the effects of the NP on the immunity protection of the VLPs, we engineered two influenza H5N1 VLPs; one contains HA, NA, and M1 (VLP3), and the other contains HA, NA, M1, and NP (VLP4). Our work demonstrated that VLP3 and VLP4 elicited vigorous humoral and cellular immune responses. Furthermore, these findings indicated that VLP4, but not VLP3, provided full protection against a heterogeneous H5N1 challenge in chickens.

2. Materials and methods

2.1. Cell line and virus

Spodoptera frugiperda Sf9 insect cells were cultured in suspension in serum-free SF900II medium (GIBCO, Grand Island, NY, USA) at 28 °C in spinner flasks at a speed of 100 rpm. The wild-type strain of influenza A/Duck/Fujian/31/2007 (H5N1) virus and inactivated influenza virus Re-1 strain (Qiao et al., 2006) were provided by Harbin Veterinary Research Institute (Harbin, China) for virus challenge in chicken model.

2.2. Generation of recombinant baculoviruses

The Bac-to-Bac baculovirus expression system was used for the generation of recombinant baculovirus vectors expressing influenza virus genes. The nucleotide sequence of segments 4, 6, 7, and 5 encoding the HA, NA, M1, and NP proteins, respectively, of A/goose/GD/1996 (H5N1), whose accession numbers were NC_007362, NC_007361, NC_007363 and NC_007360, respectively, were de novo-synthesized by Invitrogen (Guangzhou, China). The fragments containing the influenza HA, NA, M1, and NP genes were cloned into the pFastBac Dual vector (Invitrogen, Carlsbad, CA, USA), followed by PCR using specific primers annealing to the 3′ and 5′ terminus of each gene. The nucleotide sequences of the HA, NA, M1, and NP genes were confirmed by the DNA sequencing. The recombinant bacmids were generated by site-specific homologous recombination and transformation of the influenza genes-containing plasmid into *Escherichia coli* DH10-Bac competent cells that contained the AcMNPV baculovirus genome (Invitrogen). Then, 1 μg of purified recombinant bacmid DNA was transfected into Sf9 cells seeded in 6-well plates at 5×10^5 cells/ml using CellFectin reagent (Invitrogen). After incubated for 3 days, the viruses were harvested from the supernatant and subjected to three rounds of plaque purification.

2.3. Formation and purification of influenza VLPs

The influenza VLPs were obtained by co-infection of the Sf9 cells with the following baculovirus recombinants: rBacHA-NA, rBacM1-NP, or rBacM1. The Sf9 cells were seeded at a density of 2×10^6 per flask and allowed to settle at room temperature for 30 min. Subsequently, the Sf9 cells were co-infected with the rBVs expressing HA-NA, M1-NP, or M1 at multiplicities of infection (MOI) of 3–5 and incubated for 72 h at 28 °C. The culture supernatant (200 ml) from the Sf9 cells containing VLP3 and VLP4 were harvested and clarified by centrifugation for 30 min at $2000 \times g$ at 4 °C. The VLPs in the supernatant were pelleted by ultracentrifugation for 60 min at $100{,}000 \times g$ at 4 °C. The sedimented particles resuspended in 1 ml of the phosphate buffered saline (PBS) solution (pH 7.2) were loaded onto a 20–30–60% (w/v) discontinuous sucrose step density gradient and sedimented by ultracentrifugation for 60 min at $100{,}000 \times g$ at 4 °C. The VLP bands were collected and analyzed by SDS-PAGE and western blot. The functionality of HA incorporated into the VLPs was assessed by the hemagglutination activity performed as described, using 1% red blood cells (RBCs) from chickens (Matassov et al., 2007).

2.4. SDS-PAGE and Western blot analysis of the purified VLPs

The protein content and identity of the VLPs was evaluated by SDS–PAGE using 5–10% gradient polyacrylamide gels (Invitrogen) and western blot as described by Pushko et al. (2005). The expressed influenza proteins, HA, NA, M1, and NP, were detected with chicken polyclonal sera (Harbin Veterinary Institute) raised against the H5N1 influenza A virus and the HRP-conjugated donkey anti-chicken secondary antibody (PTGLAB, USA). The amount of the HA protein present in the VLPs was estimated by densitometry of the coomassie blue-stained SDS-PAGE gels (Prel et al., 2008).

2.5. Indirect immunofluorescence examination

The Sf9 cells were seeded in 24-microwell plates (Greiner Bio-One, Germany) at a density of 1×10^5 cells/well and infected with the triple/quadruple recombinant baculovirus at MOI of 3–5. After 72 h of infection, the Sf9 cells were washed three times with PBS containing 0.05% Tween-20 and fixed in 100% pre-cooled methanol at 4 °C for 10 min, then incubated with 0.5% Triton X-100 (USB, USA) at room temperature for 10 min. The RBITC/FITC-conjugated mouse monoclonal antibody of the influenza A virus M1/NP (ABCAM, England) (1:200 dilution) was used to detect the expression of the corresponding proteins. The plate was incubated at 37 °C for 1 h. At the end of the incubation, the wells were washed extensively