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H5N1 influenza virus-like particle vaccine protects mice from heterologous virus challenge better than whole inactivated virus

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ABSTRACT

The highly pathogenic avian influenza (HPAI) H5N1 virus has become highly enzootic since 2003 and has dynamically evolved to undergo substantial evolution. Clades 2.3.2.1 and 2.3.4 have become the most dominant lineage in recent years, and H5N8 avian influenza outbreaks have been reported Asia. The current approach to generate influenza virus vaccines uses embryonated chicken eggs for large-scale production, although such vaccines have been poorly immunogenic to heterologous virus challenge. In the current study, virus-like particles (VLP) based on A/meerkat/Shanghai/SH-1/2012 (clade 2.3.2.1) and comprising hemagglutinin (HA), neuraminidase (NA), and matrix (M1) were produced using a baculovirus expression system to develop effective protection for different H5 HPAI clade challenges. Mice immunized with VLP demonstrated stronger humoral and cellular immune responses than mice immunized with whole influenza virus (WIV), with 20-fold higher IgG antibody titers against A/meerkat/Shanghai/SH-1/2012 after boost. Notably, the WIV vaccine group showed partial protection (80% survival) to homologous challenge, little protection (40% survival) to heterologous challenge, and 20% survival to H5N8 challenge, whereas all mice in the VLP + CFA group survived. These results provide insight for the development of effective prophylactic vaccines based on VLPs with cross-clade protection for the control of current H5 HPAI outbreaks in humans.

1. Introduction

Influenza A virus causes acute viral respiratory disease and recurrent outbreaks, which significantly affect human health and the global economy (Quan et al., 2010). The first human outbreak of highly pathogenic avian influenza (HPAI) H5N1 was identified in 1997, and more than 649 confirmed human infections and 385 fatalities have occurred as of January 9, 2014 (http://www.who.int/csr/don/2014_01_09_h5n1/en/). HPAI H5N1 has become highly enzootic since 2003 and has dynamically evolved to undergo substantial evolution. HPAI H5N1 was found to co-circulate, particularly in China, Vietnam, Indonesia, Egypt, Cambodia, and Bangladesh, and clades 2.3.2.1 and 2.3.4 have become the predominant lineage (Le and Nguyen, 2014; Watanabe et al., 2011) (http://www.who.int/entity/influenza/vaccines/virus/201402_h5h7h9h10_vaccinevirusupdate.pdf). However, it is difficult to predict the specific isolate from different H5N1 clades with the potential to create a pandemic (Prabakaran et al., 2013). H5N8 avian influenza outbreaks have been reported in the Republic of Korea (Lee et al., 2014) and have been isolated from wild duck in China (Fan et al., 2014). Therefore, the development of a vaccine that induces cross-protection against different antigenic H5 subtypes is crucial.

The current approach to generate influenza virus vaccines uses embryonated chicken eggs for large-scale production (Robertson and Engelhardt, 2010). However, manufacturing problems,

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including limited production capacity due to insufficient egg supply and pandemic allergic reactions to egg proteins, in recent years have shown that the current methods of production are too fragile and time-consuming to ensure an adequate supply of influenza virus vaccine (Erlewyn-Lajeunesse et al., 2009). In addition, shortfalls in the response of the vaccine supply for the influenza season have been noted, and it takes months to identify new potential strains (Quan et al., 2007; Rezaei et al., 2013). More importantly, the HPAI H5N1 viruses that were responsible for recent epizootic outbreaks in Asia are lethal to chicken eggs (Choi et al., 2013a; Qiao et al., 2003). However, recombinant non-infectious virus-like particles (VLPs) produced in a baculovirus system can avoid the handling of live influenza viruses during the vaccine manufacturing process; thus, this approach represents a promising and novel technology for the creation of low-cost, safe and high-yielding commercial vaccines for influenza virus. VLPs are structurally native and immunologically relevant viral antigens because the hemagglutinin (HA) antigen is presented to the host in a native particulate form without chemical inactivation compared to the WIV vaccine (Bright et al., 2008; Bright et al., 2007; Jin et al., 2008).

Several different constructs of VLPs that contain influenza HA or HA- neuraminidase (NA) and the influenza matrix protein M1 have been shown to induce high titers of virus-specific antibodies in vaccinated mice. These VLPs provide protection against heterologous virus challenge (Choi et al., 2013a; Prabakaran et al., 2013). However, the protective efficacy of VLPs has not been evaluated for VLPs against different H5N1 virus clades and H5N8 virus or compared with that of whole inactivated virus (WIV).

This study generated H5N1 influenza VLPs with NA-HA-M1 using a baculovirus expression system, and then we investigated the immunogenicity and protective efficacy of H5N1 WIV, VLPs and VLPs plus complete Freund's adjuvant (CFA) against different H5N1 clades in mice.

2. Materials and methods

2.1. Viruses and cells

HPAI H5N1 virus A/meerkat/Shanghai/SH-1/2012 (SH-1; clade 2.3.2.1), A/duck/Jilin/JL-SIV/2013 (JL-SIV; clade 2.3.4), and H5N8 virus A/mallard duck/Shanghai/SH-9/2013 (SH-9) were originally isolated and stored in the Changchun Veterinary Research Institute. All the viruses were grown in 9-day-old embryonated hen's eggs for 24–48 h at 37 °C. Allantoic fluids were harvested from infected eggs, stored overnight at 4 °C and centrifuged to remove cell debris. The virus was purified through a discontinuous sucrose gradient (20–30–60% layers) and ultracentrifuged at 28,000 rpm for 1 h. Purified virus was mixed with formalin at a final concentration of 1:4000 (vol/vol) as described previously to inactivate the virus. Hemagglutination assays were performed to determine the virus titer. Spodoptera frugiperda Sf9 insect cells (Invitrogen, USA) were maintained in TMN insect medium (Appilchem, Germany) at 27 °C. Madin-Darby canine kidney (MDCK) cells were maintained and grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. All experiments using highly pathogenic virus were conducted in a biosafety level 3 (BSL3) facility in compliance with WHO recommendations and approved by the Changchun Veterinary Research Institute.

2.2. VLP production and protein expression

Genes encoding NA, HA, M1 (4574 bp) with the polyhedrin promoter, polyadenylation signal of A/meerkat/Shanghai/SH-1/2012 (SH-1; clade 2.3.2.1), and Not I and Sph I restriction sites were synthesized by Shanghai Generay Biotech Co., Ltd. (Fig. 1). Genes were digested with Not I and Sph I and cloned into a pFastBac 1 transfer vector (Invitrogen, USA). The final plasmid pFastBac-NA-HA-M1 was transformed into *E. coli* DH10Bac competent cells that contained the AcMNPV baculovirus genome (Invitrogen, USA), and recombinant bacmids were produced using site-specific homologous recombination, as previously described (Pushko et al., 2005). Recombinant bacmid DNA was transfected into 1×10^6 Sf9 cells seeded in 6-well plates using Cellfectin reagent (Invitrogen, USA). The pFastBac 1 transfer vector was transformed into *E.coli* DH10 cells and transfected into Sf9 cells to obtain the baculovirus (BV). The resulting recombinant baculovirus (rBV) or BV was collected from the culture medium 72 h post-infection according to the manufacturer's instructions and stored at 4 °C.

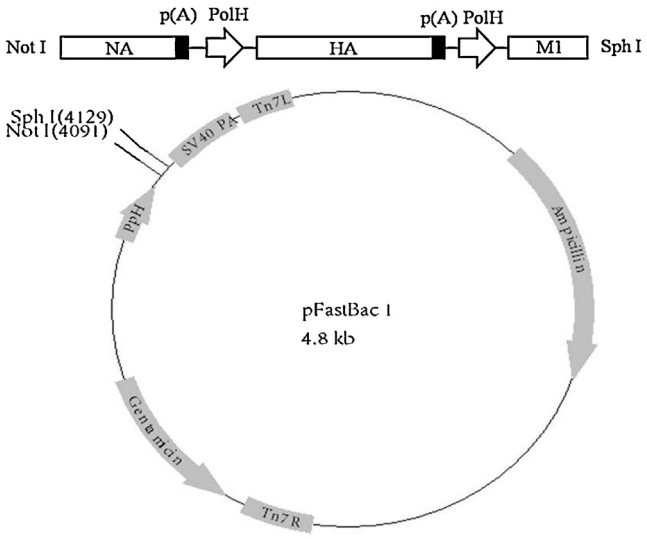

Fig. 1. Construction of pFastbac 1 plasmid. The NA-HA-M1 gene was synthesized with the Not I and Sph I restriction sites and ligated with pFastBac 1 transfer vector as indicated. The NA-HA-M1 gene was paired and cloned in bacmid recombination.

Sf9 cells were infected with the rBV or BV at a multiplicity of infection (MOI) of 3 to produce VLPs. Titers of recombinant baculovirus stocks were determined by the BacPAK Baculovirus Rapid Titer Kit (Clontech Laboratories, Inc., USA) according to the manufacturer's instructions. Culture supernatants that contained VLPs or BV were harvested 3 days post-infection and clarified by low-speed centrifugation at 2000 rpm for 20 min at 4 °C followed by ultracentrifugation at 30,000 rpm for 60 min to pellet. Sedimented particles were suspended in phosphate-buffered saline (PBS) at 4 °C overnight and further purified through a 20%–30%–60% discontinuous sucrose gradient at 30,000 rpm for 1 h at 4 °C.

Indirect fluorescence assays (IFAs) were performed to assess the expression of HA, NA and M1 proteins, as previously described (Ma et al., 2014). Sf9 cells were infected with rBV, and the cells were cultured at 27 °C for 48 h. Supernatants were discarded, and the cells were fixed with 80% pre-cooled acetone at −20 °C for 2 h. Cells were washed in PBS (0.01 mol/L) and incubated with antiserum (1:200) from chickens immunized with H5N1 WIV at 37 °C for 2 h. The cells were then washed with PBS and incubated with FITC-conjugated rabbit anti-chicken secondary antibody (Bioss, China) diluted in 0.1% Evans blue at 37 °C for 1 h. Finally, the cells were washed with PBS and observed using fluorescence microscopy (Olympus IX51).

2.3. Western blot analysis

Characterization of influenza VLPs, BV and WIV was performed using a sodium dodecyl sulfate (SDS) 12% polyacrylamide gel,