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Intramuscular and intranasal immunization with an H7N9 influenza virus-like particle vaccine protects mice against lethal influenza virus challenge

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ABSTRACT

The H7N9 influenza virus epidemic has been associated with a high mortality rate in China. Therefore, to prevent the H7N9 virus from causing further damage, developing a safe and effective vaccine is necessary. In this study, a vaccine candidate consisting of virus-like particles (VLPs) based on H7N9 A/Shanghai/2/2013 and containing hemagglutinin (HA), neuraminidase (NA), and matrix protein (M1) was successfully produced using a baculovirus (BV) expression system. Immunization experiments showed that strong humoral and cellular immune responses could be induced by the developed VLPs when administered via either the intramuscular (IM) or intranasal (IN) immunization routes. Notably, VLPs administered via both immunization routes provided 100% protection against lethal infection caused by the H7N9 virus. The IN immunization with 40 μg of H7N9 VLPs induced strong lung IgA and lung tissue resident memory (TRM) cell-mediated local immune responses. These results provide evidence for the development of an effective preventive vaccine against the H7N9 virus based on VLPs administered through both the IM and IN immunization routes.

1. Introduction

The recurrent outbreaks of the influenza A virus cause acute viral respiratory disease, significantly affecting the global economy and human health [1]. In March 2013, the first recognized human infections with avian-origin influenza A (H7N9) were reported in China, resulting in 779 documented cases and 300 deaths as of 20 April 2016 (http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation_update.html). The FDA has already started work on the preemptive and immediate development of clinical interventions and diagnostic and surveillance tools in the event that these viruses become a human pandemic [2,3].

Influenza vaccines are considered effective measures in reducing the severity of signs of the clinical disease resulting from infections with the homologous field strain [4]. The major approach for the large-scale production of influenza virus vaccines employs embryonated chicken eggs [5]. However, the emergence of manufacturing problems in recent years has illustrated that the ability of the current egg-based production methods to provide a timely supply of an adequate influenza virus vaccine is fragile [6]. In addition, identifying new, potentially infectious strains takes a long time [7]. Therefore, developing an effective vaccine production method that does not rely on propagation in embryonated chicken eggs is of considerable importance. In the past few years, many different approaches that do not require the conventional methods have been applied. Virus-like particles (VLPs) produced in a baculovirus (BV) system represent a method of vaccine production that can avoid the treatment of live influenza viruses. Commercially, this method for the production of vaccines against influenza viruses is safe, low cost, and high yield. The VLPs are structurally and immunologically relevant viral antigens but are innocuous. As the HA antigen is present in the structure of the natural particles, there is no chemical inactivation by the host [8,9].

Several different VLP constructs containing H7N9 influenza HA or a combination of HA and neuraminidase (HA-NA) and matrix protein M1.

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In addition, these VLPs have been used to vaccinate mice or chickens intramuscularly (IM), which induced high titers of virus-specific antibodies [10–12]. Influenza M1 is of prime importance in virion assembly and release. The influenza HA cytoplasmic tails are also important for the formation of budding particles. However, the protective efficacy of intranasally (IN) administered H7N9 VLPs has not been evaluated or compared with IM administration. For the IN administration of a vaccine, lung tissue resident memory (TRM) cells play a very important role in protective immune responses [13].

This study describes the use of three A/Shanghai/2/2013 (H7N9) influenza virus structural proteins, HA, NA, and M1, to develop an influenza VLP candidate vaccine. The immunogenicity and protective efficacy of IM and IN administered H7N9 VLPs in BALB/c mice were screened.

2. Materials and methods

2.1. Viruses and cells

The H7N9 A/Shanghai/2/2013 virus was kindly supplied by the Chinese Centers for Disease Control and Prevention. After being grown for 24–48 h at 37 °C in 9-day-old embryonated hen's eggs, the virus allantoic fluid was collected and inactivated by mixing it with formalin. Then, the fluid was ultracentrifuged at 28,000 g for 1 h, and the virus was further purified using a 20–30-60% discontinuous sucrose gradient. The virus titer was determined using a hemagglutination assay. *Spodoptera frugiperda* (Sf9) insect cells were purchased from Invitrogen and were cultured in TMN insect medium (Applichem, Germany) and incubated at 27 °C at a constant temperature. In compliance with WHO recommendations, the highly pathogenic virus experiments were approved by the Changchun Veterinary Research Institute at a Biosafety Level 3 (BSL3) facility.

2.2. Cloning of the H7N9 HA, NA, and M1 genes and generation of recombinant baculoviruses

To generate the VLPs, a Viral RNA Kit (BioFlux, Japan) was used to extract viral RNA under BSL3 containment, and then gene-specific oligonucleotide primers were used in reverse transcription polymerase chain reaction (RT-PCR). The HA, NA, and M1 primer pairs containing restriction sites were as follows: 5′-AAATATGCGGCCGCATGAACACTC AAATCCTGGTATTCG-3′ and 5′-ACAT GCATGCTTATATACAAATAGTG CACCGCATG-3′; 5′-AAATATGCGGCCGCAT GAATCCAAATCAGAAGAT TCTAT-3′ and 5′-ACATGCATGCTTAGAGGAA GTA CTCTATTTTA GCC-3′; and 5′-AAATATGCGGCCGCATGAGTCTTCTAACCGAG GTCG AAA-3′ and 5′-ACATGCATGCTCACTTGAACCGCTGCAGTTGCACT-3′. The H7N9 HA, NA and M1 cDNA fragments were cloned into the pFastBac1 vector (Invitrogen), which had been digested with *Not*I and *Sph*I. Then, the plasmids containing the H7N9 influenza genes were transferred into *E. coli* DH10Bac competent cells, which incorporated the BV genome.

2.3. Bacmid transfection, VLP production and protein expression

Sf9 cells were cultured in TMN insect medium. After seeding 1×10^6 Sf9 cells in 6-well plates, we used Cellfectin reagent (Invitrogen, USA) to transfect the H7N9 HA, NA, and M1 recombinant bacmid DNA.

At 72 h postinfection, the culture medium, which contains the H7N9 HA, NA, and M1 recombinant baculovirus (rBV), was harvested as recommended by the manufacturer and was stored at 4 °C. To generate the VLPs containing the H7N9 HA, NA and M1 proteins, Sf9 insect cells were co-infected with rBVs expressing HA, NA and M1, respectively, at a multiplicity of infection (MOI) of 5:1:2. At 3 days postinfection, culture supernatants were collected, centrifuged at 2000*g* for 20 min, and then ultracentrifuged at 30,000 g for 60 min. Next, the sedimented particles were suspended in PBS at 4 °C overnight, and the VLPs were purified via centrifugation using a 20–30-60% (*w/v*) discontinuous sucrose gradient.

To examine the HA, NA and M1 protein expression levels, we used an indirect fluorescence assay (IFA) as previously described [14]. H7N9 rBV-infected Sf9 cells were cultured for 48 h. Then, the supernatants were disposed of, and 80% precooled acetone was used to fix the cells at − 20 °C for 2 h. The cells were washed with PBS (0.01 mol/L) and incubated in polyserum (1:200) from a rabbit immunized with H7N9 whole inactivated virus (WIV) for 2 h at 37 °C. Next, the cells were washed with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Bioss, China) in 0.1% Evans blue for 1 h at 37 °C. The cells were washed again with PBS and imaged using fluorescence microscopy (Olympus IX51).

2.4. Western blot analysis

A sodium dodecyl sulfate (SDS) 12% polyacrylamide gel and Western blot analysis were used to characterize the H7N9 VLPs, as described previously [15]. The purified VLPs (10, 2 and 0.4 μg), 10 μg of purified BV and 10 μg of purified H7N9 WIV were loaded onto the gel, and then a Mini Trans-Blot apparatus (Bio-Rad, CA) was used to transfer the samples onto a polyvinylidene difluoride (PVDF) membrane. Then, the membrane was blocked overnight at 4 °C using Blocking Buffer, incubated with rabbit polyserum (1:500 v/v) at room temperature, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:50,000 v/v) (Sigma). Finally, the proteins were visualized using an ECL system (Thermo, USA).

2.5. Electron microscopy

To evaluate VLP budding, H7N9 HA, NA and M1 rBV-infected Sf9 cells were fixed with 1% phosphotungstic acid for 1–2 min. The excess stain was wicked away using a piece of paper, and the cells were dried for 1–3 min. The H7N9 VLPs were imaged using a transmission electron microscope at 40,000 × magnification.

2.6. Immunization and challenge

The female BALB/c mice (6–8 weeks old) used in this study were provided by Changchun Animal Breeding Center for Medical Research (Changchun, China). For the IM immunization, the mice (10 mice per group) were first vaccinated at week 0, and then boosted with the same dose with 10 μg or 40 μg of purified VLPs at week 3. Ten mice were injected with PBS as a mock (control) group. For the IN immunization, the mice (10 mice per group) were anesthetized with isoflurane and administered the purified VLPs (40 or 10 μg of total protein) by inhalation at week 0 and then were boosted with the same dose at week 3. Additionally, ten mice were immunized with PBS as a mock (control) group. For the challenge studies, at week 5 after the first immunization, each immunized mice was anesthetized with isoflurane and then infected with 10 minimal 50% lethal infectious doses (MLD_{50}) of live H7N9 A/Shanghai/2/2013 in 50 μl of PBS. Body weight changes and mortality were monitored daily. The administered vaccine doses and a schematic of the vaccine regimen are shown in Fig. 1. The experimental procedures were approved by the Animal Care and Use Committee of the Chinese People's Liberation Army (No: SYXK2009–045) and were in accordance with the ethical guidelines of the International Association for the Study of Pain.

2.7. Hemagglutination inhibition assays and antibody responses

At weeks 0, 3 and 5 after immunization, as well as 4 days post-challenge, we collected mice blood samples by retro-orbital plexus puncture. After keeping the samples at room temperature for 30 min, we collected the sera via centrifugation at 3000 g for 10 min. On day 4

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