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Virus-like particles displaying H5, H7, H9 hemagglutinins and N1 neuraminidase elicit protective immunity to heterologous avian influenza viruses in chickens

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ABSTRACT

Avian influenza (AI) viruses circulating in wild birds pose a serious threat to public health. Human and veterinary vaccines against AI subtypes are needed. Here we prepared triple-subtype VLPs that co-localized H5, H7 and H9 antigens derived from H5N1, H7N3 and H9N2 viruses. VLPs also contained influenza N1 neuraminidase and retroviral gag protein. The H5/H7/H9/N1/gag VLPs were prepared using baculovirus expression. Biochemical, functional and antigenic characteristics were determined including hemagglutination and neuraminidase enzyme activities. VLPs were further evaluated in a chicken AI challenge model for safety, immunogenicity and protective efficacy against heterologous AI viruses including H5N2, H7N3 and H9N2 subtypes. All vaccinated birds survived challenges with H5N2 and H7N3 highly pathogenic AI (HPAI) viruses, while all controls died. Immune response was also detectable after challenge with low pathogenicity AI (LPAI) H9N2 virus suggesting that H5/H7/H9/N1/gag VLPs represent a promising approach for the development of broadly protective AI vaccine.

1. Introduction

Avian influenza (AI) virus (AIV) belongs to the family *Orthomyxoviridae*, genus Influenza A virus (type A), and contains a negative-sense, segmented RNA genome. Phylogenetically, there are 18 subtypes of HA that are subdivided into two major antigenic groups (Medina and Garcia-Sastre, 2011; Tong et al., 2013). AIV represents a serious concern for the U.S. and world public health. Humans lack immunity to AIV subtypes, and infections can potentially result in a pandemic. Numerous human infections including avian-origin H5N1, H7N9 and H9N2 subtypes have been documented indicating the potential of these subtypes to adapt to humans and cause life threatening infections (Kang et al., 2009; Xiao et al., 2016). In addition to the pandemic concerns, AI represents a serious threat to the poultry industry and food safety. Wild birds, which are the natural reservoir for the AIV, occasionally transmit the virus to domesticated birds, including chickens, ducks, and turkeys, which are susceptible to AIV. Although most infections in wild birds represent a low-pathogenicity AI (LPAI), infections with the H5 or H7 LPAI viruses can result in the emergence of high-pathogenicity AI (HPAI) viruses through genetic changes of the HA gene. Multiple outbreaks of H5 and H7 HPAI in commercial poultry have been reported in the Americas over the last decade, which renewed interest for AI vaccines (Swayne, 2012). The H9N2 LPAI viruses have also been identified as AIV of concern (Lee et al., 2016).

Together with other measures, vaccines can be an effective measure to prevent AI pandemics, epidemics and epizootics (Kang et al., 2009; Kapczynski and Swayne, 2009; Swayne, 2012). In previous studies, we showed that recombinant influenza virus-like particles (VLPs) protected from pandemic influenza strains including the reconstructed 1918 virus, the 2009 swine-origin pandemic virus, and AI viruses (Kang et al., 2009; Perrone et al., 2009; Pushko et al., 2010). Recombinant VLPs represent inherently safe vaccines that are prepared by using cell culture methods and do not require live AIV for production. Recombinant VLPs comprised of hemagglutinin (HA), neuraminidase (NA) and matrix (M1) proteins have been described (Bright et al., 2007; Galarza et al., 2005; Kang et al., 2009; Perrone et al., 2009; Pushko et al., 2005; Quan et al.; Ross et al., 2009). In some cases, retrovirus gag, such as bovine immunodeficiency virus gag (Bgag), has been used in place of M1 (Kapczynski et al., 2016;

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Pushko et al., 2016; Tretyakova et al., 2016). Bgag has the advantage of a larger diameter providing more surface area to accommodate multiple HA molecules (Tretyakova et al., 2016). The HA antigen is the major vaccine component, which induces neutralizing antibodies preventing infectious virus from entering cells (Cox et al., 2015; Kang et al., 2009; Pushko et al., 2015). Expression within VLPs increases immunogenicity of the HA antigen (Bright et al., 2007; Pushko et al., 2007). NA is also often included into VLPs because of its role in VLP assembly (Chen et al., 2007) and potential contribution to immunity (Eichelberger and Wan, 2015). Recently, novel design of VLPs was described that allowed co-localization into VLP of the HA proteins derived from several influenza types and subtypes (Kapczynski et al., 2016; Pushko et al., 2011; Tretyakova et al., 2013). This approach was designed to simultaneously elicit specific immunity to multiple influenza subtypes with no requirement for blending individual vaccines. Recombinant VLPs co-localizing three subtypes of HA protected ferrets, a human influenza model, from potentially pandemic viruses of H5, H7 and H9 subtypes (Pushko et al., 2011; Tretyakova et al., 2013).

Here, we prepared H5/H7/H9/N1/gag VLPs containing HA antigens derived from several AIV and evaluated their safety, immunogenicity and efficacy in chickens, a highly sensitive bird model of AI. Immune responses to H5, H7 and H9 antigens and protective efficacy of VLPs after heterologous virus challenges were demonstrated.

2. Materials and methods

2.1. Influenza genes and expression constructs

Influenza H5/H7/H9/N1/gag VLPs were prepared in *Spodoptera frugiperda* (Sf9) insect cells using a recombinant baculovirus (rBV) expression vector system. VLPs were expressed using rBV containing three HA genes (H5, H7 and H9 subtypes), as well as NA and Bgag genes. Influenza HA gene sequences were derived from A/chicken/West Java Sbg/29/2007 (H5N1 clade 2.1.3), A/turkey/Oregon/1971 (H7N3) and A/turkey/Wisconsin/1/1966 (H9N2) viruses. N1 gene was from H5N1 strain A/chicken/Egypt/121/2012 (clade 2.2.1) (Awad et al., 2015), while Bgag gene was from BIV R-29 strain retrovirus, GenBank accession number AAA42763.

Three indicated full-length HA genes, as well as NA and Bgag genes were introduced in tandem fashion into the rBV resulting in the vector containing five VLP-relevant genes. Each gene was placed within its own transcriptional cassette that included a polyhedrin promoter upstream from each gene, as described elsewhere (Pushko et al., 2005; Tretyakova et al., 2016). Genes were codon-optimized for high-level expression in Sf9 cells and synthesized (Genscript, Piscataway, NJ). All preparations of rBV were plaque-purified and titrated using standard plaque assay in Sf9 cells.

2.2. Expression and characterization of H5/H7/H9 triple-subtype VLP vaccine

To prepare VLP vaccine, Sf9 cells were maintained as suspension cultures in SF900II-SFM insect serum free medium (ThermoFisher Scientific (Thermo), Carlsbad, CA) at 27 °C. For production of VLP vaccine, Sf9 cells (2×10^6 cells/ml) were infected in shaker flasks at a multiplicity of infection (MOI) of 0.1 for approximately 72 h with rBV expressing indicated genes. VLPs were harvested from the growth medium supernatant, clarified using centrifugation and 0.2 μm filtration, concentrated by tangential flow filtration (500 kDa MWCO), and purified by ion exchange chromatography as described elsewhere (Liu et al., 2015). Purified VLPs were further concentrated and purified by ultracentrifugation at 100 000×*g* and resuspended in the phosphate buffered saline (PBS). VLPs were characterized including SDS-PAGE and western blot, total protein and HA content, nucleic acid content, functional NA enzyme and hemagglutination activities, as well as particle morphology and size by transmission electron microscopy.

SDS-PAGE was carried out in 4–12% polyacrylamide gels (Thermo) followed by staining with GelCode Blue stain (Pierce, Rockford, IL). Western blots were done using subtype-specific primary antibodies followed by the alkaline phosphatase-conjugated goat IgG (H & L). As primary antibodies, we used mouse anti-H5 (H5N1) and anti-H7 (H7N9) (Immune Tech, New York, NY), as well as chicken anti-H9 (SEPRL, Athens, GA). Total protein in the purified VLPs was determined using Qubit 2.0 fluorometer (Thermo). The HA protein content was determined by gel densitometry of the HA bands using known amounts of reference BSA as a standard.

The nucleic acid content was determined by extracting nucleic acids from the purified VLPs using Trizol LS reagent (Thermo). The extracted nucleic acids were quantitated by using Qubit 2.0 fluorometer using RNA and DNA detection kits. In addition, nucleic acids were treated with either RNAseI or RQ1 DNAse and visualized along with untreated control on the 1% agarose gel in the presence of ethidium bromide.

To determine functional neuraminidase enzyme activity, a fluorescence-based NA assay (NA-Fluor from Thermo) was used with methyl umbelliferone N-acetyl neuraminic acid as a substrate, according to manufacturer's instructions. Unrelated antigen was used as a negative control, while unrelated H1N1 VLP (A/South Carolina/1/1918) (Perrone et al., 2009) was used a positive control. A standard curve to determine a relative fluorescence unit (R.F.U.) value within the linear range of fluorescence detection was generated using 4-methyl umbelliferone sodium salt (Sigma, St. Louis, MO).

For hemagglutination assay, VLPs were serially diluted in PBS at 2-fold increments in 50 μl volume in a 96-well plate. To each VLP dilution, 50 μl of 1% turkey red blood cell (tRBC) working solution was added as described elsewhere (Tretyakova et al., 2016). Mixtures of VLPs and tRBCs were gently agitated and the plate was incubated at 20 °C for 30–60 min before examination. The titer was calculated as the highest dilution factor that produced a positive reading.

For transmission electron microscopy, purified VLP samples were adsorbed onto a freshly discharged 400 mesh carbon parlodion-coated copper grids, negatively stained with 1% phosphotungstic acid, and visualized on a Hitachi H-7600 transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL).

2.3. Vaccinations and challenge

All study protocols were approved by the USDA Institutional Animal Care and Use Committees and all experiments were performed in accordance with the applicable guidelines for the care and use of laboratory animals. H5/H7/H9/N1/gag VLP vaccine was formulated with a commercial adjuvant (SEPPIC, Montanide 70/30, Fairfield, NJ) to contain 1536 HA units per dose of VLPs (512 HA units of each subtype). Because H5, H7 and H9 genes were expressed from the identical expression cassettes, it is expected that HA subtypes are present at comparable levels in the VLPs, which was confirmed in the previous study (Tretyakova et al., 2016). Specific Pathogen-Free (SPF) chickens (n=30) were vaccinated subcutaneously with 0.2 ml of H5/H7/H9/N1/gag VLP at day 1 of age and 0.5 ml at day 21 of age. Sham-vaccinated birds (n=30) received PBS. Both the control and vaccinated birds were arbitrarily placed into groups of 10 and challenged intranasally (10^6 EID_{50} per bird) with one of the following AI isolates at day 35: A/turkey/Minnesota/7172-1/2015 (H5N2 clade 2.3.4.4 HPAI), A/chicken/Jalisco/CPA1/2012 (H7N3 HPAI) and A/chicken/New Jersey/12220/1997 (H9N2 LPAI). After challenges, birds were monitored for clinical signs and mortality daily. HPAI causes rapid progression of disease and death in chickens (Freidl et al., 2014; Kapczynski and Swayne, 2009). If birds were found to be moribund, they were euthanized using AVMA approved methods and counted in the following day after mortality. Because birds rapidly succumbed to HPAI viruses, weight was not measured in these studies, but rather