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Supplemented vaccination with tandem repeat M2e virus-like particles enhances protection against homologous and heterologous HPAI H5 viruses in chickens

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ABSTRACT

Highly pathogenic avian influenza (HPAI) H5 viruses derived from A/Goose/Guangdong/1/96 have been continuously circulating globally, severely affecting the public health and poultry industries. The matrix 2 protein ectodomain (M2e) is considered a promising candidate for a universal cross-protective influenza vaccine that provides more effective control over HPAI H5 viruses harboring variant hemagglutinin (HA)-antigens. Here, we evaluated the protective efficacy of a tandem repeat construct of heterologous M2e presented on virus-like particles (M2e5x VLPs) either alone or as a supplement against HPAI H5 viruses in a chicken model. Chickens immunized with M2e5x VLPs alone induced M2e-specific antibodies but were not protected against HPAI H5. The homo- and cross-protective efficacy of M2e5x VLP-supplemented vaccination of chickens was also examined. Importantly, supplementation with M2e5x VLPs induced significantly higher levels of antibodies specific for M2e and different viruses as well as provided improved protection against homologous and heterologous HPAI H5 viruses. Considering the limited efficacy of inactivated vaccines, supplement vaccination with M2e5x VLPs may be an effective measure for preventing outbreaks of HPAI viruses that have the ability to constantly change their antigenic properties in poultry.

1. Introduction

Highly pathogenic avian influenza (HPAI) is a disease that poses a significant threat to public health and can cause severe economic losses to the poultry industry. In 1997, an outbreak of H5N1 HPAI virus on a poultry farm in Hong Kong was caused by the A/Goose/Guangdong/1/96 (Gs/Gd/96) strain, which was isolated from geese in China in 1996 [1]. H5 HPAI viruses have become widely distributed, and remain one of the most important infectious diseases in both poultry and humans in Asia, Africa, Europe, Southeast Asia, and the Middle East [2]. Vaccination, in conjunction with other control methods such as careful surveillance and monitoring strategies, has been used to better control H5 HPAI viruses, particularly in HPAI endemic countries [3,4].

Most conventional avian influenza vaccines are based on the hemagglutinin (HA) protein. The HA protein is a major antigenic and immunogenic target that enhances humoral immunity and prevents clinical disease. However, HA-based vaccines provide limited cross-protection against novel influenza strains expressing immunodominant surface glycoproteins such as HA and neuraminidase (NA) that have undergone point mutation (antigenic drift) and genetic reassortment (genetic shift) [5]. Therefore, to effectively control an influenza pandemic, continuous selection and updating of vaccine strains is necessary every 2–3 years.

To develop the vaccine providing broadly cross-protection against influenza A viruses, various studies have been conducted to target matrix 2 ectodomain (M2e) consisting of 24-amino acids which are exposed at viral envelope [6–10]. However, although M2e sequence is more conserved when compared to HA, M2e variation between strains can be as high as 25% [11]. In addition, M2e is known as a poor immunogen [12,13]. Due to the presence of low amounts of M2e on the viral surface and a protein coat comprising large HA and NA proteins, recognition of M2e epitopes on virions by immune cells is inefficient [14,15]. Therefore, to overcome variation between strains of M2e and enhance the immunogenicity, previous studies of M2e-based vaccines have fused the M2e of different strains of influenza virus to particular immunogenic vehicles [6,7] or linked M2e to an appropriate carrier

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to increase its immunogenicity [8–10]. Recent studies generated a novel M2e construct by genetically engineering a tandem repeat comprising M2e epitope sequences (M2e5x) from multiple host origin influenza viruses, and then presenting it with matrix 1 protein (M1) on virus-like particles (M2e5x VLPs) resulting in a significant improvement in cross-protection in mouse models [6,16,17]. M1 protein is known as an important component which is essential for VLP formation and virus budding [18,19]. In addition, recent studies showed that M1 VLP had an adjuvant effect on split vaccine and induced the Th1 type immunity [16].

Here, we aimed to overcome the limitations of HA-based vaccines by evaluating the efficacy of the M2e5x VLP, which is co-expressed with M1, vaccine in a chicken model. This study determined the immunogenicity and protective efficacy of M2e5x VLPs either as a stand-alone vaccine or as a supplement to the inactivated HA-based vaccine. M2e5x VLP-supplemented HA vaccination of chickens induced significantly higher levels of antibodies recognizing different M2e peptide antigens and viruses, and provided good protection without body weight loss after lethal challenge with heterologous H5 HPAI viruses.

2. Material and methods

2.1. Virus strains and cell lines

The HPAI virus strains, A/mandarin duck/Korea/PSC24-24/2010 (H5N1; clade 2.3.2.1; PSC24-24) [20] and A/broiler duck/Korea/Buan2 (H5N8; clade 2.3.4.4; Buan2) [21], were isolated from a wild bird and a poultry farm, respectively, and maintained by the Animal and Plant Quarantine Agency (QIA). The viruses were propagated for 48 h in 10-day-old specific pathogen free (SPF) embryonated chicken eggs. *Spodoptera frugiperda* 9 (Sf9) insect cells, used to produce M2e5x VLPs, were maintained in SF900-II SFM medium (Invitrogen, Carlsbad, CA, USA) at 27 °C in an incubator. 293T cells were obtained from the American Type Culture Collection and cultured for 72 h in MEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) in 5% CO_2.

2.2. Preparation of M2e5x VLPs and recombinant vaccine PSC24-24

M2e5x VLPs were produced in Sf9 insect cells co-infected with recombinant baculovirus expressing influenza M1 matrix protein and M2e5x (comprising a heterologous tandem repeat of M2e peptides derived from human, swine, and avian origin influenza A viruses) [6]. M2e5x VLPs were purified and characterized as previously described [6].

H5N1 reassortant viruses were generated by plasmid-based reverse genetics using the HA and NA genes of PSC24-24 and six internal genes derived from the A/Puerto Rico/8/34 (H1N1) (PR8) virus strain as previously described, with minor modifications [22]. Viral PSC2-24 RNA was isolated from allantoic fluids using a GeneSpin™ Viral RNA extraction kit (iNtRON Biotechnology, Korea). The HA and NA genes were amplified using segment-specific primers [23] and subsequently cloned into the vector, pHW2000 [22]. To modify the polybasic amino acid cleavage site in the HA genes, site-directed mutagenesis was performed using a commercial kit as described by the manufacturer (Stratagene, USA). The two plasmids containing the HA and NA segments of PSC24-24 were amplified in transformed competent cells (Invitrogen) and individual colonies were randomly picked. The correct plasmid sequences were confirmed by sequence analysis. Virus rescue was performed by culture of 293T cells (1×10^6 cells/well in 6-well plates), followed by co-transfection with 300 ng each of the eight plasmids (two HA and NA genes from PSC24-24 and six internal genes from PR8) using lipofectamine (Invitrogen) in incomplete MEM (final volume, 1 mL). At 24 h post-transfection, the cell culture supernatant was harvested and injected into 10-day-old embryonated SPF chicken eggs. After incubation for 2–3 days at 37 °C, the egg allantoic fluid was harvested and viral growth tested in a hemagglutination activity assay. The genome composition of H5N1 reassortants was confirmed by sequencing.

To obtain the recombinant vaccine, PSC24-24 (rvPSC24-24), the harvest from the first passage was injected into additional SPF chicken eggs. An H5N1 reassortant virus obtained from several egg passages showed titer of $10^{6.2}$ EID_{50}/0.1 mL. Inactivated recombinant vaccine was prepared by treating with 0.1% formalin.

2.3. M2e5x VLP vaccination and challenge of chickens

To evaluate the efficacy of M2e5x VLPs, 25 6-week-old SPF chickens were divided into five groups (five chickens per group): four immunized groups and one non-immunized group. The four M2e5x VLP vaccine groups were intramuscularly vaccinated with escalating doses (2 μg, 10 μg, and 50 μg of VLPs per chicken) of the M2e5x VLP vaccine plus the adjuvant Montanide ISA 70 VG (SEPPIC, France) or with 50 μg of M2e5x VLPs without adjuvant at 4 week intervals. The control group was inoculated with PBS and the adjuvant ISA 70 mixture. Serum samples were collected on a weekly basis to determine immune responses to M2e5x VLP vaccination. antibodies in immune sera were analyzed weekly by ELISA based on synthetic human, swine, avian type I, and avian II type M2e peptides (Peptron, Korea). Immune responses to the virus strains PSC24-24, A/chicken/Gimje/2008 (H5N1) (Gimje08), A/chicken/Vietnam/NCVD-A015/2008 (H5N1) (VNA015), A/duck/Korea/BC10/07 (H7N3) (BC10), and A/Korean native chicken/Korea/12AQ005/2012 (H9N2) (12AQ005) were determined by ELISA using the aforementioned strains as a coating antigen. The wells of the ELISA plates (Nunc, Roskilde, Denmark) were coated with 0.3 μg/well of synthetic peptides or inactivated purified virions overnight at 4 °C and subsequently blocked with 3% skim milk in PBS for 4 h at 37 °C. The coated plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST) and incubated with a 1:200 dilution of immune sera (for synthetic peptides), or with serial dilutions of immune sera (1:200 to 1:26 000) (for purified virions) in 1% skim milk in PBST for 1 h at 37 °C. The plates were subsequently washed three times with PBST and incubated with a 1:2000 dilution of HRP-conjugated anti-chicken IgG (KPL, Gaithersburg, ML, USA) for 1 h at room temperature. After washing, wells were incubated with TMB substrate (KPL) for color development and the reaction was stopped with 1 N HCL. Optical densities were read at 450 nm using an ELISA reader (Tecan, Mannedorf, Switzerland). Three weeks following boost immunization, chickens were intranasally challenged with five times of 50% chicken lethal doses (five CLD_{50}) of PSC24-24 virus ($10^{5.2}$ EID_{50}/0.1 mL).

2.4. M2e5x VLP supplement vaccination and immunogenicity

To assess the efficacy of M2e5x VLPs as a supplement vaccine, a 10 μg dose of M2e5x VLPs was added to one dose ($10^{6.2}$ EID_{50}/0.1 mL) or 1/4 dose ($10^{5.8}$ EID_{50}/0.1 mL) of rvPSC24-24. Six-week-old SPF chickens were divided into five groups (five chickens per group): four immunized groups and one non-immunized group. Immunized groups were divided according to M2e5x VLPs supplementation and dose of rvPSC24-24 as follows: rvPSC24-24 (one dose) plus M2e5x VLPs, rvPSC24-24 (one dose), rvPSC24-24 (1/4 dose) plus M2e5x VLPs and rvPSC24-24 (1/4 dose). The non-immunized group was inoculated with PBS. All groups were vaccinated using the adjuvant ISA 70. To assess immunogenicity post-vaccination, chickens were bled weekly and a