Veterinary Microbiology 203 (2017) 143–148

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Coimmunization with recombinant epitope-expressing baculovirus enhances protective effects of inactivated H5N1 vaccine against heterologous virus

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form 1 March 2017
Accepted 2 March 2017

Keywords:
H5N1
Avian influenza virus
Epitope
Inactivated vaccine
Baculovirus

ABSTRACT

H5N1, a highly pathogenic avian influenza virus (HPAIV), poses a significant threat to poultry and human health. However, currently available inactivated influenza vaccines are less efficacious against viruses that display antigenic drift. In this study, we constructed a recombinant baculovirus (BV-HMNN) expressing four conserved antigen epitopes: H5N1 hemagglutinin stem area amino acids 76–130 (HA2 76–130); three tandem repeats from the ectodomain of the conserved influenza matrix protein M2 (3M2e); nucleoprotein amino acids 55–69 (NP55–69); and nucleoprotein amino acids 380–393 (NP380–393). We evaluated the immunogenicity and protective efficacy of coimmunization with an inactivated avian influenza virus vaccine (Re6) and the recombinant baculovirus (BV-HMNN) against heterologous viral infection in specific-pathogen-free chickens. The chickens immunized with both vaccines (Re6 + BV-HMNN) achieved complete protection, was significantly greater than that of chickens vaccinated with Re6 alone. BV-HMNN-supplemented vaccination also reduced viral shedding more effectively than nonsupplemented vaccination. We conclude that coimmunization with both vaccines was superior to immunization with the inactivated vaccine alone in inducing cross-protection against heterologous H5N1 virus.

1. Introduction

H5N1 highly pathogenic avian influenza virus (HPAIV), reported to cause occasional pandemics and economic losses (Kim et al., 2014). It also sometimes crosses the species barrier, causing human infections, and therefore presents a huge threat to human health worldwide (Pappaioanou, 2009; Watanabe et al., 2011). Vaccination is the most effective strategy for preventing and controlling avian influenza virus (AIV) infections. The currently available influenza vaccines, which only induce strong neutralizing antibodies specific to the highly variable surface glycoprotein hemagglutinin (HA), provide protection against homologous viruses but not against heterologous variants (Brett and Johansson, 2005; Kim et al., 2014; Song et al., 2011; Subbarao and Joseph, 2007). In the past few years, the Chinese Government has introduced a series of H5N1 influenza vaccines derived from different H5 HA phylogenetic clades to control HPAIV H5N1, but it cannot keep up with the speed of viral mutation (Zhang et al., 2016). Recently, a newly identified HA sequence originating from H5N1 clade 2.3.4.4 viruses has been linked to outbreaks of the disease in poultry in China and other southeast Asian countries (De Vries et al., 2015; Gu et al., 2013; WHO, 2015). However, the widely used inactivated AIV vaccine provides weak protection against prevailing clade 2.3.4.4 strains (Ren et al., 2015). Therefore, the development of a new vaccine strategy that provides good heterologous protection against circulating H5N1 AIVs is a high priority.

One approach to vaccine development is the utilization of immunogenic epitopes. A combination of multiple conserved B-

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http://dx.doi.org/10.1016/j.vetmic.2017.03.004
0378-1135/

and T-lymphocyte epitopes in a single construct should stimulate both the humoral and cellular immune responses (Sette and Fikes, 2003; Sun et al., 2013). Vaccines designed to the stalk region of HA can protect against influenza virus infection (Bommakanti et al., 2010; Steel et al., 2010). An alignment of the region of HA corresponding to amino acid 76–130 of the H5N1 subtype demonstrated the high degree of conservation in this sequence. A synthetic peptide vaccine based on amino acids 76–130 of the HA2 subunit afforded protection to mice against the H3N2, H1N1, and H5N1 influenza viruses (Wang et al., 2010). The ectodomain of matrix 2 (M2e) of the influenza A virus, a highly conserved antigen, has been extensively studied in universal vaccine research (Fiers et al., 2009; Sun et al., 2013). Nucleoprotein amino acids 55–69 (NP55–69) and 380–393 (NP380–393) stimulate helper T lymphocytes (Th cells) and cytotoxic T lymphocytes (CTLs), respectively, and contributed to cross-protection in previous studies (Jeon et al., 2002; Adar et al., 2009). Conserved sequences with different constructions have shown good potential utility as vaccines against homologous and heterologous influenza A viruses in animal models (Adar et al., 2009; Dabaghian et al., 2014; Jazi et al., 2012; Sun et al., 2013; Wang et al., 2010). Although epitope vaccines have been widely demonstrated to elicit broad immune responses, strategies based on epitopes alone have shown limited protection against H5N1 HPAIV challenge in previous studies (Adar et al., 2009; Wang et al., 2010; Zhang et al., 2011). Interestingly, recent studies showed that supplementation of an inactivated viral vaccine with the M2e antigen improved the protective efficacy against heterologous AIVs or vaccine escape variants (Kim et al., 2014; Park et al., 2014; Song et al., 2011). For these reasons, coimmunization with an epitope antigen and an inactivated H5N1 vaccine might be a practical strategy with which to counter heterologous H5N1 viruses.

The main goal of this study was to develop an effective AIV vaccination strategy by combining an inactivated H5N1 vaccine and a recombinant baculovirus expressing antigen epitopes to induce cross-protection against heterologous influenza viruses. For this purpose, we constructed a recombinant baculovirus (BV-HMNN) expressing four tandem conserved antigen epitopes: the H5N1 AIV hemagglutinin stem area consensus amino acids 76–130 (HA2 76–130); three repeats of the ectodomain of the conserved influenza matrix protein M2 (3M2e); N55–69; and N380–393. BV-HMNN combined with the inactivated H5N1 vaccine significantly improved the cross-protection against challenge with a lethal heterologous H5N1 virus.

2. Materials and methods

2.1. Viruses and vaccine

Highly pathogenic avian influenza (HPAI) H5N1 viruses from clade 2.3.4.4 (A/goose/Guangdong/14079/2013, GD/14079) and clade 2.3.2.1 (A/duck/Guangdong/383/2008, GD/383) were propagated in the allantoic fluid of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The 50% egg infectious dose (EID_{50}) was calculated with the Reed–Muench method (Reed and Muench, 1938). Each experiment involving H5N1 viruses was performed in a biosafety level 3 laboratory. The inactivated H5N1 Re6 vaccine corresponding to clade 2.3.2.1 was purchased from Winsun Pharmaceutical Co. Ltd (Guangzhou, China).

2.2. Baculovirus preparation

A plasmid containing the four codon-optimized epitopes, HA2 76–130, 3M2e, NP55–69, and NP380–393 (HMNN), and a flexible linker sequence (GGGGS) (Table 1) was synthesized by Invitrogen (Carlsbad, CA, USA), and cloned into the pcDNA3.1(+) vector (Invitrogen) under the control of the human cytomegalovirus (CMV) immediate early (IE) enhancer/promoter ($P_{CMV\text{-}IE}$). The cassette consisting of the CMV-IE promoter and the HMNN sequence was amplified by PCR and inserted downstream from the polyhedrin promoter (P_{PH}) in the pFastBac-VSV-G baculovirus transfer vector after restriction digestion with *Sal*I/*Hin*dIII, to generate pFast-G-HMNN.

The recombinant baculovirus (BV-HMNN) and the wild-type baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (BV-WT) were produced and propagated in *Spodoptera frugiperda* cells (Sf9), cultured at 27 °C in Sf-900™ II SFM serum-free medium (Invitrogen), according to the manufacturer's manual, using the Bac-to-Bac® Baculovirus Expression System (Invitrogen). The viral particles were purified with sucrose-gradient ultracentrifugation using the standard protocol (O'Reilly et al., 1994), and the viral titers were determined with the BD BacPAK™ Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA, USA).

2.3. Immunofluorescence assay (IFA)

IFA was used to detect the expression of the recombinant baculovirus in vitro. Briefly, chicken embryo fibroblast (CEF) cells, prepared according to the standard protocol (Ping et al., 2006), were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 100 μg/mL streptomycin, and 100 IU/mL penicillin. The CEF cells were seeded at a concentration of 2.5×10^5 cells/well in six-well tissue culture plates (Nunc, Rochester, NJ, USA) and transfected with baculovirus particles at a multiplicity of infection (MOI) of 10. After incubation for 48 h, the cells were fixed with absolute methanol for 15 min and incubated with the primary anti-M2 monoclonal antibody 14C2 (Santa Cruz Biotechnology, Inc., CA, USA), and then with the secondary fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibody (Sigma St. Louis, MO, USA). Fluorescent images were examined under an inverted fluorescence microscope (Leica, DM5000B, Germany).

2.4. Animal experiments

Three-week-old SPF chickens were purchased from the Experimental Animal Center (Xinxing Dahuanong Eggs Co., Ltd, Guangdong, China) and were maintained according to the South China Agricultural University's guidelines for the care and use of

Table 1
Antigen epitopes included in BV-HMNN.

Epitope	Sequence
HA2 76–130	RIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNA
M2e 1–24	MSLLTEVETPTRNGWECKCSDSSD
NP55–69	RLIQNSLTIERMVLS
NP380–393	ELRSRYWAIRTRSG

Note: Hemagglutinin stem area amino acids 76–130, HA2 76–130; the ectodomain of matrix protein M2, M2e; nucleoprotein amino acids 55–69, NP55–69; nucleoprotein amino acids 380–393, NP380–393.