Vaccine 31 (2013) 3268–3273

Contents lists available at SciVerse ScienceDirect

Vaccine

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

Virus-like particle vaccine protects against H3N2 canine influenza virus in dog

Dong-Hun Lee[a,1], Sang-Woo Bae[b,1], Jae-Keun Park[a], Jung-Hoon Kwon[a], Seong-Su Yuk[a], Jae-Min Song[c], Sang-Moo Kang[d], Yong-Kuk Kwon[e], Hwi-Yool Kim[b], Chang-Seon Song[a,*]

[a] Avian Disease Laboratory, College of Veterinary Medicine, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[b] Department of Veterinary Surgery, College of Veterinary Medicine, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[c] Department of Global Medical Science, Sungshin Women's University, Seoul 136-742, Republic of Korea
[d] Center for Inflammation, Immunity & Infection, and Department of Biology, Georgia State University, Atlanta, GA 30303, United States
[e] Animal, Plant & Fisheries Quarantine & Inspection Agency, Ministry for Food, Agriculture, Forestry and Fisheries, Anyang, Gyeonggi 430-824, Republic of Korea

ARTICLE INFO

Article history:
Received 22 January 2013
Received in revised form 30 April 2013
Accepted 8 May 2013
Available online 22 May 2013

Keywords:
Virus-like particle
Canine influenza
Vaccine
H3N2

ABSTRACT

In the present study, virus-like particles (VLPs) were evaluated as a candidate veterinary vaccine against canine influenza virus (CIV) subtype H3N2. Specific pathogen-free (SPF) beagle dogs received a single injection of a VLP vaccine containing hemagglutinin (HA) and M1 protein of CIV H3N2 (H3 HA VLP). The vaccine was tested at 3 different doses with an adjuvant and 1 dose without an adjuvant. To evaluate the immunogenicity and protective efficacy of the H3 HA VLP vaccine, we performed hemagglutination inhibition tests to determine serological immune responses and conducted challenge studies using SPF beagle dogs. The addition of Montanide ISA 25 adjuvant significantly increased the immunogenicity of the H3 HA VLP vaccine. The experimental infection study showed that a single dose of H3 HA VLP vaccine induced protection against wild-type virus challenge in dogs. These results provide support for continued development of the VLP as an animal vaccine against influenza virus.

1. Introduction

Canine influenza virus (CIV) belongs to the genus *Influenza-virus A* of the family *Orthomyxoviridae*. An equine-origin H3N8 influenza virus was first isolated from racing dogs affected with acute respiratory disease in the United States in 2004 [1]. Subsequent outbreaks were reported, and the infection spread rapidly across the United States [2]. Between May 2006 and March 2010, sporadic cases of a different subtype of CIV, namely, H3N2, were identified from sick dogs at animal clinics in China [3,4]. In 2007, CIV H3N2 also caused an outbreak of contagious canine respiratory disease in South Korea [5]. This virus appears to be entirely of avian origin and is, to the best of our knowledge, the first low pathogenic avian influenza (LPAI) virus reported to cause respiratory disease in dogs. CIV H3N2 infection results in clinical outcomes ranging from mild respiratory illness to death. Previous studies found that experimentally infected and contact-exposed beagle dogs shed virus through nasal excretion, seroconverted, and became ill with severe necrotizing tracheobronchitis and bronchioalveolitis with accompanying clinical signs [5,6].

Recently, Lee et al. reported that CIV H3N2 can replicate efficiently in the respiratory system of experimentally infected ferrets and cause acute necrotizing bronchioalveolitis and non-suppurative encephalitis [7]. Furthermore, a novel reassortant CIV H3N1 between pandemic H1N1 and Korean CIV H3N2 has been reported [8]. Therefore, epidemics of CIV H3N2 among dogs in Asian countries have raised concern over the potential for zoonotic transmission of this virus.

Virus-like particles (VLPs), which resemble infectious virus particles in their structure and morphology and display multiple antigenic epitopes, have been proposed as a new generation of non-egg-based, cell culture-derived vaccine candidates against influenza virus [9]. VLP vaccines containing influenza surface proteins are produced easily in insect or mammalian cells via simultaneous expression of hemagglutinin (HA) and neuraminidase along with a viral core protein such as the influenza matrix protein (M1) [10]. The protective mechanism of influenza VLP vaccines is known to be similar to that of the commercial inactivated full virus influenza vaccine; they induce neutralizing antibodies and hemagglutination inhibition (HI) activities and have a solid safety profile [9]. Previous studies have demonstrated strong immunogenicity and high protective efficacy of VLP vaccines in animal influenza challenge models [11–16]. However, there have been no studies of VLP vaccines against influenza virus in dogs.

In the present study, we developed a canine influenza H3 HA VLP vaccine by using a baculovirus expression system and evaluated its

* Corresponding autho at: Avian Disease Laboratory, College of Veterinary Medicine, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea. Tel.: +82 2 450 3712; fax: +82 2 455 3712.
E-mail address: songcs@konkuk.ac.kr (C.-S. Song).
[1] These authors contributed equally to this work.

0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.05.023

immunogenicity and protective efficacy in beagle dogs for the first time.

2. Materials and methods

2.1. Preparation of canine influenza H3 HA VLPs

VLPs containing H3 HA and M1 proteins were produced as described previously [17]. Briefly, to generate the recombinant baculoviruses (rBVs) expressing the influenza proteins, full-length HA cDNA derived from influenza A/canine/Korea/LBM412/2008 (H3N2) virus and M1 cDNA were cloned into the pFastBac vector (Invitrogen, Carlsbad, CA), and then transferred into a bacmid (baculovirus shuttle vector) by transforming the recombinant plasmid DNA into competent DH10Bac *Escherichia coli* cells (Invitrogen, Carlsbad, CA). Sf9 insect cells were transfected with the recombinant bacmid DNA, and rBVs expressing HA and M1 proteins were plaque purified from culture supernatants of the transfected Sf9 cells. To generate H3 HA VLPs, Sf9 cells (ATCC, CRL-1711) were co-infected with rBVs expressing H3 HA and M1 at multiplicities of infection of 3 and 1, respectively. Culture medium was collected and clarified by low-speed centrifugation (2000 × *g*, 30 min, 4 °C) at 2 days post infection. Culture supernatants were concentrated and filtrated using a QuixStand benchtop system (GE Healthcare, Waukesha, WI) with a hollow fiber cartridge (300,000 Da as the nominal molecular weight cutoff). Further purification was performed by 30% and 60% sucrose gradient ultracentrifugation (28,000 × *g*, for 60 min) followed by dialysis with HEPES-buffered saline solution, and then, the H3 HA VLP solution was concentrated using a Viva spin (Sartorius, Bohemia, NY) protein concentrator. The final protein concentration of H3 HA VLPs was determined using a protein assay kit (Bio-Rad, Irvine, CA) and the biological activity was determined by a hemagglutination assay as described previously [18]. Units of hemagglutination activity are presented as a factor of dilution that prevents the precipitation of red blood cells. The electron microscopic examination of purified VLPs was carried out. Briefly, VLPs were absorbed onto carbon coated grids and negatively stained with 2% uranyl acetate. The grids were observed by transmission electron microscopy (TEM) at 100,000× magnification.

3. Vaccine and viruses

Vaccines were prepared at 3 different protein concentrations (3.75 μg, 7.5 μg, and 15 μg). Preparations with adjuvant were made by emulsifying VLP solution with Montanide ISA 25 VG (SEPPIC, France) oil adjuvant at a ratio of 75:25 (w/w) or aluminum hydroxide gel adjuvant at a ratio of 80:20 (v/v). To evaluate vaccine efficacy, dogs were challenged intranasally with 10^7 50% egg infectious dose (EID_{50}) of homologous H3N2 influenza A virus (A/canine/Korea/LBM412/2008).

3.1. Animals and experimental design

To select an effective adjuvant and to determine the immunogenicity of the VLP vaccine, 21 influenza-seronegative adult beagle dogs were divided into 7 groups. Dogs were immunized by intramuscular injection with a single dose of VLP vaccine (3.75 μg, 7.5 μg, or 15 μg) with oil or gel adjuvant or 15 μg of VLP vaccine without an adjuvant. As a non-vaccinated control group, another 3 beagle dogs were injected with PBS. Serum samples were collected prior to vaccination and 2, 3, 4, and 12 weeks after vaccination, and treated with a receptor-destroying enzyme for HI tests. HI tests were performed according to the OIE standard method, by using formalin-inactivated homologous antigen and turkey erythrocytes. The cut-off HI antibody titer for seropositivity was 16.

To determine the protective efficacy of the VLP vaccine, 12 specific pathogen-free beagle dogs were divided into 4 groups. Dogs were immunized with a single dose of VLP vaccine (3.75 μg, 7.5 μg, or 15 μg) with ISA 25 VG oil adjuvant. In the non-vaccinated control group, dogs were injected with an emulsified solution of distilled water and ISA 70 prepared at the same ratio as the VLP vaccine. At 4 weeks after vaccination, under Animal Biosafety Level 2 enhanced conditions, dogs were challenged intranasally with the homologous H3N2 virus. We observed the mortality and clinical signs daily for 4 days post inoculation (dpi) and determined viral shedding pattern by using real-time reverse transcriptase polymerase chain reaction (rRT-PCR). At 4 dpi, necropsies were performed on the dogs for pathologic examination. All animal procedures performed in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of Konkuk University.

4. Virus quantification

To determine viral shedding in the respiratory tract, nasal swab samples were collected at 1, 2, 3, and 4 dpi, and suspended in 1 mL of phosphate-buffered saline (PBS). At 4 dpi, lung tissues were collected from each dog and homogenized in PBS (10% w/v). The suspensions were centrifuged and a clear liquid supernatant was collected. A 0.2-mL aliquot of each sample was used for RNA extraction with an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. CIV RNA was quantified by the cycle threshold (Ct) method using matrix gene-based rRT-PCR as described previously [19]. For extrapolation of the Ct values to infectious units, known titers of CIV from egg allantoic fluid, measured as EID_{50}, were serially diluted 10 fold. Viral RNA was extracted from these dilutions and quantified by rRT-PCR as described above. To generate a standard curve, Ct values of the viral dilutions were plotted against viral titers. The resulting standard curve was highly correlated ($r^2 > 0.99$) and was used to convert Ct values to EID_{50}.

5. Lung histopathology

The cranial, middle, and caudal lobe tissues were fixed in 10% neutral buffered formalin, processed, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

6. Statistical analysis

Statistical analysis of differences between the vaccinated groups and the unvaccinated control group was performed. One-way ANOVA with a Dunnett's post hoc test was used for statistical analysis of body temperature and viral shedding. Statistical significance was designated for differences with p values less than or equal to 0.05.

7. Results

7.1. Production of CIV H3 HA VLPs

VLPs containing the HA protein from influenza A/canine/Korea/LBM412/2008 (H3N2) virus were produced in insect cells by using a baculovirus expression system and purified by ultrafiltration and sucrose gradient ultracentrifugation. Purified CIV H3 HA VLPs were found to have approximately 8000 units of hemagglutination activity at a protein concentration of 1.2 mg/mL. This result indicates that the HA protein maintains a biologically