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The immune response of a recombinant fowlpox virus coexpressing the HA gene of the H5N1 highly pathogenic avian influenza virus and chicken interleukin 6 gene in ducks

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ABSTRACT

Ducks have played an important role in the emergence of H5N1 subtype of highly pathogenic avian influenza (HPAI), and the development of an effective vaccine against HPAI in ducks is a top priority. It has been shown that a recombinant fowlpox virus (FPV)-vectored vaccine can provide protection against HPAI in ducks. In this study, a recombinant fowlpox virus (rFPV-AIH5AIL6) coexpressing the haemagglutinin (HA) gene of the H5N1 subtype of the avian influenza virus (AIV) and chicken interleukin 6 gene was constructed and tested in Gaoyou and cherry valley ducks to evaluate the immune response in ducks. These animal studies demonstrated that rFPV-AIH5AIL6 induced a higher anti-AIV HI antibody response, an enhanced lymphocyte proliferation response, an elevated immune protection, and a reduction in virus shedding compared to a recombinant fowlpox virus expressing the HA gene alone (rFPV-SYHA). These data indicate that rFPV-AIH5AIL6 may be a potential vaccine against the H5 subtype of avian influenza in ducks and chicken interleukin 6 may be an effective adjuvant for increasing the immunogenicity of FPV-vectored AIV vaccines in ducks.

1. Introduction

The H5 subtype of the highly pathogenic avian influenza virus (AIV) has caused outbreaks in poultry and deaths in human for more than a decade [1,2]. Conventional inactivated vaccines have proven to be effective for the control and prevention of avian influenza outbreaks. However, outbreaks of H5 AIV still continue to occur in poultry [3]. To develop an improved vaccine against AI, recombinant DNA technology has been used to generate live virus vaccines. Fowlpox virus (FPV) [4–9], adenovirus [10], infectious laryngotracheitis virus [11,12], Newcastle disease virus [13,14], and duck enteritis virus [15] have been used as vectors to develop recombinant vaccines. It has been reported that a FPV-vectored vaccine against AIV can be used in both permissive hosts, such as chickens [16,17], as well as non-permissive hosts, such as ducks [18–20]. However, recombinant FPVs (rFPVs) generally require administration at high titres with a booster injection at a later time to achieve sufficient protection in a non-permissive host [19]. Therefore, an effective adjuvant may be needed to help increase the immune response. A number of avian cytokines have been shown to be effective immunomodulators in animal models [21–24]. It has been reported that interleukin 6 (IL-6) augmented both systemic and mucosal antibody responses when an rFPV coexpressing murine IL-6 gene and hemagglutinin (HA) gene of influenza virus A/PR/8 was used to immunize in a mouse model [25]. However, whether chicken IL-6 (ChIL-6) can be used as an adjuvant to improve the immune response and provide protection against AI in ducks is still unknown.

Wild waterfowl are considered a reservoir for AIV, and wild ducks and domestic ducks play an important role in the epidemiology of influenza [26–30]. Because highly pathogenic AIVs can cause an asymptomatic infection in ducks, it is important to control the H5 subtype of AI in ducks to prevent transmission of the virus from ducks to other susceptible animals and humans [31]. To improve the immune response induced by the FPV-vectored vaccine against AIV infection in ducks, we constructed a recombinant FPV coexpressing the ChIL-6 gene and the HA gene of the H5N1 subtype of AIV, and evaluated its immunogenicity in Gaoyou and cherry valley ducks.

Abbreviations: AI, avian influenza; AIV, avian influenza virus; CEF, chicken embryo fibroblast; ChIL-6, chicken IL-6; EID50, 50% chicken embryo infection dose; E-rosette, erythrocyte rosette; FBS, fetal bovine serum; FPV, fowlpox virus; HA, haemagglutinin; HI, hemagglutination inhibition; HPAI, highly pathogenic avian influenza; IFA, indirect immunofluorescence assay; IL, interleukin; MOI, multiple of infection; PFU, Plaque forming units; rFPV, recombinant fowlpox virus; RT-PCR, reverse transcriptase-polymerase chain reaction; SPF, specific-pathogen-free.

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2. Materials and methods

2.1. Plasmids, viruses, cells, and experimental animals

The fowlpox virus vector plasmid, $_P$12LS was developed by Sun et al. [32]. The plasmid $pP_{E/L}NA$ was obtained from Chen et al. [33]. The plasmid, p12LSH5A, containing the expression cassette of the Ps-HA gene was obtained from Yun et al. [23]. The FPV vaccine strain, 282E4 (wt-FPV), was purchased from the China Institute of Veterinary Drug Control (Beijing, China). The H5N1 AIV A/mallard/Huadong/SY/2005 (clade 2.3.4) was isolated in eastern China [34] and its HA gene was used as expression gene in p12LSH5A to construct the rFPV-SYHA. Fertilised White Leghorn specific-pathogen-free (SPF) eggs were purchased from Shandong Institute of Poultry Science (Shandong, China). Chicken embryo fibroblast (CEF) cells were prepared and maintained in DMEM medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, UT, USA). The Plaque forming units (PFU) of FPVs were calculated by inoculation of 10-fold serial dilutions onto CEF cells. The 50% chicken embryo infection dose (EID_{50}) of the AIV was determined by inoculating serial 10-fold dilutions of virus onto 10-day-old SPF embryonated chicken eggs. One-day-old Gaoyou ducks and cherry valley ducks were purchased from two duck farms (Jiangsu, China). All of the ducks were housed in an animal facility, and all of the animal studies were performed under a standard animal study protocol.

2.2. Construction and characterisation of rFPV-IL6 and rFPV-AIH5AIL6

ChIL-6 gene was amplified from chicken splenocytes by reverse transcriptase-polymerase chain reaction (RT-PCR) using the following primers:
5′-AAAGGATCCGCCACCATGAACTTCACCGAGGG-3′ (A *Bam*HI site was underlined and the Kozak sequence was indicated by the box) and
5′-CCCTCTAGAAAGCTTATAAAAATCAGGCACTGAAACTCCT-3′ (*Xba*I and *Hin*dIII sites were underlined and the early transcription termination signal of FPV was indicated by the box). The resulting PCR product was cloned into pGEM-T easy vector (Promega, WI, USA), and its sequence was confirmed by sequence analysis. After digestion with *Bam*HI and *Sal*I, the ChIL-6 gene was inserted into plasmid, p12LS, which had been digested with the same restriction enzyme, to generate the transferring vector, p12LSIL6 (Fig. 1A). For construction of the transferring vector p12LSAIH5AIL6, the ChIL-6 fragment was first digested with *Bam*HI and *Xba*I, and inserted into plasmid $pP_{E/L}NA$, in which the ChIL-6 gene was regulated under promoter $P_{E/L}$ [35]. Then, the *Hin*dIII digestion fragment containing $P_{E/L}$-ChIL6 was inserted into plasmid 12LSH5A to form p12LSAIH5AIL6 (Fig. 1A). The positive plasmids were confirmed by sequence analysis.

The rFPVs were generated by homologous recombination using previously published procedures [23]. The two rFPVs (rFPV-AIH5AIL6 and rFPV-IL6) were obtained and passaged in CEF cells. Expression of AIV HA and ChIL-6 was confirmed by indirect immunofluorescence assay (IFA) using a chicken polyclonal antibody against the H5 subtype AIV whole virus or a monoclonal antibody against ChIL-6 protein as the primary antibodies, respectively.

To determine the growth kinetics of FPVs, CEF cells was infected with each FPV at a multiple of infection (MOI) of 0.05. The infected cells were harvested by three freeze/thaw cycles at different timepoints and PFUs of FPVs were determined. The supernatants of above suspension were collected by centrifugation followed the filtration with a 0.22-μm filter and bioactivity of expressed ChIL-6 was detected by its ability to stimulate proliferation of the IL-6-dependent murine hybridoma cell line 7TD1 [36]. Briefly, 100 μl of 7TD1 cells in RPMI 1640 (Sigma, MO, USA) medium containing 10% FBS were adjusted to 2×10^4 cells per well of 96-well microtiter plate, 100 μl of serial 5-fold dilutions of the supernatants and 5 ng/ml recombinant human IL-6 (PeproTech, NJ, USA; as positive control) were added to each well, and the cells containing medium only was used as negative control. After incubation at 37 °C in 5% CO_2 for 72 h, 10 μl (5 mg/ml) of MTT (Sigma, MO, USA) was added into the media. Then, 10 μl of HCl–SDS (10% SDS, 0.01 mol HCl) was added into the media after 3 h, followed by an addition 2 h of incubation. The optical density at 570 nm (OD_{570}) of the samples was measured using a Microplate Reader 680 (Bio-Rad, CA, USA). $OD_{ChIL6}/OD_{negative}$ value of 1.5 or above was defined as positive [37]. One unit means that the OD value induced by ChIL-6 was comparable with that induced by 1 ng/ml recombinant human IL-6.

2.3. Immunisation and challenge study

2.3.1. Experiment 1

CEF cells were infected with each FPV at an MOI of 0.05. The infected cells were incubated at 37 °C in 5% CO_2 for 72 h and harvested by three freeze/thaw cycles for vaccine production. Then, the PFUs and bioactivity for ChIL-6 of FPVs were determined. A total of 108 24-day-old Gaoyou ducks were randomly divided into 6 groups and each group included 18 ducks. The ducks in groups 1 through 4 were immunised subcutaneously with rFPV-SYHA, rFPV-AIH5AIL6, rFPV-IL6 and wt-FPV, respectively, at a titre of 10^5 PFU in a 0.2-ml inoculum. The ducks in group 5 were immunised with 0.5 ml of H5 inactivated vaccine (H5N1 AIV Re-5, clade 2.3.4, QYH Biotech Company limited. Jiangsu, China). The ducks in group 6 were inoculated with 0.2 ml of sterile PBS. The sera of each group were collected at 0, 7, 14, and 21 days post-vaccination. Hemagglutination inhibition (HI) antibody titres of sera were determined by HI assay with AIV Re-5 antigen [38]. The thymuses, spleens, bursas, and peripheral blood of ducks in each group were collected at 0, 9, 16, and 23 days post-vaccination. At day 23 post-vaccination, each duck was challenged with 0.2 ml of $10^{5.75}$ EID_{50} of H5 AIV A/mallard/Huadong/SY/2005 by nose/eye drop. The ducks were monitored daily for 2 weeks for survival and clinical signs of infection. Furthermore, oropharyngeal and cloacal swabs were collected for virus isolation from each group at 3, 5, and 7 days post-challenge. The swabs were placed in PBS and an aliquot was titrated by inoculation of embryonated eggs.

2.3.2. Experiment 2

A total of 108 10-day-old cherry valley ducks were randomly divided into 6 groups and were immunised subcutaneously as described in experiment 1. The thymuses, spleens, bursas, and peripheral blood of ducks in each group were collected at 0, 7, 14, and 21 days post-vaccination. At day 21 post-vaccination, each duck was challenged with 0.2 ml of $10^{5.75}$ EID_{50} of H5 AIV A/mallard/Huadong/SY/2005 by nose/eye drop. The sera and swab collection were performed according to the method described above.

2.4. Lymphocyte proliferation assay

Lymphocytes of thymus, spleen, bursa, and peripheral blood were collected as described previously [39,40]. Briefly, thymuses, spleens, and bursas were grinded and filtered by wire mesh (200 μm) into a 100-mm petri dish containing HBSS (Sigma, MO, USA). The cell suspension was then loaded onto the surface of the lymphocytes separation medium (Sigma, MO, USA) and centrifuged at 400 × *g* for 20 min at 4 °C. Lymphocytes were collected and suspended in RPMI 1640 medium. Peripheral blood samples