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Inactivated vaccine with adjuvants consisting of pattern recognition receptor agonists confers protection against avian influenza viruses in chickens

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ABSTRACT

Use of adjuvant containing pathogen pattern recognition receptor agonists is one of the effective strategies to enhance the efficacy of licensed vaccines. In this study, we investigated the efficacy of avian influenza vaccines containing an adjuvant (CVCVA5) which was composed of polyriboinosinic polyribocytidylic, resiquimod, imiquimod, muramyl dipeptide and levomisole. Avian influenza vaccines adjuvanted with CVCVA5 were found to induce significantly higher titers of hemagglutiniton inhibition antibodies ($P \leq 0.01$) than those of commercial vaccines at 2-, 3- and 4-week post vaccination in both specific pathogen free (SPF) chickens and field application. Furthermore, virus shedding was reduced in SPF chickens immunized with H9-CVCVA5 vaccine after H9 subtype heterologous virus challenge. The ratios of both $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes were slowly elevated in chickens immunized with H9-CVCVA5 vaccine. Lymphocytes adoptive transfer study indicates that $CD8^+$ T lymphocyte subpopulation might have contributed to improved protection against heterologous virus challenge. Results of this study suggest that the adjuvant CVCVA5 was capable of enhancing the potency of existing avian influenza vaccines by increasing humoral and cellular immune response.

1. Introduction

Both H5 and H9 subtype avian influenza viruses (AIV) not only cause seriously economic loss in poultry industry, and even endanger human public health (Tang et al., 2009; Zhao et al., 2013). Vaccination is one of the primary strategies to prevent and control these infectious diseases spread in poultry industry in China and other countries (Swayne, 2012). However, influenza viruses with antigenic mutations in the hemagglutinin (HA) gene may escape host immunity elicited by the vaccine strain, and cause outbreaks (Connie Leung et al., 2013; Liu et al., 2011a; Sun et al., 2012). Furthermore, the pivotal steps of the traditional vaccine development processes involving identification of antigenicity match of the vaccine strain to the predicted circulating viruses in the coming season are time-costing and labor-consuming procedures

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(Sun et al., 2012). In addition, inactivated vaccines primarily induce systemic humoral immune response, and only marginally cell-mediated immune response (Xu et al., 2011).

Combination of adjuvant and the licensed vaccine is one of the fast and efficient ways toward improving its efficacy. The agonists to pathogen-recognition receptors (PRRs) are potential immunopotentiators in vaccine development. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), two subclasses of the PRRs, are important components of the innate immune system that play a crucial role in the first line host defense against microbial pathogens (Kawai and Akira, 2009). Some agonists of the TLRs or NLRs are used as vaccine adjuvants. The poly I:C (TLR3 agonists) (Strayer et al., 2012), flagellin (TLR5 agonists)(Liu et al., 2011b), imidazoquinolines (TLR7 and TLR8 agonists) (Raman et al., 2012), CpG (TLR9 agonists) (Gupta and Cooper, 2008) are tested as vaccine adjuvants in human. The adjuvant AS04 containing ingredients derived from TLR4 agonists is licensed for use in human vaccines (Rappuoli et al., 2011). Muramyl dipeptide (MDP) and its derivatives (NLR agonists) also showed immune boosting potential in vaccine development (Kanneganti et al., 2007). Besides the agonists of PRRs, some chemical compounds also possess immuno-stimulatory capabilities. The levamisole, an imidazole–thiazole group derivate which is an anthelminthic commonly used in veterinary and human medicine, is able to enhance both humoral and cellular immune responses in animal (Oladele et al., 2012).

In this study, an adjuvant containing poly I:C, resiquimod, imiquimod, MDP and levamisole hydrochloride was prepared and named as CVCVA5. Addition of CVCVA5 adjuvants to the avian influenza vaccines significantly elicited antibody immune responses against H5 or H9 subtype virus antigen in experimental tests or in field applications and long-lasting antibody against H9 antigen in layer, and provided robust protection against H9 heterologous virus challenge.

2. Material and methods

2.1. Viruses

H9 subtype AI viruses strain, A/Chicken/NJ/02/2001 (NJ02/01) and A/Chicken/SD/YH01/2011 (SDYH01/11), were isolated from dead chicken. Viruses were passaged in 10-day-old specific pathogen free (SPF) embryonated chicken eggs and cloned by three limiting dilutions. The infective allantoic fluid was aliquoted and stored at −80 °C. The HA subtype was determined by hemagglutinination inhibition (HI) tests with a panel of reference antisera provided by the China Institute of Veterinary Drug Control (Beijing, China) and hemagglutinin (HA) sequences were confirmed by reverse transcription–polymerase chain reaction. Nucleotide sequences are available in databases under the accession numbers: KF844248 and KF844249.

The hyperimmune serum against the virus strain NJ02/01 or SDYH01/11 were obtained from chickens receiving two shots of vaccines, with each dose of vaccine containing $10^{7.8}$ EID_{50} (median embryo infectious dose) corresponding virus antigen and formulated to water-in-oil emulsion.

2.2. Preparation of vaccines and adjuvants

The H9 subtype AI vaccine was prepared as a water-in-oil form. In brief, the H9 subtype virus strain NJ02/01 was propagated in allantoic cavities from 10- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs. The viral allantoic fluids (EID_{50}, $10^{8.0}$/0.1 ml) were purified by centrifugation (28,000 × *g*, 30 min, 4 °C) and inactivated with β-propiolactone (v/v 0.5‰, 24 h, 37 °C, Sigma, St. Louis, MO). The purified virus suspended in the same volume of phosphate buffer solution (pH 7.2, PBS) buffer was added into Marcol 52 mineral oil (ESSO, Paris, France) to manufacture a water-in-oil emulsion vaccine (v/v, 1:3). The H5 vaccine (H5-Re5, Weike Biotechnology Co., Ltd., Harbin, China) is a commercial product.

The preparation process of adjuvant, CVCVA5, was similar to the procedure as vaccine in a water-in-oil emulsion form. The adjuvant components of poly I:C (InvivoGen, San Diego, CA), MDP in L–D isoform (InvivoGen) and levamisole hydrochloride (Sigma) were dissolved in PBS as aqueous phase. Additional adjuvant components resiquimod (InvivoGen) and imiquimod (InvivoGen) were dissolved in Marcol 52 mineral oil (ESSO) as oil phase. One volume aqueous phase adjuvant was mixed with three volume oil phase adjuvant. The recipe of adjuvants was followed as described in the Chinese patent license with the registered number 201210235427.0. One volume of adjuvant was mixed with nine volumes of AI vaccine by vortex violently (5 min) before injection. The H5 or H9 vaccine mixed with CVCVA5 were named as H5-CVCVA5 or H9-CVCVA5.

2.3. Immunization of chickens

Specific pathogen free (SPF) white Leghorn chickens (*G. gallus domesticus*) were grown in isolation units from day 1. Six groups of ten 14-day-old SPF chickens were included in this trial. Chickens from each group ($n = 10$) via subcutaneous route received only a single dose injection (0.3 ml) of H5, H9, H5-CVCVA5 or H9-CVCVA5 vaccines, respectively. Chickens in the challenge control group did not receive any vaccine. All birds were bled on day 14, 21 and 28 post-vaccination (dpv) to collect sera. Serum antibody levels were measured by hemagglutinin inhibition (HI) assay.

2.4. Virus challenge of immunized chickens

At 28 dpv, all birds in each group were intranasal challenged with 0.1 ml of $10^{7.0}$ EID_{50} dose of a heterologous H9 subtype AI virus SDYH01/11 strain. Chicken were observed clinically for 14 days and after this observation period, all surviving chickens were killed humanely and subjected to check gross lesions. Oropharyngeal and cloacal swab samples were collected at 3, 5 and 7 days post-challenge (dpc), or collected when chickens died within the clinical observation period. Virus isolation from