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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 levamisole. Avian influenza vaccines adjuvanted with CVCVA5 were found to induce significantly higher titers of hemagglutinin inhibition antibodies ($P = 0.01$) 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.

Keywords

Avian influenza virus; Inactivated vaccine; Adjuvants; Pattern recognition receptor; Agonist; Toll-like receptor

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 (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 anthelmintic 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 hemagglutination 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 $10^{7.8}$ EID₅₀ (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 (EID₅₀, $10^{8.0}/0.1$ ml) were purified by centrifugation ($28,000 \times g$, 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 $10^{7.0}$ EID₅₀ 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 the swab samples was performed as previously described (Tang et al., 2009).

2.5. Monitoring of long term immune persistence

The commercial Hy-Line variety brown chicken (obtained from the Shuangyu Poultry Farm, Haian, China) that maternal derived HI antibodies against H9 subtype AI viruses were lower than 2 log₂, were used to perform the test of persistence of immune response. Three groups of twenty birds were tested in this trial, including H9 AI vaccine group, H9-CVCVA5 vaccine group and control group. The birds in each group were bled on 2-, 3- and 4-week post-vaccination and then at 2-week intervals thereafter, until 32-week post-vaccination.

2.6. Field application studies

The field application test included two groups (named as A and B) of one thousands of the commercial yellow broiler chicken that were reared under commercial chicken management condition in two different chicken houses (Dingyan Poultry Farm, Haian, China). The 10-day old chickens in the group A were vaccinated with 0.3 ml of the bivalent AI commercial vaccine (H5 Re5 + H9 Re-2) (Weike), and the B group were vaccinated with bivalent AI commercial vaccine (Weike) plus CVCVA5 adjuvant with the same volume as in the group A. Five percent of the total vaccinated chickens in each group were randomly chosen for blood sampling and detection of HI antibody titer against commercial H5 (Re5) and H9 (Re2) subtype AI virus antigen (Weike) at 14 and 21 dpv.

2.7. Flow cytometry analysis

The peripheral blood lymphocytes from the SPF chicken in immune efficacy test of H9 subtype vaccine containing groups were analyzed by fluorescent-activated cell sorting (FACS) with FACS Calibur fluorospectrometer (BD Biosciences, Franklin Lakes, NJ, USA). For sorting, 6×10^7 cells from birds were triple-stained with mouse anti-chicken CD3-R-PE (Southern Biotech, Birmingham, AL), mouse anti-chicken CD4-FITC (Southern Biotech) and mouse anti-chicken CD8 α -chain-PE/CY5 (Southern Biotech). FACS controls (1×10^6 cells) included unstained cells and cells only stained with anti-CD3-R-PE or anti-CD4-FITC, anti-CD8 α -chain-PE/CY5 or appropriate isotype controls.

2.8. Adoptive transfer of immune lymphocytes

Groups of five 14-day-old inbred SPF chickens (homozygous for the B19 MHC haplotype, Harbin Veterinary Research Institute, Harbin, China) were housed in isolation with HEPA filtered air-flow for this trial. Splenocytes from donor chickens, H9-CVCVA5 vaccine or H9 vaccine immunized chickens or unvaccinated control chickens, were collected 10-day after vaccination, separated with a chicken lymphocyte separation medium (HaoYang Co., Ltd.,

Nankai, China) before grouped into T and B cell populations with nylon wool columns (Polysciences, Inc., Warrington, PA). Unbound T cells and macrophages were resuspended in RPMI 1640 with 10% chicken serum (Invitrogen, Carlsbad, CA, USA) and incubated in tissue culture flasks for 3 h to collect the non-adherent T cells. Nylon wool-bound B cells were also collected for use in adoptive transfer study (Seo and Webster, 2001). Purified T lymphocytes were further separated into CD4⁺ or CD8⁺ subtypes by FACS Aria II (BD) which was similar to the 'flow cytometric analysis'.

The donor T, B, CD4⁺ or CD8⁺ subtypes lymphocytes were injected into the wing veins of 14-day old naïve B¹⁹/B¹⁹ inbred recipients with 4×10^7 cells in 0.5 ml volume per chicken. All recipients were then challenged with 10×10^7 EID₅₀ of heterologous viruses SDYH01/11. Chickens were monitored daily for clinical symptoms, death and weight gain. Oropharyngeal and cloacal swabs were collected as described above.

3. Results

3.1. Virus antigenic variation

The cross-reactive HI assay was carried out to characterize antigenic variation between the H9 challenge virus (SDYH01/11) and the vaccine virus (NJ02/01). The cross-reactive HI antibody titers of hyper immune sera showed high HI titer in reaction with homologous virus antigen, in contrast, low HI titer against heterologous virus antigen (Table 1). Combined the results of cross reactive HI assay and unpublished cross challenge test indicated that SDYH01/11 virus was a heterologous H9 virus to NJ02/01 virus.

3.2. Immunization of chickens with adjuvanted AI vaccines induce enhanced antibody responses

Groups of chickens ($n = 10$) were subcutaneously immunized one time with H5 or H9 inactivated vaccine only or in the adjuvanted formulation. The levels of serum antibody titers against homologous H5 (Re5) or H9 (NJ02/01) subtype AI virus antigen were determined by HI assay. The antibody titers of chickens in the adjuvanted H5-CVCVA5 or H9-CVCVA5 group were significantly higher ($P = 0.01$) than those in chickens that were immunized with H5 or H9 commercial vaccine group at time points 2-, 3- and 4-week post vaccination. No HI titers were detected in sera of birds in the control group (Fig. 1).

3.3. Adjuvanted H9 AI vaccine confers improved protection against H9 subtype heterologous virus challenge

Challenge was carried out at 28 dpv with heterologous virus SDYH01/11 in a dose of 10^7 EID₅₀. No chickens died during the 14-day observation period in the vaccinated groups of H9-CVCVA5 and H9 after heterologous H9 subtype AI virus challenges. Two chickens died in the control group on day 9 post-challenge. The only clinical signs of depression lasted three days in one bird of the H9-CVCVA5 group. Three birds in the H9 group showed signs of depression or anorexia, and lasted four to eight days. All chickens in the control group showed depression, anorexia and comb cyanosis, and five birds showed disequilibrium, only three birds with disequilibrium symptom were recovered at the end of 14-day observation period, and other two birds with disequilibrium symptom were died at 9 dpc.

Oropharyngeal and cloacal swab samples were collected at 3, 5 and 7 dpc for virus isolation. One bird out of ten at 3 dpc in the H9-CVCVA5 group was positive for virus isolation. Three birds on day 3, four birds on day 5 and two birds on day 7 post-challenge in the H9 group were not free from infection. No bird in the control groups was free from infection at 5 dpc and afterwards (Table 2).

3.4. Chickens with adjuvanted H9 AI vaccine maintains long lasting antibodies at high levels

Serum antibodies were monitored as an index of the immune persistence induced by the vaccine in Hy-Line variety brown commercial chickens (Fig. 2). The level of antibodies specific for H9 virus elicited by the vaccine of H9-CVCVA5 in chicken was higher than that of the commercial H9 vaccine during the 30-week monitoring period. The HI antibody titer of the chickens in H9 commercial vaccine group was declined to a lower level below the 6 log₂ at 26-week post vaccination. In contrast, the HI serum titer of chicken in H9-CVCVA5 vaccination group was postponed four weeks that declined to 6 log₂ below. The levels of maternally-derived antibodies against H9 AI viruses were barely detectable in control group chickens (Fig. 2).

3.5. Adjuvanted bivalent AI vaccine induces higher antibody titers in the field application

The broilers were raised commercially and immunized with either commercial bivalent vaccine (H5 + H9) or commercial bivalent vaccine mixed adjuvant CVCVA5. Chickens in the group of adjuvanted bivalent vaccines (H5 + H9-CVCVA5) showed significantly higher levels of antibody titers against H9 AI antigen ($P = 0.01$) than those in chickens with the commercial vaccine (H5 + H9) in the absence of adjuvant at the time points of detection at 2-, 3- and 4-wpv (Fig. 3). A similar rise in H5 antibody levels was observed in HI test with H5 subtype viral antigen. The H5 antibody levels induced by the H5 + H9 vaccine containing adjuvants were higher than those of the commercial vaccine alone group. However, the overall levels of H5 antibodies were lower than those of antibodies against H9 viral antigen.

3.6. Adjuvanted H9 AI vaccine induces higher immune cells upon challenge infection

We determined the ratios and kinetics of T lymphocytes in chickens with flow cytometry analysis. These birds were vaccinated with H9 vaccine in the presence or absence of adjuvant components (Fig. 4).

Percentages of CD3⁺CD4⁺ lymphocyte subsets of peripheral blood lymphocytes in birds of group H9-CVCVA5 were higher than those in the groups of chickens with H9 AI vaccine alone or control (no vaccine) (Fig. 4A). Levels of CD3⁺CD4⁺ T lymphocytes were increased during the 7-day analyzed periods post vaccination in the H9 vaccine adjuvanted group but not in other two groups (Fig. 4A).

Similar results were detected in CD3⁺CD8⁺ lymphocyte subsets. The percentages of CD3⁺CD8⁺ lymphocyte subsets in birds of the H9-CVCVA5 AI vaccine group were slightly higher than those in the commercial H9 AI vaccine group and control group, and remained

in an escalating trend during the 7-day monitoring periods (Fig. 4B). A subtle rising magnitude in H9 AI vaccine group was also detected.

3.7. Adoptive transfer of T immune lymphocytes confers protection against heterologous virus

In order to explore the impact of the adjuvants in the H9 AI vaccine on cellular immunity, the B¹⁹/B¹⁹ inbred chickens were employed as a model to study the adoptive transfer of immune lymphocytes. All the recipients were challenged with heterogeneous virus strain SDYH01 after transfer of lymphocytes (4×10^7 cells per chicken).

In recipients that received total T lymphocytes from H9-CVCVA5 AI vaccinated-chickens, only two recipient chickens showed depression and detectable virus shedding during the time of post challenge observation. We observed virus shedding in all chickens of recipients that received total T lymphocytes from the H9 AI vaccine alone group and the control group, and one dead chicken was founded in each group of recipients (Table 3). The gross lesion was similar to that observed in the common SPF chicken mock control group post-challenge, which included depression, anorexia, ruffled feathers and comb cyanosis. The dead chickens in the H9 AI vaccine group and control group showed disequilibrium during the period of dying. The body weight gaining of recipient chickens that received T lymphocytes from H9-CVCVA5 AI vaccinated chickens was higher than the other two challenge groups. Recipient chickens with T lymphocytes from the H9 AI vaccine group and the control group did not show an increase in body weight at the end of the observation period (Fig. 5A).

No chicken was free from infection in the B lymphocytes transferred recipients as determined by clinical symptoms and virus shedding detection. One chicken died in each group (Table 3). The clinical symptoms were more serious than those chickens in T lymphocyte transferred-recipients. Similar to T lymphocyte transfer groups, body weight gaining of chickens in H9-CVCVA5 AI vaccine lymphocyte transferred group was slightly higher than those chickens in the other two groups, the H9 AI vaccine group and control group. Chickens in the latter two groups exhibited scarcely body weight increment (Fig. 5B).

Similar to observations from the B lymphocyte transferred groups, all chickens in three groups that received transfer of the CD4⁺ T lymphocytes were infected post challenge as determined by virus shedding test and gross lesion (Table 3). Each group of recipient chickens with CD4⁺ T lymphocytes resulted in a dead chicken during the monitoring period and showed significant weight loss (Fig. 5C).

In the CD8⁺ T lymphocyte transferred groups, two birds were free from infection in the H9-CVCVA5 AI vaccine transferred group. All chicken receivers with CD8⁺ T lymphocytes from H9 AI vaccinated and control chickens were infected upon infection with H9 virus, and one bird died in chickens that received CD8⁺ T lymphocyte from H9 AI vaccine group and two birds died in control CD8⁺ T lymphocyte transferred group during the period of monitoring (Table 3). Consistent with observations in the B lymphocytes transfer groups, body weight gain in chickens of H9-CVCVA5 AI vaccine lymphocytes transferred group

was higher than those in the other two CD8⁺ T lymphocytes transferred groups, H9 AI vaccine group and control group (Fig. 5D).

Among the four type lymphocyte transfer groups, the efficacy of total T lymphocytes transferred groups was superior to that of CD8⁺ T lymphocytes transferred groups, and the latter's efficacy was slightly preceded over that of CD4⁺ T and B lymphocytes transferred groups (Table 3 and Fig. 5).

4. Discussion

The inactivated H5 or H9 subtype AI vaccines have been being used in China as one of the strategies to control and prevent these diseases (Swayne, 2012). A previous study demonstrated that H9 commercial vaccine was not highly effective in preventing vaccinated chicken from wild virus infection and shedding virus in farms (Lee et al., 2011). One of the criteria to assess the efficacy of H5 or H9 avian influenza vaccine was HI antibody titer which was adopted both in field application and in new vaccine candidate selection. Improving the antibody response will be a benefit in field application. TLRs and NLRs agonists are effective immunopotentiators to up regulate a level of antibody responses (Modlin, 2012; O'Neill et al., 2009). The CVCVA5 adjuvants were composed of poly I:C, resiquimod, imiquimod and MDP, which were recognized by TLR3 or RIG-1, TLR7, TLR8 and NOD2 of NLR.

In this study, the HI antibody titers in chickens with H5 and/or H9 subtype AI vaccine which contained adjuvants (CVCVA5) were significantly ($P < 0.01$) higher than those in chickens immunized with H5 and/or H9 AI vaccine only. Antibodies specific for H5 subtype virus antigen were induced faster at a higher level in SPF chickens of H5-CVCVA5 group than those in control chickens with H5 vaccine only. The morbidity and mortality in chickens with H9-CVCVA5 adjuvanted vaccine were milder than those in chickens with H9 vaccine alone or control group after H9 challenge infection. The total numbers of birds with virus shedding in the H9-CVCVA5 vaccinated group were twofold lower than those in the H9 vaccine only group post challenge. It is an encouraging outcome that combination application of CVCVA5 with H5-H9-bivalent vaccine to the commercial birds in the fields showed similar reproducible results as observed in SPF chickens studies with monovalent H5 or H9 vaccine in the laboratory. Levels of antibody titers of each vaccination group in the field application test were slightly lower than those in SPF chickens tests. The minor discrepancies are probably due to different raising environments and conditions which might influence chicken's immune responses.

The test of vaccine immune sustain periods is targeted to application in layers. The production period of layers is approximately 500 days depending on raising conditions. Elongation of immune persistence with one injection is desirable in decreasing infection risks, reducing vaccination frequency and stress, and saving labor cost. The 6 log₂ of the serum HI titer is a recommended guideline for the qualification of the commercial H9 vaccine in China that enable birds acquire protective immune response on 4-week post-vaccination. The qualified HI antibody levels in chickens with the H9-CVCVA5 vaccine were one month period longer than those of the commercial H9 vaccine in the absence of

adjuvants. The results were consistent with previously report that the *Leishmania infantum* promastigotes vaccine antigen combination with MDP as adjuvant which conferred 100% protection and stimulated a long lasting immunity to dogs experimentally infected with *L. infantum* (Lemesre et al., 2007). A boost immunization would be needed to maintain high levels of HI antibody titers beyond the period of over 30 weeks.

An ideal vaccine needs to elicit both potent humoral immune response and robust cellular immune response against infectious pathogens. However, the current killed virus vaccines are poor in stimulating cellular immunity. The seasonal trivalent inactivated influenza vaccine administered together with poly (I):poly (C12U) as an adjuvant via intranasal route afforded cross-protection against H5N1 influenza virus (Ichinohe et al., 2007). In this study, the clinical signs, body weight and virus shedding in birds of the H9 commercial vaccine only group were more severe than those birds in the H9-CVCVA5 group after the H9 subtype heterologous virus challenge. The poly (I:C) was reported to contribute to diminishing virus shedding after homologous virus challenge (Ichinohe et al., 2005). Co-administration of poly (I:C) and CL097 (TLR7/8 ligands) with killed porcine reproductive and respiratory syndrome virus vaccine conferred protection against challenge (Zhang et al., 2013).

In order to determine the effect of CVCVA5 adjuvanted inactivated vaccines on cell-mediated immune responses, the kinetic changes in the CD3⁺/CD4⁺ and CD3⁺/CD8⁺ lymphocyte subpopulations were monitored in chickens after vaccination with H9-CVCVA5 or only H9 vaccine alone (Fig. 4). The percentages of both CD3⁺/CD4⁺ and CD3⁺/CD8⁺ lymphocyte subpopulations in chickens of the H9-CVCVA5 group were increased up to 7 days test post vaccination, which was higher than those in chickens with H9 vaccine alone and control. This study suggests that the CVCVA5 adjuvant in the inactivated H9 AI vaccine plays an important role in inducing cell-mediated immune responses. The inflammation of the mineral oil vaccine immunization may lead to the temporal increment in T lymphocyte populations in chickens with H9 AI vaccine formulated in mineral oil. Ingredients of the compound immunopotentiator CVCVA5, such as poly I:C, resiquimod, imiquimod is capable of boosting innate and adaptive immune response (Hart et al., 2005; Ichinohe, 2010).

The lymphocytes adoptive transfer assay was conducted to further investigate in vivo protective roles of different phenotypic immune cells that were induced by vaccination with CVCVA5 adjuvanted AI vaccine. Transfer of total T lymphocytes was slightly superior to that of CD8⁺ T lymphocyte subpopulation in conferring protection in recipient chickens. Also, protective efficacy of CD8⁺ T lymphocyte subpopulation slightly surpassed that of B lymphocytes or CD4⁺ T lymphocytes as shown by adoptive transfer experiments. T lymphocytes from H9-CVCVA5 immunized chickens were more effective in improving the overall health status of recipient chickens after challenge infection than those from H9 vaccine alone or control chickens. The results suggest that CVCVA5 adjuvant present in H9 vaccines induced CD8 T cell immune responses contributing to protection. No significant contribution to protection from B lymphocyte adoptive transfer might be due to the lack of antibody secreting plasma cells that are likely to be present in bone marrows. Consistent with results in this study, combination using MDP and TLR3, or TLR4 or TLR9 agonists

was shown to synergistically induce T helper type 1 lineage immune responses (Tada et al., 2005). A modified levamisole in combination use with inactivated Newcastle disease virus antigen was also reported to improve both humoral and cellular mediated response in chickens (Yin et al., 2006). The overall efficacy of each individual components of the CVCVA5 on commercial vaccine in chickens is lower than that of the compound form of CVCVA5 (data not shown).

In this study, we demonstrated that H5 and/or H9 subtype inactivated viral vaccines adjuvanted with CVCVA5 significantly enhanced humoral and cell-mediated immune responses, and prolonged immune persistence periods, slightly broadened protection against heterologous virus challenge in chickens. CVCVA5 adjuvanted vaccination can induce CD8 T lymphocytes contributing to protection. CVCVA5 can be developed as an effective adjuvant in improving the efficacy of existing vaccines in chickens.

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Fig. 1. HI antibody titer against H5 (Re5) and H9 (NJ02/01) AI antigen post vaccination
H5-CVCVA5, H5 commercial vaccine (H5-Re5) mixed with CVCVA5 adjuvants. H5, H5 commercial vaccine (Re5). H9-CVCVA5, H9 commercial vaccine (NJ02/01) mixed with CVCVA5 adjuvants. H9, H9 commercial vaccine. C, control group. wpv, Week post vaccination. The asterisk indicates a significant difference between groups, *** $P < 0.01$.

Fig. 2.

Immune persistence period. The horizontal dash line is the qualified antibody titer according to the requirements of commercial vaccine. H9-CVCVA5, H9 commercial vaccine (NJ02/01) mixed with CVCVA5 adjuvants. H9, H9 commercial vaccine. C, control group. wpv, Week post vaccination.

Fig. 3.

Antibody titer of the adjuvant application in field. A, HI antibody titer to H5 viral antigen. B, HI antibody titer to H9 viral antigen. H5 + H9-CVCVA5, bivalent commercial vaccine (H5-Re5 + H9-Re-2) mixed with CVCVA5 adjuvants. H5 + H9, bivalent commercial vaccine (H5-Re5 + H9-Re-2). The asterisk indicates a significant difference between groups, *** $P < 0.01$.

Fig. 4.

The lymphocyte subset ratio of CD3⁺CD4⁺ and CD3⁺CD8⁺. The peripheral blood lymphocytes (6×10^7 cells) collected from the SPF chickens immunized with H9-CVCVA5 (H9 commercial vaccine mixed with CVCVA5), H9 vaccine only or control, were analyzed by fluorescent-activated cell sorting (FACS) with FACS Calibur fluorospectrometer. Cells were triple-stained with mouse anti-chicken CD3-R-PE, mouse anti-chicken CD4-FITC and mouse anti-chicken CD8 α -chain-PE/CY5. FACS controls (1×10^6 cells) included unstained cells and cells only stained with anti-CD3-R-PE or anti-CD4-FITC, anti-CD8 α -chain-PE/CY5 or appropriate isotype controls.

Fig. 5.

Body weight of lymphocyte transferred recipient chickens after challenge with heterogeneous virus. The inbred recipient chickens were transferred lymphocyte with amount of 4×10^7 cells in a volume of 0.5 ml from donor chickens which vaccinated with H9-CVCVA5 or H9 subtype vaccine or control, respectively. All recipients were then challenged with 10×10^7 EID₅₀ of H9 subtype AIV SDYH01/11 strain. A, all recipients were transferred total T lymphocyte. B, total B lymphocyte. C, total CD3⁺CD4⁺ lymphocyte. D, total CD3⁺CD8⁺ lymphocyte. ■, Recipients were transferred lymphocyte derived from donors with vaccination of H9-CVCVA5 vaccine. ▲, From H9 vaccine donors. ●, From control donors.

Table 1

Cross reactive HI antibody titer of NJ02/01 and SDYH01/11.

Antigen	Serum of HI titer ($n\log_2$)	
	NJ02/01	SDYH01/11
NJ02/01	12	7
SDYH01/11	9	11

Table 2

Virus isolation post-challenge.

Vaccine	Number of chickens shedding H9 virus/total number			Number of sick chickens post challenge/total number ^a
	3 dpc	5 dpc	7 dpc	
H9-CVCVA5	1/10	0/10	0/10	1/10
H9	3/10	4/10	2/10	4/10
Control	8/10	10/10	10/10	10/10

Three groups of 10 specific pathogen free 14-day-old white Leghorn chickens (*G. gallus domesticus*) received one injection (0.3 ml) of each vaccine accordingly via subcutaneous route.

^aClinical signs were recorded during a 14-day period of observation post challenge which included one or more of the following symptoms, depression, anorexia, comb cyanosis, disequilibrium and death. H9-CVCVA5, H9 commercial vaccine (NJ02/01) mixed with CVCVA5 adjuvants. H9, H9 commercial vaccine. C, control group. dpc, Day post challenge.

Table 3

Morbidity, mortality and virus shedding of inbred chicken post-challenge.

Ratio	Recipients	Transferred lymphocyte			
		T total	B total	CD4 ⁺	CD8 ⁺
Sick no./total	H9-CVCVA5	2/5	5/5	5/5	3/5
	H9	5/5	5/5	5/5	5/5
	Control	5/5	5/5	5/5	5/5
Shedding no./total	H9-CVCVA5	2/5	5/5	5/5	3/5
	H9	5/5	5/5	5/5	5/5
	Control	5/5	5/5	5/5	5/5
Death no./total	H9-CVCVA5	0/5	1/5	1/5	0/5
	H9	1/5	1/5	1/5	1/5
	Control	1/5	1/5	1/5	2/5

The inbred recipients chickens were transferred lymphocyte with amount of 4×10^7 cells in a volume of 0.5 ml from donor chickens which were vaccinated with H9-CVCVA5, H9 subtype vaccine, or control, respectively. All recipients were then challenged with 10×10^7 EID₅₀ of H9 subtype AIV SDYH01/11 strain. Sick no./total, sick number/total number. Shedding no./total, shedding number/total number. Death no./total, death number/total number. H9-CVCVA5, H9 commercial vaccine (NI02/01) mixed with CVCVA5 adjuvants. H9, H9 commercial vaccine. T total, total T lymphocyte. B total, total B lymphocyte.