INTRODUCTION

Avian influenza (AI), caused by avian influenza virus (AIV), has entered the public spotlight as a significant infectious disease of poultry and is a serious threat in animal husbandry and public health. Avian influenza can cause high morbidity and mortality in both poultry and waterfowl (Stallknecht and Shane, 1988; Alexander, 2000; Chen et al., 2005). In recent years, most studies about AIV focused on highly pathogenic avian influenza. However, some subtypes of low-pathogenic avian influenza, such as H9, can also cause high morbidity and mortality in poultry, although the threat to waterfowl is much lower. Furthermore, AIV can be shed in the stool after replicating in the intestinal tract, resulting in a serious threat to the nearby environment and other birds and mammals, particularly sensitive poultry, such as chicken. Therefore, both low-pathogenic avian influenza and waterfowl have become important factors in AI prevention.

Intramuscular immunization with the influenza vaccine (oil emulsion) is the major means of prevention of AI in the world. However, stress caused by injection could potentially slow down the growth of the animals, and the deposit of a stable emulsion could reduce the quality of the meat (Xiaowen et al., 2009). Special features of the waterfowl's body, such as the thick and slippery feathers, can present difficulties during intramuscular injection. The nasal cavity of the respiratory tract is the primary entry site of various pathogenic microorganisms, and this route of AIV infection could be discontinued if intranasal immunity is established. Previous reports have demonstrated that intranasal inoculation of vaccines against respiratory diseases has the potential to elicit protective immune responses and to effectively prevent respiratory infection (Zuercher, 2003; Chiou et al., 2009; Baaten et al., 2010). However, the induction of an immune response seemed to be enhanced if the antigens were administered together with an appropriate immunostimulant compared with the antigens alone (Scheerlinck et al., 2006; Ichinohe et al., 2009; Rauw et al., 2010). Administration of immunostimulants, such as CpG oligodeoxynucleotides (CpG-ODN), liposomes, or cholera toxin, intranasally with inactivated influenza virus has been shown to in-

ABSTRACT

To evaluate the effects of co-administration of inactivated avian influenza H9N2 virus and adjuvants in waterfowls, 10-d-old ducks were immunized intranasally with inactivated avian influenza virus (IAIV) combined with CpG DNA and sodium cholate. Immunoglobulin A and IgG antibody levels in throat and tracheal tissues increased significantly, as did specific IgA and IgG antibody levels in the serum after intranasal immunization with IAIV combined with CpG DNA and sodium cholate, compared with immunization with IAIV only. Furthermore, enhanced hemagglutination inhibition titers were also detected in serum samples taken between the third and seventh weeks after immunization with IAIV and both adjuvants compared with IAIV alone. The expression of IL-2 and IL-6 in tracheal and lung tissues increased significantly in the early period after booster immunization. However, the enhancement induced by a single adjuvant was insignificant, and no significant change was detected in the antibody titers or cytokine levels between the ducks that received IAIV alone or saline. In the viral challenge study, prior administration of both CpG DNA and sodium cholate with IAIV reduced the viral titers in the oropharynx and cloaca swabs. Our study suggests that the combination of CpG DNA and sodium cholate could be beneficial to immunization with inactivated H9N2 virus by enhancing the local and systemic immune responses.

Key words: inactivated avian influenza virus, CpG DNA, sodium cholate, intranasal immunization, respiratory tract

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duce effective immune responses (Tamura et al., 1988; Prabakaran et al., 2008; Chiou et al., 2009; Xiaowen et al., 2009). Therefore, inactivated avian influenza virus (IAIV), together with CpG DNA and sodium cholate, was used to immunize ducks through the nasal cavity. Following immunization, systemic and local immune responses in the respiratory tract were followed. The aim of this study was to evaluate a convenient and practical method to prevent AIV in ducks.

**MATERIALS AND METHODS**

**Virus and Adjuvants**

Influenza A/Duck/NanJing/01/1999 (H9N2) virus, isolated from healthy ducks, was supplied by Jiangsu Academy of Agricultural Sciences (Nanjing, China). After inoculation of 10-d-old chick embryos with the live virus, the allantoic fluid was concentrated 10-fold (10^9 ELD_{50}/0.1 mL, where ELD_{50} is 50% of the embryo lethal dose) and then inactivated by formalin (final concentration 0.025%) during a 96-h incubation at 37°C. The hemagglutination assay (HA) titer of the viral concentration 0.025%) during a 96-h incubation at 37°C. The hemagglutination assay (HA) titer of the virus was 1:1024 before inactivation and the measurement of ELD_{50} was performed on 10-d-old chick embryos. The CpG DNA used in our study was bacterial genomic DNA extracted from the *Escherichia coli* poephagus grunniens strain, provided by the laboratory of Animal Disease Diagnostic and Immunology, Ministry of Agriculture, Nanjing Agricultural University (China). The CpG DNA was dissolved in TE buffer, and the concentration was estimated by measurement of the absorbance ratio A260/A280 nm (A260/A280 = 1.99) and by agarose gel electrophoresis after extraction, as described (Lin-lin, 2007). The IAIV was mixed with CpG DNA or sodium cholate for 2 h before immunization. The vaccine was composed of 2 × 10^9 ELD_{50} IAIV, 50 μg of CpG DNA, and 2 mg of sodium cholate per duck.

**Immunization Schedule**

In total, 150 one-day-old Shaoxing laying ducks were obtained from Ningbo Poultry Farm (Ningbo, China) and reared in isolation. Feed and water were provided ad libitum. Ducks were randomly divided into 5 groups and immunized at 10 d of age. Nonimmunized control ducks received intranasal administration of 0.2 mL of physiological saline. Groups of ducks were immunized intranasally with one of the following: IAIV only, IAIV mixed with CpG DNA or sodium cholate, or IAIV mixed with both adjuvants. All ducks were immunized again 7 d after the primary immunization. The vaccine was dropped into the ducks’ nostrils with a dropping bottle while the rostrum was gently kneaded.

**Collection of Samples**

Blood samples were taken weekly for 8 wk from 6 ducks in each group following the first immunization. Serum was collected after a 3,000 × g centrifugation for 10 min at 20°C and stored at −30°C. The ducks were anesthetized and killed at wk 3, 5, and 7 after the first immunization and 28 h after the booster immunization. Tissue samples were taken from the throat, trachea, and lung and stored at −30°C after freezing in liquid nitrogen for 2 h.

**ELISA for IgA and IgG**

Throat, trachea, and lung samples were triturated in a mortar with liquid nitrogen, and the homogenate was weighed and dissolved in PBS (0.01 M, pH 7.4) at a dilution of 1 mg per 8 μL. The supernatant of the homogenate was then collected after a 5,000 × g centrifugation for 10 min at 4°C and stored at −30°C. The wells of ELISA plates were coated with 0.1 mL of IAIV (10^8 ELD_{50}), 1:800 diluted in carbonate buffer, pH 9.6, and incubated overnight at 4°C. Following the virus being removed, the plates were incubated with blocking buffer (0.6% BSA in PBS) for 2 h at 37°C and then incubated with 0.1 mL of supernatant of the samples for 1 h at 37°C. After washing with PBST (0.01 M, pH 7.4), 0.1 mL of rabbit anti-duck IgA or IgG hyperimmune serum (BioSun Sci & Tech Co. Ltd., Shanghai, China) was added in 1:400 dilutions and incubated for 1 h at 37°C. Then, the plates were washed and incubated with HRP-linked goat anti-rabbit IgG (1:20,000 diluted in PBS; Boster Biological Technology Ltd., Wuhan, China) which acted as second antibody for 1 h at 37°C. After washing, 0.1 mL of o-phenylenediamine (Solarbio Science & Technology Co. Ltd., Shanghai, China) was added, incubated 10 min at 20°C and stored at −30°C. The ducks were anesthetized and killed at wk 3, 5, and 7 after the first immunization and 28 h after the booster immunization. Tissue samples were taken from the throat, trachea, and lung and stored at −30°C after freezing in liquid nitrogen for 2 h.

**Radioimmunoassay for Cytokines**

Tracheal and lung samples were collected 28 h after booster immunization, homogenized in a mortar with liquid nitrogen, and dissolved in NP-40 lysis buffer (Beyotime Biotechnology Institute, Nantong, Jiangsu, China). The expression of *IL-2* and *IL-6* in the homogenate was detected by radioimmunoassay (Huaying Biotecbiology Institute, Beijing, China). Briefly, 100 μL of sample supernatant was mixed with 100 μL of anti-human *IL-2* or *IL-6* monoclonal antibody and 100 μL of 125I-labeled *IL-2* or *IL-6* in a glass tube. The mixture was vortexed and incubated for 24 h at 4°C. Separation buffer (500 μL) was then added to the mixture, vortexed, and then incubated at room temperature for 15 min. The supernatant was aspirated immediately after a 3,000 × g centrifugation for 20 min at 4°C, and the counts per minute of the pellet was measured using a gamma counter.
Hemagglutination Inhibition Assay

The neutralization activity of serum antibody was measured by hemagglutination inhibition (HI) assays. Serum samples were treated overnight with receptor-destroying enzyme at 37°C to eliminate nonspecific HI factors, the assays were performed as described by WHO (WHO, 2002). In brief, 50 μL of PBS was added to each well of 96-well plates, and 50 μL of serum was transferred in and serially diluted 2-fold in PBS. Then, 4 hemagglutination units of A/Duck/NanJing/01/1999 was added and the mixture was incubated for 30 min at room temperature. Plates were incubated for 30 min at room temperature after the addition of 50 μL of 1% chicken erythrocytes. The HI endpoint titer was determined as the reciprocal of the highest serum dilution that inhibited hemagglutination completely.

Virus Challenge

A total of 45 one-day-old ducks was randomly divided into 3 groups. The ducks were immunized as described above and challenged intranasally with live H9N2 avian influenza virus (A/Duck/NanJing/01/1999, 5 × 10^6 ELD50) 21 d after the final immunization. This virus strain was isolated from a healthy duck and had low pathogenicity; no significant weight reduction and 0% mortality were observed in a previous challenge study. Thus, oropharyngeal and cloacal swabs were collected at 3, 6, and 9 d postchallenge. Clinical symptoms were also monitored for 14 d postchallenge.

Virus Isolation

The swab samples collected after the virus challenge were inoculated into 10-d-old specific-pathogen-free chicken embryos (3 embryos per sample; Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, China). Embryos that died within 24 h postinoculation were removed from the analysis to eliminate the effects of nonviral factors on the data. Allantoic fluids were collected from the embryos that died between 24 and 120 h postinoculation and from the embryos that were still alive after 120 h. The virus titers in the allantoic fluids were measured by HA assay. The allantoic fluids that were negative in the HA assay were given blind passages (3 embryos per mixed sample), with a maximum of 2 passages. If the final HA titer was greater than or equal to 4 (reciprocal log2 transformed), we considered the duck to be positive for virus isolation.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL). The significance of the data was determined by one-way ANOVA. P-values less than 0.05 were considered statistically significant.
RESULTS

IgA and IgG Levels in Respiratory Tract

Specific IgA and IgG levels were determined by ELISA (Figure 1). The levels of mucosal antibody were significantly enhanced after intranasal immunization with CpG DNA and sodium cholate ($P < 0.01$) at almost all sampling points, compared with ducks immunized with a single adjuvant or IAIV only. The addition of either CpG DNA or sodium cholate induced higher levels of mucosal antibodies at the third or fifth week after the first immunization, but the antibody levels were much lower than with both adjuvants together. The CpG DNA had a stronger effect on the levels of IgA than on those of IgG. However, no significant changes were detected at most sampling points between groups that received either CpG DNA or sodium cholate. No significant differences were found in ducks immunized with IAIV alone, compared with ducks in the control group.

Specific IgA and IgG Levels in the Serum

The specific IgA and IgG antibody levels in the serum were detected weekly between wk 2 and 7 after the first immunization (Figure 2). No changes were observed at wk 2, but from wk 3 to 7, both IgA and IgG antibody levels increased significantly ($P < 0.01$) in ducks immunized with both adjuvants. The serum antibody level was only marginally increased ($P < 0.01$ or $P < 0.05$) after immunization with a single adjuvant after 4 to 6 wk compared with animals without the adjuvant.

High levels of maternal antibody, which has been documented to interfere with a live virus vaccine, were detected in the control group at wk 2 (Siegrist, 2003). The neutralization of the virus by maternal antibodies would result in diminished amounts of vaccine antigen, potentially leading to a failure to elicit an effective immune response (Sakaguchi et al., 1998). However, the administration of the vaccine directly to the mucosa was unaffected by maternal antibodies in the serum. No significant difference was found between ducks immunized with IAIV and those immunized with physiological saline.

IL-2 and IL-6 Expression in the Trachea and Lung

As members of the interleukin family, IL-2 and IL-6 are capable of promoting the proliferation of T lymphocytes and B lymphocytes, respectively. In addition, as an important cytokine in the mucosa, IL-6 is correlated with IgA antibody responses in the respiratory tract. As shown in Figure 3, the expression of IL-2 and IL-6 in both the trachea and lung increased significantly at 28 h after intranasal immunization with IAIV combined with CpG DNA and sodium cholate ($P < 0.01$, with 2- to 5-fold). These data indicate that the IAIV was sufficiently immunogenic to induce a local immune response in the respiratory tract. The expression of IL-2 in the lung also increased significantly after immunization with CpG DNA, compared with the group without adjuvant ($P < 0.05$).
HI Titer in the Serum

The antibody titer in the serum was detected by the HI assay (Figure 4). The serum titers from the group that received IAIV combined with both adjuvants increased by wk 3 and peaked at wk 4. Both groups without adjuvants showed low antibody titers, and the addition of a single adjuvant led to a significantly higher titer. No significant changes were observed between single adjuvant groups in either serum IgA or IgG levels. The highest titer (mean titer, 7.6) was detected at wk 4 in the group immunized with IAIV and both adjuvants and it persisted for over 7 wk after the primary immunization.

Virus Titer After Challenge

The number of ducks that tested positive for the isolated virus is shown in Table 1. The peak of virus shedding was approximately 3 d postchallenge, and no live virus was isolated from the group immunized with IAIV combined with both adjuvants. In addition, the total number of positive ducks by cloacal swab was greater than by oropharyngeal swab, indicating that the virus was preferentially excreted through the cloacal.

DISCUSSION

The nasal cavity is one of the primary entry sites of pathogenic microorganisms. The nasal mucosa is an attractive site for the induction of mucosal immunity because of the low threshold for initiation of an immune response and the mild physiological environment. It has been reported that intranasal administration of attenuated virus was capable of preventing infectious diseases in poultry, such as Newcastle Disease in chickens (Tseng et al., 2009; Rauw et al., 2010). However, mucosal immunity induced by inactivated virus is customarily ineffective, as the inactivated virus cannot replicate in the epithelium of the respiratory tract to induce effective immune responses. In recent years, the application of mucosal immunopotentiators had brightened prospects for the induction of mucosal immunity. Several immunopotentiators, such as interferons and cholera toxin, could enhance the immune response effectively by mucosal immunization with inactivated AIV (Tamura et al., 1988; Couch et al., 2009; Yang et al., 2010). Our previous research indicated that the local and systemic immune responses in chickens were
significantly enhanced when CpG DNA was added to inactivated avian influenza virus (H5N1) and administered intranasally (Xiaowen et al., 2009). Many other studies have demonstrated that inactivated virus can be used to induce intranasal immunity and that the effects can be significantly enhanced when combined with appropriate adjuvant.

The CpG-ODN have been used as a potent adjuvant for intramuscular immunization (Chu et al., 1997). Most vaccination studies were performed using CpG-ODN (Heike Weighardt, 2000; Klimman, 2004; Kwant and Rosenthal, 2004), but the high cost of CpG-ODN has hindered its widespread use, particularly in the animal industry. A less expensive alternative to CpG-ODN, CpG DNA has adjuvant activity similar to CpG-ODN. It can enhance an immunological response in the mucosa by activating the NF-κB pathway, stimulating the leukocytes, increasing the number of dendritic cells, promoting the expression of major histocompatibility complex class II molecules, and enhancing the specific local immune response (Qian, 2009). Research has shown that CpG-ODN with different motifs can have unique effects on immune responses in different animal species. The effects of CpG-ODN on immune-related gene expression and serum antibody titer in White Leghorn chickens following subcutaneous immunization with formalin-inactivated avian influenza H9N2 virus were inferior to water-in-oil or hydroxide [Al(OH)₃] adjuvants (Reemers et al., 2010). Our observations suggested that the tight pseudostratified columnar ciliated epithelium and the thick mucus layer in the nasal cavity may restrict the absorption of antigen and CpG DNA from the mucosal surface and inhibit antigen recognition and presentation by antigen presenting cells. This poor performance of CpG DNA as an adjuvant may be due to a low sensitivity in ducks, which could be overcome with more antigen and adjuvant. As an absorbefacient, sodium cholate is able to disrupt the tight junction between epithelial cells, enhance the permeability of the plasmalemma, and increase the absorption of proteins and polypeptides (Sawicki, 2001). In this study, the ducks that were immunized intranasally with IAIV combined with sodium cholate alone showed no specific immune response even though the antigen absorption may have been enhanced, indicating that both immunopotentiators and absorbefacients were necessary for induction of mucosal immunity.

The IgA- and IgG-secreting cells are widely dispersed in the respiratory tract of poultry. And sIgA is a combination of IgA produced by B lymphocytes and secretory components synthesized by epithelial cells (Macpherson et al., 2008). Regarded as the major component of the first immunological barrier encountered by a microorganism that infects through the mucosal surface, sIgA is capable of reducing viral adhesion and capturing virus that has invaded in the mucosa (Macpherson et al., 2008). In our study, the IgA antibody levels in the respiratory tract tissues were elevated after intranasal immunization with CpG DNA and sodium cholate. This may have increased the sIgA level in the airway, which is important to inhibit the incursion of the virus. In addition, levels of AIV-specific antibodies and functional antibodies in the serum increased significantly and persisted for over 7 wk, indicating that intranasal immunity was an efficient countermeasure against AIV.

Cytokine production by lymphocytes after antigen stimulation can regulate the immune response, and the cytokines IL-2, IL-6, and IL-10 play important roles in local immune responses in the mucosa (Ilona Kryczek, 2007; Rauw et al., 2010). The IL-2 cytokine can be used directly as an adjuvant (Min et al., 2001; Sabbatini et al., 2010), whereas IL-6 is capable of inducing the proliferation of B cells and promoting the secretion of IgA (Ramsay et al., 1994). The results of our study show that the secretion of IL-2 and IL-6 was enhanced after booster immunization, indicating that cellular immunity was induced and may play a role in preventing AIV infection. This was not only beneficial to ducks used for meat but also for laying ducks. In the case of laying ducks, which have a rearing period that may last 2 yr or even more, the levels of AIV-specific antibody may be below a critical threshold required for protection. In case of an AIV outbreak, an additional immunization through the nasal route is more effective than an intramuscular immunization, as the latter required a longer period of time to develop an immune response.

The low pathogenic avian influenza virus, H9N2 (A/Duck/Nanjing/01/1999), used in our study is nonlethal to ducks in nature (Liu et al., 2005). However, a series of clinical symptoms were observed after virus challenge in groups that were previously immunized without adjuvant, such as head droop, neck tremor, severe weakness, loss of appetite, and listlessness. In contrast, among ducks immunized with IAIV combined with CpG DNA and sodium cholate, only 2 exhibited a loss of appetite. In addition, the results of the virus isolation indicated that the combination of IAIV and both adjuvants helped to inhibit viral replication after the challenge. This inhibitory effect has important significance in AIV prevention because it not only protects the immunized ducks against infection but also reduces the threat to other animals.

Conclusions

Inactivated low-pathogenic AIV was capable of inducing effective immune responses through the intranasal route in duck. Intranasal immunization of IAIV combined with CpG DNA and sodium cholate significantly increased the mucosal and humoral immune responses and reduced virus shedding, which are beneficial to preventing AI in waterfowl.

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