Adjuvant Effect of Ginsenosides on the Recombinant SO7 Antigen Against *Eimeria tenella* in Chickens

Jinjun Xu, Jinbiao Peng and Jianping Tao
Key Lab for Avian Preventive Medicine, Ministry of Education, Yangzhou University,
Yangzhou, 225009 Jiangsu Province, P.R. China

Abstract: An experiment was conducted to study the adjuvant effect of ginsenosides on the recombinant refractile body antigen SO7 in coccidian-infected AA broilers. Five, days old chickens were subcutaneously immunized with SO7 antigen (100 µg per chick) mixed with various doses of ginsenosides or without adjuvant. A boost of the same dose was given at 12 and 19 days of age. The specific antibody response and lymphocyte proliferation response to SO7 antigen were measured periodically postimmunization. Chickens in all groups were orally challenged with 1×10^5 homologous sporulated oocysts of *E. tenella* at 26 days of age and killed at eight days postchallenge. Results showed that when immunized by antigen adjuvanted with ginsenosides, lymphocyte proliferation was significantly enhanced at 19, 26 days of age and antibody response was obviously observed at 12, 19 and 26 days of age. All groups administered with antigen mixed in adjuvant showed higher protective immunity compared to PBS or nonadjuvanted antigen control in terms of lower mortality, oocyst output, lesion score and higher weight gain. A linear dose relationship was observed in terms of lesion score among three groups immunized with various doses of ginsenosides (p<0.05) in which the group given adjuvanted antigen with 1.0 mg ginsenosides showed significantly stronger adjuvant effects. These results suggest that ginsenosides has a strong adjuvant effect at a appropriate dose and may potentially serve as a vaccine adjuvant against avian coccidiosis.

Key words: *Eimeria tenella*, SO7 antigen, ginsenosides, adjuvant, dose

INTRODUCTION

Avian coccidiosis is one of the most economically diseases in the poultry industry. It is caused by 7 species of Eimeria which develop within intestinal epithelial cells and produce varying degrees of morbidity and mortality (McDonald and Shirley, 2009). Currently, prophylactic feeding of coccidiostat drugs is the major control method. However, drug resistance is a serious problem and along with public demand for chemical-free meat, these issues have led to the requirement for an effective vaccine strategy (Sharman *et al.*, 2010). Live vaccines containing low doses of virulent or attenuated oocysts have been developed and used worldwide (Williams and Catchpole, 2000) but live vaccines also have disadvantages: production of live vaccines is relatively expensive; attenuated vaccines have the potential to revert to the pathogenic wild type; low dose of virulent oocysts also have the risk of causing disease. Therefore, with the progress of molecular biology, non-infectious vaccines such as recombinant subunit vaccines and/or DNA vaccines may be good alternatives (Dalloul and Lillehoj, 2005).

Refractile body antigen SO7 is located in the sporozoites of *E. tenella* (Miller *et al.*, 1989) which has been found to be highly immunogenic with both B and T cell epitopes. It is a good candidate antigen for a subunit vaccine, an has also been successfully used to construct live vector vaccines (Pogonka *et al.*, 2003; Konjufca *et al.*, 2008; Yang *et al.*, 2010). A single dose of the recombinant fusion protein CheY-SO7' not only partially protected against severe coccidiosis induced by *E. tenella* but also protected chickens challenged with the heterologous species *E. acervulina*, *E. maxima* and *E. necatrix* however, this therapy was still not sufficient to compete with prophylactic chemotherapy (Crane *et al.*, 1991). In addition, when SO7 protein was mixed in Freunds Incomplete Adjuvant an almost nonprotective antibody response was observed (Kopko *et al.*, 2000). Difference in antigen delivery, especially in the adjuvant that was used may explain the differences between these two experiments that have used the same recombinant antigen (Kopko *et al.*, 2000). Therefore, it is important to select appropriate adjuvants to maximize the efficacy of coccidial subunit vaccines.

Corresponding Author: Jianping Tao, Veterinary College of Yangzhou University, No. 12 Wenhui Road, Yangzhou,
225009 Jiangsu Province, P.R. China

In the poultry industry, mineral oil or aluminum based adjuvants are widely uesd (Newman and Powell, 1995). Side effects especially local reactions such as swellings, indurations, erythemas and cutaneous nodules of these adjuvants may persist for up to 8 weeks or sometimes longer (Leenaars *et al.*, 1998). Moreover, government clearance for oil-based adjuvants used in food animals is absent, so other novel adjuvants such as cytokines and traditional. Chinese medicine with greater safety and efficacy are needed.

In an earlier study, researchers have expressed SO7 protein in *E. coli* and its immune efficacy adjuvanted with recombinant Chicken Interferon-gamma (ChIFN-gamma) was evaluated (Xu *et al.*, 2007, 2012). In the present study, chickens were subcutaneously injected with the protein mixed with various doses of ginsenosides, the immune response was evaluated by measuring specefic antibody response and lymphocyte proliferation, the immune efficacy was further evaluated by measuring survival rate, Body Weight Gain (BWG), lesion score and oocyst output after a challenge with *E. tenella* oocyts. The data lay a foundation for the improvement of subunit vaccine against coccidiosis in chickens.

MATERIALS AND METHODS

Animals and parasites: The 1 day old, newly hatched AA broilers were obtained from Jiangsu Institute of Poultry Science (Yangzhou, Jiangsu, China). Chickens were raised in a coccidian-free environment in wire cages and feed and water were supplied *ad libitum*. Animal breeding, care and all experiments were performed according to the rules of the Animal Experiment Ethic Committee of Yangzhou University. *E. tenella* Yangzhou strain with high virulence was originally isolated from Yangzhou, Jiangsu Province. Parasites were maintained and propagated in chickens free of infection with *Eimeria* sp.

Ginsenosides: Ginsenosides (Nanjing Qingze Herbal Technology Development Co., Ltd.) which are the active components in the root of *Panax ginseng* C.A. Meyer were extracted under ethanol reflux. After removing the ethanol, the extract was then dissolved in water and washed with ether and then extracted with n-butanol saturated with water. The contents of ginsenosides in the extract were >95%.

Recombinant SO7 protein: Recombinant SO7 protein was produced and purified as deacribed by Xu *et al.* (2007). Briefly, the cDNA of the *E. tenella* gene SO7 of Yangzhou strain were obtained by Reverse Transcriptional Polymerase Chain Reaction (RT-PCR) of total RNA of sporocysts. PCR products were cloned into the plasmid vector pGEM-T-easy (Promega) and then subcloned into the expression vector pGEX-6P-1 (Pharmacia). After induced by isopropyl-β-D-thiogalactopyranoside (IPTG, AMRESCO), reconbinant SO7 protein was expressed in *E. coli*. The solubilized GST-SO7 protein was purified by affinity chromatoprapy using a Glutathione Sepharose 4B column and 10 mM glutathione elution buffer according to the manufacturer's instructions (Amersham Biosciences), followed by cleavage with PreScission™ protease (Amersham Biosciences) by a bath method. The protein concentration was spectrophotometrically estimated at A_{280nm} and A_{260nm} then the purified protein was stored in aliquots at -20°C until further use.

Immunization and challenge: Five, days old AA broilers were divided into eight groups (n = 40, 20 for immunization and challenge, 20 for immunization and detection of lymphocyte proliferation and antibody responses) and subcutaneously (s/c) immunized with SO7 antigen (100 μg per chick) with various adjuvants or without adjuvant. Chickens in group I were each injected with 200 μL PBS; chickens in group II were given antigen without adjuvant; chickens in groups III-V were given antigen mixed with 0.25, 0.5 and 1.0 mg ginsenosides, respectively. All chickens were boosted at 12 and 19 days of age. The 7 days after the third boost immunization, all chickens were weighted and challenged orally with 100,000 *E. tenella* sporulated oocysts. The clinical signs and mortality of each group were observed and documented daily post challenge. Feces of each group were collected separately on days 5-8 post infection (dpi). Chickens in all groups were weighted and euthanized on day 8 post challenge. The experimental design and immunization protocol are outlined in Table 1.

Lymphocyte proliferation assay: Peripheral blood was collected from five chickens in each group at 5, 12, 19 and

Table 1: Experimental design and immune program

Groups	Number of Chickens	SO7 protein (mg/chick)	Adjuvant and dose per chick	Primary	Secondary	Tertiary	Days of challenge	Challenge dose for sporulated oocysts per chick
I	40	PBS	None	5	12	19	26	1×10^5
II	40	100	None	5	12	19	26	1×10^5
III	40	100	0.25 mg ginsenosides	5	12	19	26	1×10^5
IV	40	100	0.5 mg ginsenosides	5	12	19	26	1×10^5
V	40	100	1.0 mg ginsenosides	5	12	19	26	1×10^5

26 days. Lymphocytes were isolated adjusted to 1.0×10^7 cells mL^{-1} in RPMI-1640 medium (Gibco) containing 10% fetal calf serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. For lymphocyte proliferation assay, 100 uL $well^{-1}$ lymphocyte suspension was added to 96 well flat-bottomed plates (Costar, USA) and cultured at 37°C in 5% CO_2 with 20 μL SO7 protein (final concentration: 10 μg mL^{-1}) for 56 h. The 10 μL of MTT (5 mg mL^{-1}, AMRESCO) solution was added to each well and incubated for 4 h. The reaction was stopped by addition of 100 μL Dimethyl Sulfoxide (DMSO) and the optical density was measured in an enzyme-linked immunosorbent reader (Tecan, Switzerland) at 570 nm. Each sample was analyzed in triplicate.

Enzyme linked immunosorbent assay: Sera from chickens used in lymphocyte proliferation were collected and tested for the SO7-specific antibody response with Enzyme-Linked Immunosorbent Assay (ELISA). In brief, 96 well plates were coated with 1 μg $well^{-1}$ of purified recombinant SO7 protein. Plates were blocked with PBS buffer containing 10% fetal calf serum for 1 h at 37°C. The 100 μL of serum sample diluted 1:200 was added per well and incubated for 1.5 h at 37°C. After washing three times with PBS containing 0.05% Tween, 100 μL peroxidase-conjugated goat anti-chicken IgG (Sigma) diluted 1:10, 000 was added per well and incubated for 1 h at 37°C. After washing, 100 μL orthophenylene diamine (Sigma) (0.4 mg mL^{-1} in 0.05 M phosphate citrate buffer, pH 5.0) was added per well and incubated for 15 min at 37°C to detect peroxidase activity. When the reaction was stopped by the addition of 50 μL of 2 M sulfuric acid, optical density at 490 nm (OD_{490}) was measured with an automated microplate reader. All samples were analyzed in triplicate.

Evaluation of immune protection: Efficacy of immunization was evaluated on the basis of survival rate, BWG, lesion score and oocyst output. Survival rate was estimated by the number of surviving chickens divided by the number of initial chickens. BWG was determined at the beginning of the challenge and at the end of the challenge. The lesion score of caecum in each group was investigated according to Johnson and Reid (1970) by double-blind examination (minimum score 0, maximum score 4). Total oocysts in feces of each group were counted using the McMaster Method modified by Talebi and Mulcahy (1995) and each sample was counted three times. The reduction in oocyst output was calculated as follows: (The average number of oocysts from challege control group the average number of oocysts from vaccinated group/The average number of oocysts from challenge control group)×100%.

Statistical analysis: Data were analyzed by SPSS Software (SPSS 15.0 K for Windows). The ANOVA test was used to test for differences between the groups. Duncan's multiple range test was used to analyze differences between the mean values and differences were considered statistically significant at p<0.05.

RESULTS

Proliferation of blood lymphocytes: The cell-mediated proliferation patterns to SO7 protein as indicated by MTT in immunized and control chickens are described in Fig. 1. There was no significant difference among the five groups at 5 and 12 days of age (p>0.05). At 19 days of age, the cellular proliferation response to SO7 in groups immunized with antigen added with adjuvant were significantly higher than that in the PBS control group or the group immunized with antigen alone (p<0.05) however, there was no significant difference among groups immunized with antigen plus adjuvant (p>0.05). At 26 days of age, the cellular proliferation response in groups immunized with antigen plus adjuvant increased also no significant difference was observed in the immunized groups (p>0.05).

Serum antibody responses: Eimeria-specific antibody responses to SO7 antigen in immunized and control chickens are depicted in Fig. 2. There was no significant difference among the five groups at 5 days of age (p>0.05). At 12 days of age, antibody responses were significantly higher in groups immunized with antigen added with adjuvant than in PBS control group or group
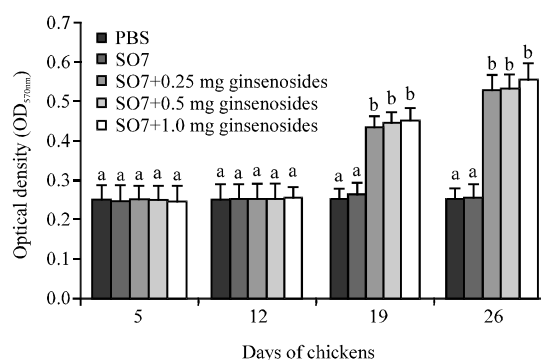

Fig. 1: Lymphocyte proliferation in response to recombinant SO7 protein immunization. Shown are mean OD_{570} of stimulated lymphocyte cultures measured by MTT (n = 5). Result was detected at 570 nm using an ELISA reader. Values with different letters differ within the same time point at p<0.05
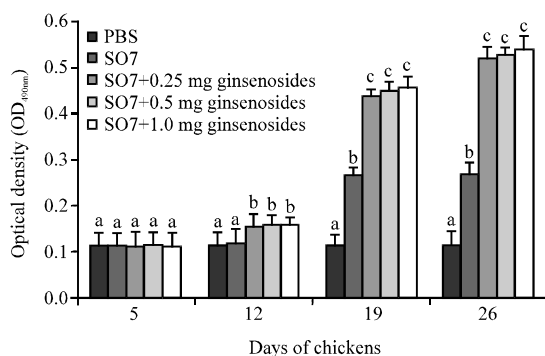
Fig. 2: Antibody response in chickens immunized with recombinant SO7 protein. Values with different letters differ within the same time point at p<0.05

immunized with antigen alone (p<0.05) however, there was no significant difference among groups immunized with antigen plus adjuvant (p>0.05). Compared with the PBS control group, significant increases in mean absorbance values of immunized chickens were noted at 19 or 21 days of age (p<0.05). Among chickens immunized with antigen with adjuvant, antibody responses in chickens immunized with antigen mixed with the highest dose of ginsenosides was higher than in the other two groups at 19 or 21 days of age but no significant difference was observed (p>0.05).

Immunizing efficacy: Chickens immunized with antigen alone had a 50% mortality which was the same as the PBS control group. In the chickens immunized with antigen mixed with various dose of ginsenosides, there was a 45, 40 and 30% mortality in the 0.25, 0.5 and 1.0 mg ginsenosides groups, respectively. Among the immunized groups, the mortality in chickens immunized with antigen mixed with the highest dose of ginsenosides (1.0 mg) was the lowest.

The immunizing efficacy of recombinant SO7 protein in terms of BWG is shown in Fig. 3. There was no significant difference in BWG among the PBS control group and the group immunized with antigen alone. However, BWG in the group with adjuvant was higher than the challenged control group or the group immunized with antigen alone (p<0.05). Although, BWG of chickens immunized with antigen mixed with the highest dose of ginsenosides was higher than in the other two groups, no significant difference was observed (p>0.05).

The immunizing efficacy of recombinant SO7 protein in terms of mean caecal lesion score is shown in Fig. 4. The lesion score in the control group and the group immunized with antigen alone was significantly higher
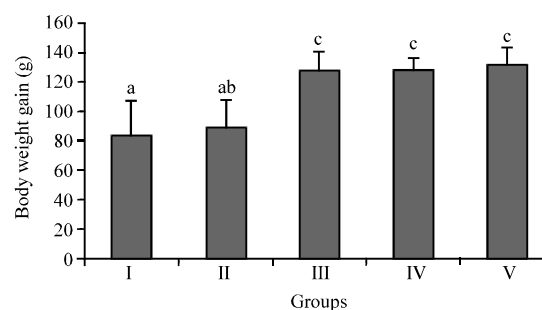

Fig. 3: Mean body weight gains of chickens immunized with recombinant SO7 protein following *Eimeria tenella* challenge infection. Values with different letters differ significantly at p<0.05
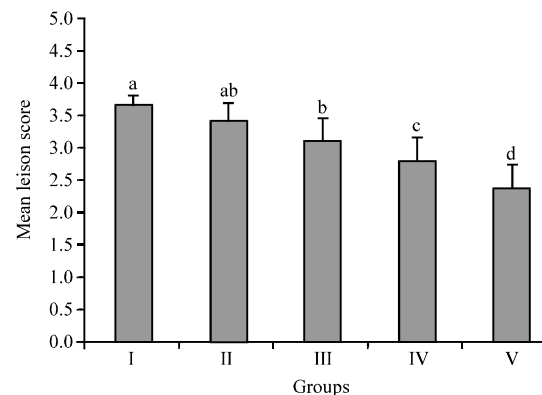

Fig. 4: Mean lesion scores of chickens immunized with recombinant SO7 protein following *Eimeria tenella* challenge infection. Values with different letters differ significantly at p<0.05

than in other immunized groups (p<0.05). Among groups immunized with antigen plus adjuvant, lesion scores of chickens immunized with antigen mixed with the highest dose of ginsenosides was significantly lower than other groups (p<0.05) and a linear dose relationship was clearly observed.

Compared with the PBS control group, immunized chickens showed varied reduction in the number of oocysts in feces after infection with *E. tenella*. There was only 1.66% reduction in the number of oocysts in the group immunized with antigen alone which was the lowest among the immunized groups. Among groups immunized with antigen plus adjuvant, the highest reduction in the number of oocysts was seen in the chickens immunized with antigen mixed with the highest dose of ginsenosides which was up to 48.80%. Other two groups had reductions of 40.11% (0.25 mg ginsenosides) and 46.95% (0.5 mg ginsenosides).

DISCUSSION

Recombinant proteins are often weakly immunogenic or non-immunogenic on their own and adjuvant is usually needed to enhance the resultant immune responses. The root of Panax ginseng C.A. Meyer (ginseng) is one of the most popular traditional Chinese medicines. As a general tonic, ginseng modulates blood pressure, metabolism and immune functions and has been offcially written in the Chinese Pharmacopoeia. Ginsenosides are the biologically ingredients in ginseng extracts and morethan 40 ginsenosides have been identied in *P. ginseng* (Leung and Wong, 2010). Recent investigations have shown that ginsenosides has adjuvant effects on the immune responses to viral, bacterial and parasitic vaccines (Hu *et al.*, 2003; Song *et al.*, 2010; Qu *et al.*, 2011). The application of ginsenosides as adjuvant of subunit vaccines against coccidiosis has also been reported with the targeted antigens include profilin and 5401 antigen of *E. tenella* and the advances are very encouraging (Du *et al.*, 2005; Zhang *et al.*, 2012).

Both cellular immunity and humoral immunity are involved in the protective immunity in chickens against Eimeria infection (Jang *et al.*, 2011). Results of this study confirmed that ginsenosides not only increasing specific antibody level but also enhancing specific cellular immune response to SO7 antigen which have been reported in orther two recombinant subunit antigens earlier (Du *et al.*, 2005; Zhang *et al.*, 2012). The survival rate, BWG, reduction in oocyst output and reduction of caecal lesion score all also showed a greater level of improvement compared to the anitgen immunization alone. Obvious linear dose relationship was only observed in terms of lesion score among three doses of ginsenosides, the increasing tendency with the higher dosage ofadjuvant is similar to those reported by others (Yang *et al.*, 2008; Qu *et al.*, 2011). The reason for such a dose-response relationship is not clear and needed to further elucidate.

Compared to the PBS control group and the group immunized with antigen alone, injecting chickens with 1.0 mg ginsenosides alone did not show any protection or specific immune response, this further confirmed adjuvant effects of ginsenosides. Gnsenosides boosting of both cellular and humoral immune responses has been reported in various studies (Du *et al.*, 2005; Yang *et al.*, 2008; Qu *et al.*, 2011; Zhang *et al.*, 2012). To date, the mechanisms that gnsenosides mediates its adjuvant effects are not fully understood and may be related to its activation of the innate immunity. The increased Th1 and Th2 immune responses might be attributed to the enhanced activity of T helper cells and NK cells responsive to given antigens (Sun *et al.*, 2008). Recent investigations have shown that TLR4 signaling pathway was involved in the adjuvant activities of gnsenosides (Su *et al.*, 2012). Although, ginsenosides appear to be suited as adjuvant for subunit vaccines, future research is necessary to identification the mechanisms.

CONCLUSION

The studies suggest that ginsenosides has strong adjuvant effects when mixed with *E. tenella* recombinant SO7 antigen and has potential as a vaccine adjuvant. Therefore, it is necessary to continue large scale laboratory tests as well as in the field to determine the appropriate dosage and procedure of this adjuvant in the prevention of avian coccidiosis.

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