

Interferon-Gamma Enhance Immune Response and Efficacy of Recombinant Subunit Vaccine 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: To effectively control coccidiosis with a subunit vaccine, the refractile body antigen SO7 adjuvanted with Chicken Interferon-gamma (ChIFN-gamma) was administered to chickens and its ability to protect against virulent challenge by *E. tenella* was evaluated. About 5 days old chickens were subcutaneously immunized with SO7 antigen (100 µg per chick) mixed with various doses of ChIFN-gamma 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 at 5, 12, 19 and 26 days of age. 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 8 days postchallenge. Results showed that when immunized by antigen adjuvanted with ChIFN-gamma, specific lymphocyte proliferation in chickens was obviously observed at 19 and 26 days of age and specific 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 control, non-adjuvanted antigen control in which the group given adjuvanted antigen with 5,000 units ChIFN-gamma showed significantly stronger adjuvant effects in terms of lower mortality, oocyst output, lesion score and higher weight gain ($p < 0.05$). Protective immunity was affected by dose of the adjuvant, no linear dose relationship was observed. These results suggest that ChIFN-gamma 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, subunit vaccine, adjuvant, interferon-gamma

INTRODUCTION

Eimeria sp. are the causative agents of coccidiosis, a major disease affecting many intensively-reared livestock, especially poultry. The chicken is host to 7 species of *Eimeria* that develop within intestinal epithelial cells and produce varying degrees of morbidity and mortality (McDonald and Shirley, 2009). Control of coccidiosis in the poultry industry is dominated by prophylactic chemotherapy. 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; Lillehoj and Choi, 1998) 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).

The SO7 protein which was described to be located in the refractile body of *E. tenella* sporozoites (Miller *et al.*, 1989) has been found to be highly immunogenic with both B and T-cell epitopes. SO7 is a good candidate antigen for a subunit vaccine (Tennyson, 1994), an it 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 Freund's 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.

Veterinary adjuvants which are mostly mineral oil or aluminum-based have been successfully applied to enhance antigen-specific immune responses for >80 years (Newman and Powell, 1995). However, side effects especially local reactions such as swellings, indurations, erythemas and cutaneous nodules of these adjuvants (which may persist for up to 8 weeks or sometimes longer) have been reported (Leenaars *et al.*, 1998). Moreover, government clearance for oil-based adjuvants used in food animals is absent so other novel adjuvants with greater safety and efficacy are needed.

In a earlier study, researchers have expressed SO7 protein in *E. coli* and its immune efficacy adjuvanted with Freund's adjuvant, ginsenosides and Chicken Interferon-gamma (ChIFN-gamma) was preliminary evaluated (Xu *et al.*, 2007). In the present study, chickens were subcutaneously injected with the protein mixed with various doses of ChIFN-gamma, the immune response was evaluated by measuring specific 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* oocysts. The data lay a foundation for the improvement of subunit vaccine against coccidiosis in chickens.

MATERIALS AND METHODS

Animals and parasites: About 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.

Chicken interferon-gamma: Recombinant ChIFN-gamma was purified from the supernatant of recombinant baculovirus infected Sf9 cells. Its biological activity was assayed by its ability to inhibit the cytopathic effects of

Vesicular Stomatitis Virus on chicken embryo fibroblast cells by Methylthiazolotetrazolium (MTT) assay (Kong *et al.*, 2005).

Recombinant SO7 protein: Recombinant SO7 protein was produced and purified as described 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), recombinant SO7 protein was expressed in *E. coli*. The solubilized GST-SO7 protein was purified by affinity chromatography using a Glutathione Sepharose 4B column and 10 mM glutathione elution buffer according to the manufacturer's instructions (Amersham Biosciences) followed by cleavage with PreScissionTM protease (Amersham Biosciences) by a bath method. The protein concentration was spectrophotometrically estimated at A280 and A260 nm then the purified protein was stored in aliquots at -20°C until further use.

Immunization and challenge: About 5 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 μ g per chick) with various adjuvants or without adjuvant. Chickens in group I were each injected with 200 μ L PBS; chickens in group II were given antigen without adjuvant; chickens in groups III-V were given antigen mixed with 1,000, 5,000 and 10,000 units ChIFN-gamma, respectively. All chickens were boosted at 12 and 19 days of age. About 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 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 mL⁻¹ penicillin and 100 μ g mL⁻¹

Table 1: Experimental design and immune program

Groups	Number of chickens	SO7 protein (µg/chick)	Adjuvant and dose per chick	Days of immunization			Days of challenge	Challenge dose for sporulated oocysts per chick
				Primary	Secondary	Tertiary		
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	1000 units ChIFN-gamma	5	12	19	26	1×10 ⁵
IV	40	100	5000 units ChIFN-gamma	5	12	19	26	1×10 ⁵
V	40	100	10000 units ChIFN-gamma	5	12	19	26	1×10 ⁵

streptomycin. For lymphocyte proliferation assay, 100 µL/well lymphocyte suspension was added to 96 well flat-bottomed plates (Costar, USA) and cultured at 37°C in 5% CO₂ with 20 µL SO7 protein (final concentration: 10 µg mL⁻¹) for 56 h. About 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) 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 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 (OD₄₉₀) 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 challenge 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 15.0K 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 AND DISCUSSION

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 and the values in chickens immunized with antigen mixed with the middle dose of ChIFN-gamma (5000 units) were significantly higher than other 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 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

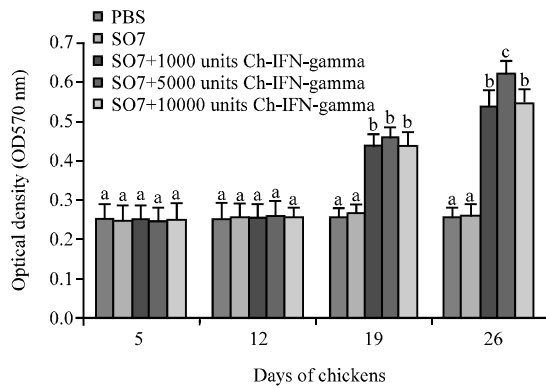


Fig. 1: Lymphocyte proliferation in response to recombinant SO7 protein immunization. Shown are mean OD₅₇₀ of stimulated lymphocyte cultures was measured by MTT (n = 5). Result was detected at 570 nm using an ELISA reader. Values with different letters differ within the same time point at p<0.05

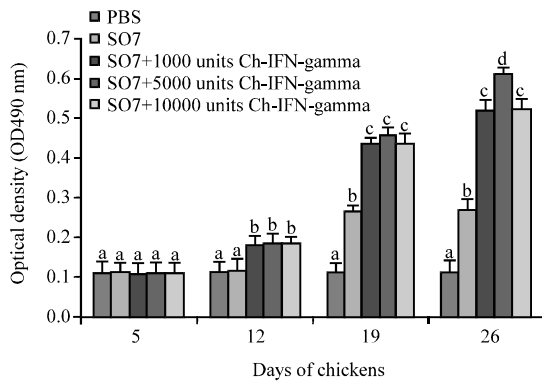


Fig. 2: Antibody response in chickens immunized with recombinant SO7 protein. Values with different letters differ within the same time point at p<0.05

values of immunized chickens were noted at 19 or 21 days of age (p<0.05). Among chickens immunized with antigen with adjuvant, a significantly higher level in mean absorbance values was observed in chickens immunized with antigen mixed with middle dose ChIFN-gamma (5000 units) (p<0.05) at 21 days of age.

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 ChIFN-gamma, there was a 40, 20 and 40% mortality in the 1,000, 5,000, 10,000 units ChIFN-gamma groups, respectively. Among the immunized groups, the mortality in chickens immunized with antigen mixed with the middle dose of ChIFN-gamma (5,000 units) was the lowest.

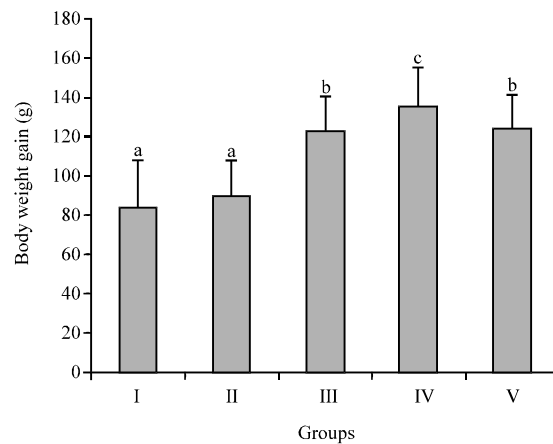


Fig. 3: Mean body weight gains of chickens immunized with recombinant SO7 protein following *Eimeria tenella* challenge infection. Values with different letters differ significantly at p<0.05

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). Among groups immunized with antigen plus adjuvant, BWG of chickens immunized with antigen mixed with the middle dose of ChIFN-gamma was significantly higher than in the other groups (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 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 middle dose of ChIFN-gamma was significantly lower than other groups (p<0.05).

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 middle dose of ChIFN-gamma which was up to 50.09%. Other two groups had reductions of 43.07 (1,000 units ChIFN-gamma) and 48.98% (10,000 units ChIFN-gamma).

Both cellular immunity and humoral immunity are involved in the protective immunity in chickens against

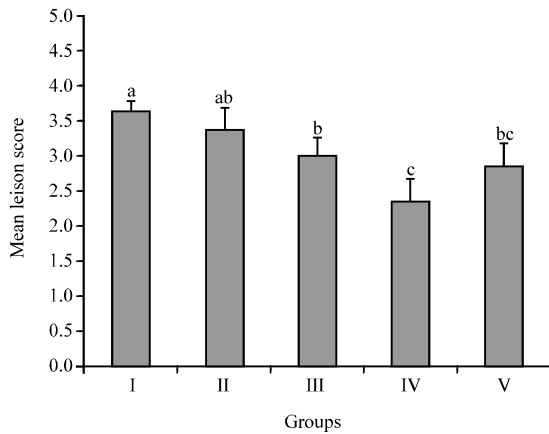


Fig. 4: Mean lesion scores of chickens immunized with recombinant SO7 protein following *Eimeria tenella* challenge infection. Values with different letters differ significantly at $p < 0.05$

Eimeria infection (Jang *et al.*, 2011). Earlier studies showed that cellular immunity is critical to protection against *Eimeria* whereas the humoral immunity plays a very minor role in resistance against infection (Yun *et al.*, 2000). In contrast, there are also reports pointing towards the ability of antibodies to block parasite invasion, development and transmission and to provide passive and maternal immunity against *Eimeria* infection (Wallach, 2007; Ding *et al.*, 2008; Subramanian *et al.*, 2008; Lee *et al.*, 2009). Due to the inefficient process and presentation of antigen, intramuscular or subcutaneous injection of the subunit antigen without adjuvants normally won't produce the desired protection effect against avian coccidiosis (Jenkins, 2001; Jang *et al.*, 2011), the results also confirmed this concept. On the other hand, the non-specific immunization from immunopotentiators only provide very limited protection against the disease, it is not effective in resisting infection by a large population of oocytes (Xie *et al.*, 2002). Therefore, developing highly effective and feasible new vaccine adjuvants to enhance immune response of a subunit vaccine is becoming an urgent task in fighting avian coccidiosis.

Various vaccine adjuvants such as oil, cytokine, traditional Chinese medicine, microorganisms and their derivate have been developed. The cytokine adjuvants are easy to mass-produce using genetic engineering technology, they have short half-life period and require low dosage in administration. In this study, the recombinant ChIFN-gamma was evaluated for the efficiency of enhancing immune response and respective optimal dosage. At the same time, compared to the PBS control group and the group immunized with antigen

alone, injecting chickens with middle dose of ChIFN-gamma alone did not show any protection and specific immune response. These results confirmed the effect of the adjuvant in increasing antibody level and enhancing cellular immune response against SO7 antigen in chickens. The survival rate, BWG, reduction in oocyst output and reduction of caecal lesion score all showed a greater level of improvement. However, ChIFN-gamma adjuvant did not exhibit linear dose-immune-response relationship, instead the greatest impact was observed at the medium dosage level (5,000 units). Results from this study confirmed the dual effects of cytokines which have been reported previously (Heriveau *et al.*, 2000).

Since, the gene encoding for ChIFN-gamma was firstly cloned and then recombinant ChIFN-gamma has been expressed *in vitro* (Digby and Lowenthal, 1995), developing ChIFN-gamma into a vaccine adjuvant or using it as a pharmaceutical reagent to treat avian diseases have received great attention. Muscle injection of 5,000 units recombinant ChIFN-gamma raised greatly the antibody level against antigen of sheep red blood cells in chicken and abdomen injection also increased chicken body weight albeit the amount of difference was not statistically significant (Lowenthal *et al.*, 1998). Recombinant ChIFN-gamma also exhibited an inhibitory effect against *Eimeria in vitro* (Lillehoj and Choi, 1998). In this study, the ChIFN-gamma was used as an adjuvant for the subunit vaccine to protect against coccidiosis in chickens and the results have confirmed the function of the adjuvant effect of ChIFN-gamma. The immune enhancement function of ChIFN-gamma was likely achieved through the following mechanisms. Firstly, cytokines induce expression of MHC I and MHC II molecules whereby promoting the efficiency of presentation of SO7 antigen. Secondly, cytokines induce the activation of macrophages which then release NO and other effector molecules (Song *et al.*, 1997; Heriveau *et al.*, 2000). The future research will focus on identification of those effective mechanisms.

CONCLUSION

The studies suggest that ChIFN-gamma has strong adjuvant effects when mixed with *E. tenella* recombinant SO7 antigen at a dose of 5000 units 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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