Immunoprotection of recombinant Eg.myophilin against *Echinococcus granulosus* infection in sheep

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Abstract. The aims of the present study were to investigate the immunoprotection of recombinant *Echinococcus granulosus* myophilin (rEg.myophilin) against the establishment of a challenge oral infection with *E. granulosus* eggs, as well as to determine the mechanisms underlying this protection. Sheep were subcutaneously immunized two times with rEg.myophilin, followed by the challenge with *E. granulosus* eggs orally. The animals were sacrificed 44 weeks after infection and the immunoglobulin (Ig) and cytokine levels were analyzed using ELISA. The results identified significant changes in several indexes of animal immune response subsequent to immunization with rEg.myophilin. These changes included reduced number of formed cysts, as well as elevated levels of IgG, IgA and cytokines. The present data suggest that immunization with rEg.myophilin in sheep can successfully reduce the formation of cysts caused by challenge *E. granulosus* infection and stimulate immune response, suggesting that rEg.myophilin a has potential value as a candidate vaccine against *E. granulosus*.

Introduction

Cystic hydatid disease (CHD) is a result of infection with *Echinococcus (E.) granulosus* at the larval stage. CHD affects sheep, cattle and humans, and occurs throughout the world (1-2). In endemic regions, approximately 50/100,000 individuals have cystic echinococcosis, and prevalence is between 5 and 10% in regions of Argentina, Peru, East Africa, Central Asia and China (3). At present, the major methods for the treatment of CHD include early prevention, drug administration, which typically involves the administration of benzimidazoles such as albendazole and mebendazole, and surgery (4-6). The disadvantage of these methods is the high cost, which is particularly challenging in remote areas and undeveloped countries. Therefore, the development of a treatment method that is relatively cheap and highly efficient at the same time is of critical importance.

Vaccination of livestock may provide an additional approach for the management of CHD. Various studies have reported vaccines for the protection of certain animals, such as sheep, goats and cattle, against hydatid disease caused by the cysts of *E. granulosus* (7-12).

In a previous study of our group, *E. granulosus* myophilin (Eg.myophilin) was isolated from a strain of the parasite present in China (13). Furthermore, the immune response and the induced immunoprotection of recombinant Eg.myophilin (rEg.myophilin) were investigated using an experimental model of hydatidosis in mice challenged with *E. granulosus* protoscoleces (14). It is also known that cytokines and serum antibodies serve an important role in CHD. Cytokine response indicates Th1/Th2 polarization, which is related to the cystic localization, the clinical stage and evolution. Hydatid infection can induce Th1 and Th2 cytokines. Th1 cytokines are associated with protective immunity in echinococcosis, while Th2 cytokines have been suggested to induce susceptibility to the disease. If cytokine response skews Th1/Th2 ratios towards a preferentially immunopathology-associated Th2 polarization, the immune response will benefit parasite growth and development. In addition, serum antibody response is associated with (or is a marker for) cystic development, growth and disease progression (1,15). The present study aimed to investigate the immunoprotection of rEg.myophilin against the establishment of a challenge oral infection using *E. granulosus* eggs in sheep. In addition, the study investigates the underlying protection mechanisms in order to assess the value of rEg.myophilin as a potential molecular vaccine.

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Materials and methods

Ethics statement. The present study was performed in strict accordance with the recommendations reported in the Guidelines for Animal Experimentation of Ningxia Medical University (Yinchuan, China). The experimental protocol was approved by the Ethics Committee of Ningxia Medical University.

Recombinant antigens. The recombinant myophilin antigens to protect sheep against hydatid CHD caused by the *E. granulosus* was expressed and purified by Ni^{2+} affinity chromatography as described previously (14) Briefly, protein expression was induced at 37˚C by cultivation of the transformed *E. coli* BL21 overnight in the presence of 0.4 mM isopropyl-β-D-thiogalactoside (Promega Corporation, Madison, WI, USA). The recombinant six His-tagged rEg. myophilin was purified from the extract of transformed *E. coli* BL21 (DE3) by Ni^{2+} chelate affinity chromatography (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Purified six His-tagged protein was analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Promega Corporation).

Parasite eggs. Adult tapeworms of the *E. granulosus* species were obtained from five dogs (gender, female; age, 18 months; obtained from Lanzhou Veterinary Research Institute, Lanzhou, China) using arecoline hydrobromide (Shanghai Seebio Biotech, Inc., Shanghai, China) and isolated from the mature proglottids under an XSP-37XB inverse microscope (Qianke, Shanghai, China). The procedure was performed as previously described by Lamberti *et al* (16). Briefly, dogs were given an oral dose (4 mg/kg) of arecoline hydrobromide. Purgation followed for ~30 min. The purged samples were collected, mixed in 5% formal saline then passed through a sieve. The tapeworms were examined and collected. After the proglottids of tapeworms were opened with a scalpel and the eggs were carefully removed with tweezers. The eggs were then packed in capsules, with 1,500 eggs per capsule, which were used for challenging oral *E. granulosus* infection.

Animals and immunization. Sheep of mixed gender (n=18; Lanzhou Veterinary Research Institute) were used in the present study. Weaning of the sheep was performed at 3-4 months of age and vaccination was performed at 5-6 months. All sheep were raised in the same farm of Lanzhou Veterinary Research Institute with a natural environment, and were numerically identified using ear-tags. The animals were divided into three random groups (n=6 in each) as follows: i) rEg.myophilin group, in which sheep were immunized twice with 50 μg rEg.myophilin in 1 ml phosphate-buffered saline (PBS) emulsified in Freund's Adjuvant solution (Sigma-Aldrich, St. Louis, MO, USA), with the first immunization initially conduced in Freund's Adjuvant, Complete (week 0) and then in Freund's Incomplete Adjuvant after 4 weeks; ii) control group, in which sheep were injected with corresponding adjuvant plus PBS; and iii) negative control group (healthy, untreated). Animals were injected intramuscularly in the neck region. The rEg.myophilin and control groups were challenged orally with 3,000 parasite eggs at 8 weeks after the first vaccination.

Serum collection and protective immunity. Blood samples were obtained from the animals through venipuncture of the jugular vein using disposable syringes at different time intervals after the first immunization (0, 1, 2, 4, 8, 12, 20, 28, 36 and 44 weeks), with infection performed at week 8. The blood samples were left to clot for 1 h at 37˚C, and serum was removed following centrifugation at 2,200 x g for 5 min at 4˚C, and then stored at -84˚C.

At 44 weeks after challenge infection, the sheep were sacrificed by captive bolt stunning followed by exsanguination. The liver and lungs of each animal were removed and sectioned (3 mm specimens for liver and 5 mm specimens for lungs), and each slice was inspected and palpated to identify the cyst. Subsequently, the percentage of protection in sheep was determined according to the method reported by Dempster *et al* (17), as follows: Protective immunity in vaccinated sheep (%) = (1 - mean number of cysts in test group / average number of cysts in control group) x 100%.

Antibody measurement by ELISA. Serum antibody responses were quantified by ELISA using the a polyclonal primary antibody purchased from Bio-Rad Laboratories, Inc. (1:1,000; Hercules, CA, USA) at 0, 1, 2, 4, 8, 12, 20, 28, 36 and 44 weeks after the first immunization: Mouse anti-bovine (cat no. MCA627) ; with infection performed at week 8. Briefly, 96-well microtitre plates (Sino-American Biotechnology Co., Luoyang, China) were incubated overnight at 4˚C with rEg.myophilin (10 μg per 100 μl per well) in 0.1 M carbonate buffer (pH 9.6). Next, PBS plus 0.05% Tween-20 (PBST) was used to dilute the serum samples by 1:1,000. Bound antibody was detected using the following secondary antibodies diluted in PBST: Horseradish peroxidase (HRP)-conjugated rabbit anti-sheep immunoglobulin (Ig)G (heavy/light chains; 1:10,000; cat no. 51842504), mouse anti-bovine IgG1 (1:20,000; cat no. MCA627), mouse anti-bovine IgG2 (1:20,000; cat no. MCA6269) and HRP-conjugated rabbit anti-sheep IgA (1:100; cat no. AHP949P). Goat Anti-Mouse IgG conjugated to HRP (cat no. ZB-2305; Zhongshan Jinqiao,Inc. Beijing, China) was used to detect antibodies following IgG1 and IgG2. Antibody titers were read at 450 nm using an ELISA Model 680 Microplate Reader (Bio-Rad Laboratories, Inc.). The enzyme substrate used for detection was 3,3'5,5'-Tetramethylbenzidine (cat no. CW0050S; Kangwei Biotech Company, Beijing, China). All samples were tested in duplicate.

Cytokine measurements by ELISA. The optical density value of cytokines was determined by ELISA in accordance with the manufacturer's instructions. Briefly, serum samples were added into microplate ELISA kits pre-coated with rEg.myophilin (cat nos. CSB-E15963Sh, CSB-E11217Sh, CSB-E12817Sh and CSB-E14018Sh for IL-4, IL-2, IL-10 and IFN-γ kits, respectively; Wuhan Huamei Biotech Co., Ltd., Wuhan, China) and maintained at 37˚C for 2 h. Subsequent to washing with PBST, 50 ng (100 μl) biotin-conjugated antibodies were added per well and cultured for 2 h at 37˚C. Following additional washing, horseradish peroxidase-labeled streptavidin was added for 1.5 h at 37˚C. Next, the plates were washed again and substrate was added for 0.5 h at 37˚C. Finally, the reaction was stopped by adding 100 μl

Table I. Number of hydatid cysts and protective immunity in vaccinated and control groups of sheep.

Groups	Eggs for challenge	No. of cysts	Protective immunity (%)
Negative control	0	0	-
Control	3,000	25.00±16.59	-
rEg.myophilin	3,000	2.00±1.22[a]	92.00

[a]P<0.05 vs. control group. Data are expressed as mean ± standard deviation (n=6 in all groups). rEg, recombinant *Echinococcus granulosus*.

2 M sulphuric acid, and the optical density was measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data comparisons were performed using SPSS statistical software (version 19.0; IBM SPSS, Armonk, NY, USA) and were tested for significance using one-way analysis of variance (ANOVA). Data are presented as mean ± standard deviation. A P-value of <0.05 was considered to show a statistically significant difference.

Results

Antibody levels in sheep, measured by ELISA. The animals were challenge-infected 8 weeks after the first immunization. Sera from animals treated with adjuvant plus PBS and rEg.myophilin were prepared and tested using the ELISA method. As shown in Fig. 1A, the total IgG rapidly increased following immunization, when compared with the control and negative control groups, reaching a maximum level at week 12. The antibody levels were maintained until week 44 (Fig. 1A). In addition, the levels of IgG1 and IgG2 tended to increase following rEg.myophilin immunization, particularly between weeks 4 and 12 after the beginning of immunization. After week 12, the level of IgG1 was maintained high (Fig. 1B), whereas the level of IgG2 declined slowly (Fig. 1C). Notably, the levels of IgA increased subsequent to the first immunization; however, a declining trend was observed in week 2, followed by further increase between weeks 8 and 28, after which the levels declined quickly (Fig. 1D).

Analysis of cytokine levels in sheep immunized with rEg.myophilin, measured by ELISA. The response of animals to rEg.myophilin vaccination was determined by measuring the levels of cytokines, including interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4 and IL-10. These levels were measured at week 8 (corresponding to the time point prior to infection) and week 12 (corresponding to 4 weeks after infection), as shown in Fig. 2. The levels of IL-2 (Fig. 2A) and IFN-γ (Fig. 2B) in the immunized group were found to be significantly higher when compared with those in the control group prior to and following infection. Furthermore, the levels of IL-4 were significantly higher compared with those in the control group in week 8, while no statistically significantly difference was observed in week 12 (Fig. 2C). By contrast, the levels of IL-10 were not found to be significantly different from those of the control group at weeks 8 and 12 (Fig. 2D). The aforementioned results suggest that rEg.myophilin immunization continuously induces the production of IL-2 and IFN-γ, whilst only temporarily inducing the production of IL-4, and has no effect on IL-10 level.

Protective immunity in sheep. Sheep in each group were sacrificed 44 weeks after immunization followed by challenge-infection. The internal organs of all sheep were carefully examined for the presence of hydatid cysts. In sheep vaccinated with rEg.myophilin, the number of hydatid cysts was found to be significantly lower compared with that in the control group (Table I; P<0.05). The protective immunity induced by rEg.myophilin was determined based on the formula described earlier and was found to be 92%.

Discussion

E. granulosus parasites are known to undergo numerous development stages in their life cycle, including oncosphere, protoscoleces, adult worm and egg. The life cycles involve two mammalian hosts. The adult cestode inhabits the small intestine of a definitive host, such as a dog or fox, and produces eggs containing infective oncospheres. Following the oral uptake of eggs by an intermediate host animal, such as a human or sheep, a metacestode develops in the internal organs of the host, which is the larval stage of development (18).

The research group of the present study has previously reported that the recombinant protein 14-3-3 (19), ferritin (20) and p29 (21) derived from the *E. granulosus* exhibit strong immunogenic properties. In addition, rEg.myophilin has been reported to show an effectively protective immunity in mice (14). However, these experiments present two deficiencies: *E. granulosus* protoscoleces were intraperitoneally inoculated (secondary infection) due to the experimental condition at the time of the experiments, and thus the natural infection mode was peroral with *E. granulosus* eggs. Furthermore, infection commonly affects sheep, cattle and humans, not mice; thus, secondary infection in mice may not accurately reflect the *E. granulosus* immune mechanism.

In the present study, we attempted to investigate the immunoprotection properties of rEg.myophilin against CHD following oral infection of *E. granulosus* eggs in sheep. The study examined whether immunization with rEg.myophilin leads to effective immunity protection compared with the control group, which may provide a foundation for the use of rEg.myophilin as a valuable vaccine.

The results of the current experiments showed that immunization with rEg.myophilin induced a high level of humoral antibodies, and a mixed IgG1 and IgG2 response. Notably,

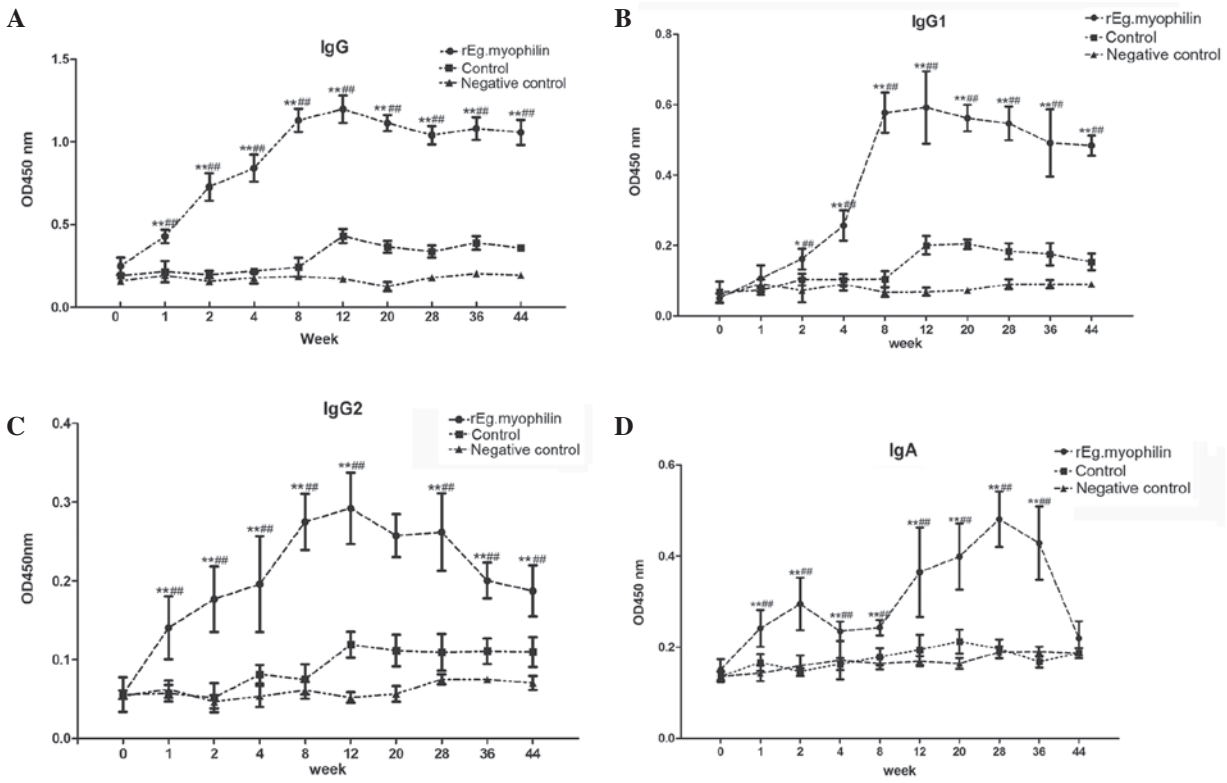

Figure 1. (A) Total IgG, (B) IgG1, (C) IgG2 and (D) IgA antibody response as detected by ELISA in the serum. OD, optical density; Ig, immunoglobulin; rEg, recombinant *Echinococcus granulosus*. *P<0.05 and **P<0.01 control vs. rEg.myophilin groups. $^{\#\#}$P<0.01 negative control vs. rEg. myophilin groups.

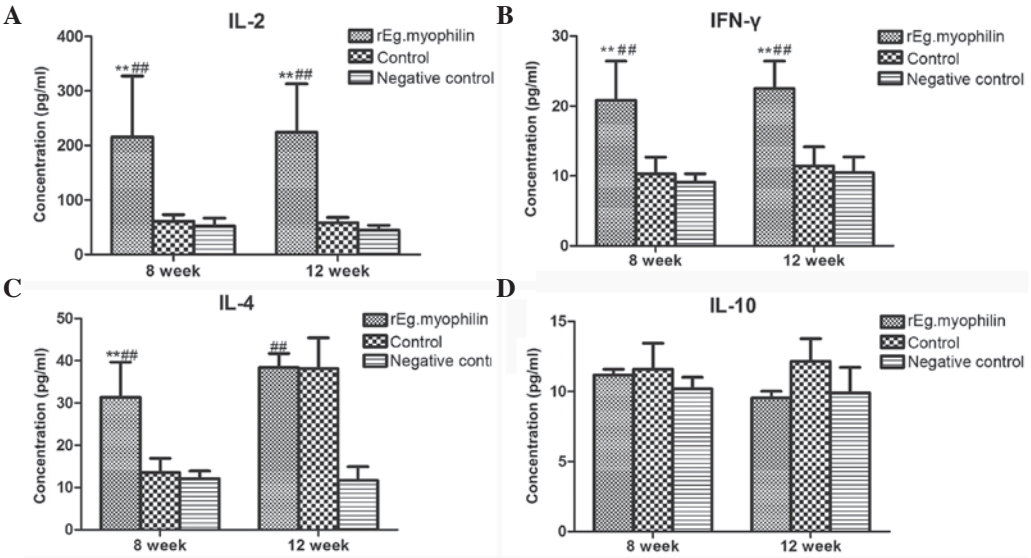

Figure 2. Detection of cytokine levels at different stages in sheep. (A) IL-2, (B) IL-4, (C) IL-10 and (D) IFN-γ. rEg, recombinant *Echinococcus granulosus*; IL, interleukin; IFN, interferon. *P<0.05 and **P<0.01 control vs. rEg.myophilin groups. $^{\#\#}$P<0.01 negative control vs. rEg. myophilin groups.

the analysis of IgG subtypes revealed a gradual shift from a predominant IgG2 to a predominant IgG1 isotype at week 4 post-immunization (which corresponds to 4 weeks before infection). With the extension of infection time, IgG and IgG1 were maintained at higher levels compared with the control, whereas the levels of IgG2 declined slowly. The results may suggest that protection against *E. granulosus* in the rEg.myophilin group sheep was correlated with the increased levels of IgG1 and total IgG, but not with IgG2. In addition, the level of IgA increased subsequent to the first immunization, while tended to decline at week 2. Next, a rapid increase was observed following infection, reaching a maximum level at week 28 (corresponding to 20 weeks after infection), after which IgA levels quickly declined.

Although echinococcus IgA has been extensively studied in dogs and several experimental models have been described (22-24), little is known regarding *E. granulosus* infection in sheep. Carol and Nieto (25) demonstrated that

nasal immunization of immunostimulating complexes produced from protoscoleces showed significant induction of the secretory IgA antibody response, as detected in the saliva and serum of dogs infected with *E. granulosus*. Kouguchi *et al* (26) observed a sharp increase in serum IgA responses immediately after challenge infection with *E. multilocularis* in dogs (26). The results of the present study showed that IgA may be involved in protective immune response. However, the detailed mechanisms through which this process leads to protective immunity remain unknown.

Cytokines are commonly produced by T cells in the regulation of parasitic diseases of humoral immunity. Th1 cells produce IL-2 and IFN-γ to promote IgG2 production, while Th2 cells produce IL-4 and IL-10 to promote a humoral response, particularly by inducing IgG1. Th1 protective response mediates protective immunity and helps the host to clear hydatid disease. Furthermore, Th2 response promotes the humoral immune response and is beneficial to parasitism (27-29). In the present study, four types of cytokines were selected (IFN-γ, IL-2, IL-10 and IL-4), and their dynamic changes in sheep were examined at weeks 8 (prior to infection) and week 12 (4 weeks following infection). The levels of Th1-type cytokines (IFN-γ and IL-2) were significantly higher compared with that in the control group; however, Th2-type cytokine (IL-10) were not significantly changed in sheep prior to and following infection. The levels of IL-4 were significantly higher compared with that of the control group after the second immunization, while no significantly different was detected at 4 weeks after egg challenge.

These results suggested that rEg.myophilin likely induced Th1-type immune response following immunization and infection. The changes in cytokine levels appeared to be associated with changes in the levels of specific antibodies. Furthermore, after rEg.myophilin immunization, the increase of IgG2 levels was coordinated with the increase of Th1-type cytokines. However, the increase in IgG1 levels was not associated with an increase in the levels of Th2-type cytokines. Furthermore, after rEg.myophilin immunization, the increase in IgG2 levels was coordinated with the increase of Th1-type cytokines (IL-2 and IFN-γ). However, the increase in IgG1 levels was not associated with an increase in the levels of Th2-type cytokines (IL-4 and IL-10). In addition, the levels of IgG subtypes showed that IgG2 was predominant following the second immunization. However, the mechanism through which rEg.myophilin stimulates such an immune response in sheep remains unclear.

In conclusion, the results of the present study showed that immune reaction can be effectively activated by treatment with rEg.myophilin. The results also demonstrated that 92% protection can be induced by rEg.myophilin against a challenge oral infection with *E. granulosus* eggs in sheep. However, the associated mechanism detailing how rEg.myophilin induces protection requires further investigation prior to its development as a practical vaccine.

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