

***Echinococcus granulosus*: Immunoprotection accompanied by humoral and cytokine response against secondary hydatidosis in mice immunized with rEg.myophilin**

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Abstract

Objects: This study aimed to investigate the immunoprotection of the recombinant Eg. myophilin (rEg.myophilin) and tentatively analyze mechanism of this protection.

Materials and Method: Three groups of female mice were immunized subcutaneously with rEg.myophilin and contrasts, after two boosters, mice were challenged with *Eg* protoscoleces (PSCs) intraperitoneally, and then analyzed the humoral immune response with specific IgG, IgG1, IgG2a, IgG2b, IgG3 and IgE, and also evaluated the cellular immune with IFN- γ , IL-4, IL-10.

Results: Mice immunized with rEg.myophilin produced effective immunity protection (reductions in cyst load more than 86.11%). Mice immunized with rEg.myophilin showed specific IgG, IgG1, IgG2a and IgE accompanied by IFN- γ and IL-4 significantly increased after one or more times of immunization, but neither IgG2b nor IgG3 increased. Nevertheless, IL-10 significantly increased in the control groups but a decline trend in rEg.myophilin group.

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Conclusions: *rEg.myophilin* showed a effectively protective immunity, which may attribute to: (1) IgGs and IgE-mediated humoral immune response, as well as cell-mediated immunity which was marked by IFN- γ . (2) IL-4 assists to stimulate type transformation of IgE which plays an important role in Eosinophils-related anti-parasite immunity. (3) Immunosuppressive effect (marked by IL-10), the key obstacle to clean of parasites in the patient, was inhibited in the *rEg.myophilin* group compared with control groups.

Keywords Cystic echinococcosis (CE) · *rEg.myophilin* · Immunoprotection · Vaccine candidate · Immunity response

Introduction

Cystic echinococcosis, caused by the larval form of the cestode *Echinococcus granulosus*, is one of the most serious emerging zoonotic parasitic diseases throughout the world. Despite efforts to educate persons at risk and the success of control programs in a few countries, cystic echinococcosis continues to be a substantial cause of morbidity and mortality in many parts of the world (Eckert et al. 2000; Torgerson and Heath 2003).

Vaccine of livestock may provide an additional approach to the control of echinococcosis. It was observed that the intermediate hosts of *Eg* are long-lived and infection by eggs provokes a high degree of protective immunity. This important characteristic has been used for the development of highly effective vaccines. Any success in recognizing the immunologic reactions of intermediate hosts and finally in producing an effective vaccine could lead to a decline in human infections and also in economic loss of animals. In addition, most grazing animals are already vaccinated successfully against viral or bacterial diseases, and it indicated that vaccine against parasitic diseases can greatly helpful to normal farm practice (Heath et al. 2003). Ongoing investigations will be emphasized on the biological roles played by the proteins within the parasites and the mechanism by which they make the parasites vulnerable to vaccine-induced immune responses (Lightowlers et al. 2003). Vaccination to intermediate host is a burgeoning area that has moved forward considerably in recent years following the development of a recombinant vaccine against *Taenia ovis* infection in sheep (Lightowlers et al. 2000). Recombinant *Eg* proteins and synthetic peptides have been proven to be more reliable for immunodiagnostic purposes than native antigens and their purified subunits/fractions (Carmena et al. 2006). So we want to found a recombinant protein as a vaccine candidate to prevent *Eg* infection. Myophilin antigen of *Eg* proteins was reported to be essential for signal transduction and regulating cells shapes (ncbi conserved domain web site. 2009). In our preliminary studies, we successfully constructed plasmid *Eg.myophilin*/pET28a and transformed it into *E. coli* BL21(DE3) pLysS (Ma et al. 2008). In the present study, *rEg.myophilin* was expressed in *E. coli* and purified for antigen preparation, then immunoprotection and immunity mechanism after vaccination of mice with *rEg.myophilin* were investigated and analyzed to assess the value of its vaccine potential.

Materials and methods

Animals and parasite

111 male ICR mice (4–6 weeks old, obtained from the Experimental Animal Centre of Ningxia Medical University, Yinchuan, China which was proved by science and technology

department of Ningxia Hui Autonomous Region, China) were randomly allocated into 3 groups (5 mice/group was killed in every time point, and 2 mice for accidental death in each group). *Eg* protoscoleces (PSCs) were collected aseptically from fertile *Eg* cysts from the livers and lungs of infected sheep. The collected PSCs were washed in phosphate buffered saline (PBS-1%) and Hanks' balanced salt solution (Sigma, St. Louis, USA) containing 100 U/ml of penicillin G and 100 mg/ml of streptomycin sulfate. The viability of PSCs was determined by trypan blue exclusion assay. Only those batches containing more than 90% viable PSCs were used for mice infection.

Preparation of antigens

Plasmid *Eg.myophilin/pET28a* was transformed into *E. coli* BL21 (DE3) pLysS and recombinant *E. coli* was constructed in our preliminary studies. Protein expression was induced at 37°C for 5 h in the presence of isopropyl- β -D-thiogalactoside (IPTG, Promega) at a final concentration of 0.4 mM. The recombinant His6-tagged *Eg* Myophilin was purified from the extract of transformed *E. coli* BL21(DE3) by nickel chelate affinity chromatography (Novagen) according to the manufacturer's instructions. The purified His6-tagged protein was analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Protein concentration was determined by Bradford method (Bradford 1976).

Vaccination and challenge infection

Mice in group A were vaccinated subcutaneously with 100 μ l PBS and group B with 50 μ l FCA in 50 μ l PBS respectively and group C vaccinated with 50 μ g of *rEg.myophilin* emulsified with an equal volume FCA. Two additional boosters were manipulated with the same preparation except that FCA was replaced by Freund's incomplete adjuvant (FIA) at the 2nd and 4th week. Two weeks after the third vaccination, mice were challenged with 1000 PSCs intraperitoneally.

Determination of cytokines

Spleen cells were isolated from mice before and after challenge infection for in vitro assays. 5×10^6 lymphocytes per ml were maintained at 37°C with 5% CO₂ in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, and 25 mM 2-mercaptoethanol. Cells were stimulated with concanavalin A 5 mg/ml. Supernatants from 72 h cultures were collected and frozen until cytokine determination.

Optical density value of cytokine was determined by ELISA (Jingmei Biotech Company). Supernatant of lymphocytes cultures was added into the coated wells of 96-well plates, and maintained at 37°C for 2 h. After washed with PBS-0.05% Tween 20, 50 ng of biotin-conjugated antibody was added to each well for another 2 h at 37°C. Then washed again, after which peroxidase-labeled streptavidin was added and incubated for 1.5 h at 37°C. This was followed by washings and substrate addition and incubation for 0.5 h at 37°C. Finally, the reaction was stopped by addition of 100 μ l of 2 M sulphuric acid. The optical density was measured at 490 nm in ELISA reader (Bio-Rad).

Determination of specific antibodies

Serum antibody responses were quantified by ELISA at 0, 2, 4, 6, 7, 10 and 31 week from the commencement of experiments which covered the stages of immunization and post-

challenge. 96-well microplates (Sino-American Biotechnology Company) were coated with *rEg.myophilin* (10 µg/100 µl/well) by overnight incubation in 0.1 M carbonate buffer (pH 9.6) at 4°C. Serum samples were diluted 1:100 in PBST (PBS with 0.05% Tween-20) and tested in duplicate. Bound antibody was detected by HRP-conjugated goat anti-mouse IgG and IgG subclass (Novagen) at a 1:1000 dilution in PBST, and OD values of antibody were read at 490 nm in ELISA reader (Bio-Rad).

Statistical analysis

Statistical analysis for differences among groups was done using the non-parametric (Mann–Whitney) t test or Student's t-test. Results were considered significant at $P < 0.05$.

$$\text{reduction in cyst load} = 1 - \frac{\text{average of cysts in test group}}{\text{average of cysts in control group}} \times 100\%$$

Results

REg.myophilin immunization reduced the numbers and sizes of developing cysts

Significant reduction in cyst load was found in the group immunized with *rEg.Myophilin*, compared with the control groups which immunized with PBS or FCA in PBS at 6 months post-infection (Table 1). The reduction in cyst load is 89.86% and 86.11% (compared with group A and group B).

REg.myophilin immunization effectively induced humoral response

The immunization of mice with *rEg.myophilin* induced higher level of specific IgG, IgG1, IgG2a and IgE (Fig. 1 a, b, c and f), but it didn't induce higher level of specific IgG2b and IgG3 (Fig. 1 d and e). Surprisingly, despite a higher level of IgG2a was induced, it showed no persistence high after secondary infections, which is contrary to other *rEg.myophilin*-induced antibodies (Fig. 1 c). The level of *rEg.myophilin* specific IgG2b didn't increased until after 1 week of infection of PSCs (Fig. 1 d). There was no

Table 1 Numbers of hydatid cysts in vaccinated and control groups

Group	No. of cysts in individual mice							Mean ± SD
Group A	3	4	0	2	3	6	5	3.29 ± 1.976 *C
Group B	4	0	3	2	3			2.40 ± 1.517 *C
Group C	0	1	1	0	0	0		0.33 ± 0.516 *AB

*AB shows significantly different from group A and group B by Student's t-test, *C shows significantly different from group C by Student's t-test, ($P < 0.05$). We planned to obtain the material each time at the expense of five mice, and two more for in case of accidental death. Finally, 7, 5, 6 mice in 3 groups lasted in order to determine the reduction in cysts load. Mice infected with 1,000 PSCs after immunization with *rEg.myophilin* in group C, with PBS or FCA in PBS in group A or group B as control. The peritoneal cavity was inspected for cysts in each mouse at the termination of experiment (6 months after infection). Cysts count were determined, and statistically significant difference ($P < 0.05$) was found compared with the two control groups.

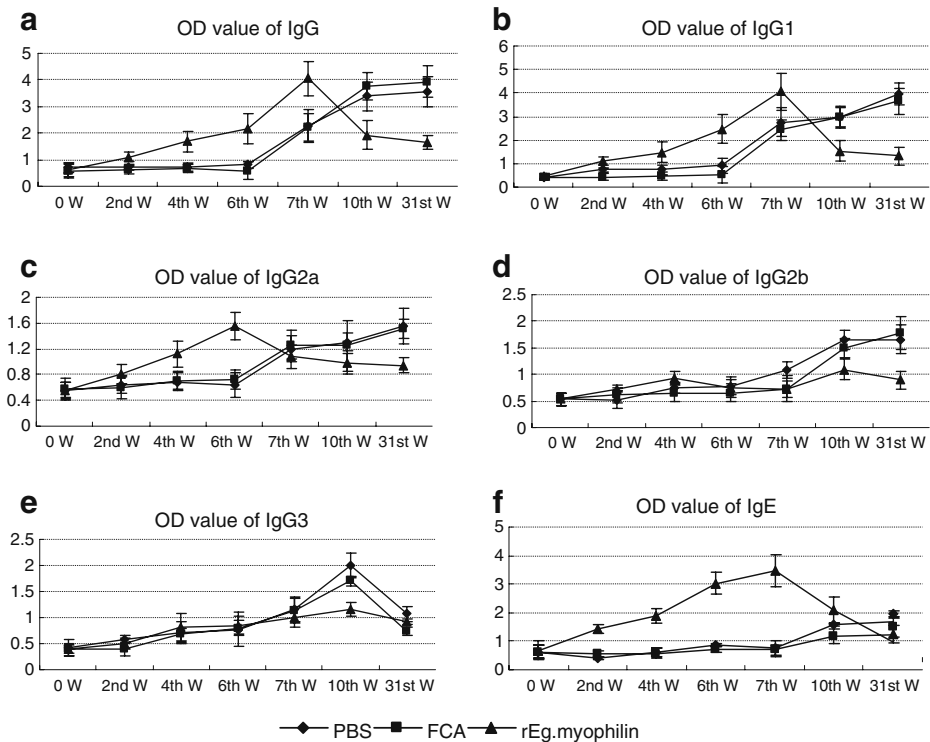


Fig. 1 O.D. value profiles of IgGs and IgE in immunized mice before and after infection Different stages were selected for antibodies examination: 0 week(before immunization), 2nd(2 weeks after first immunization), 4th(2 weeks after 2nd immunization), 6th(2 weeks after 3rd immunization), 7th(1 week after infection), 10th(4 weeks after infection) and 31st(25 weeks after infection)

significant difference in specific IgG3 level between *rEg.myophilin* group and control groups (Fig. 1 e).

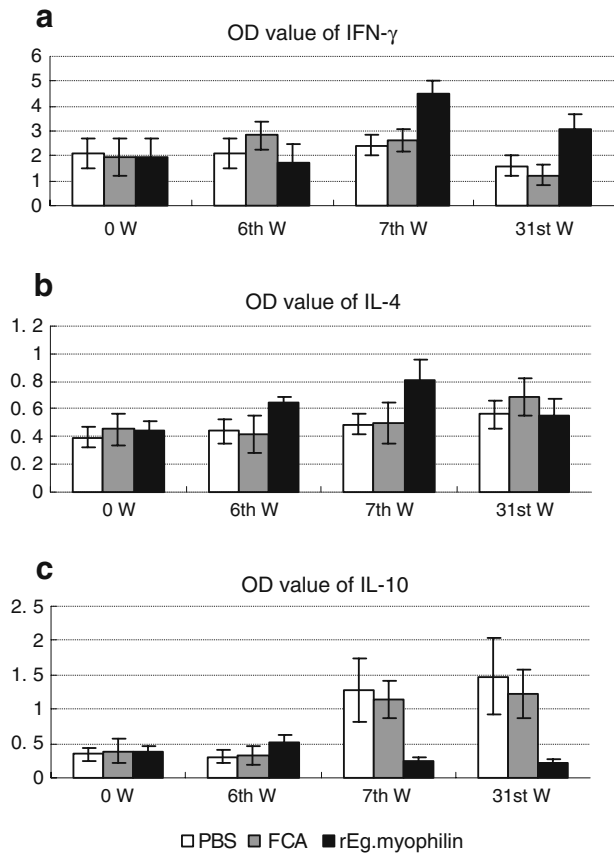
Cytokine response caused by *rEg.myophilin* immunization

IFN- γ and IL-4 were measured in the supernatant of spleen cells 6 months after the infection as indicators of Th1 and Th2 immune responses, and IL-10 as indicators of immunosuppression. Cells taken from *rEg.myophilin*-immunized mice and stimulated with same antigen secreted higher Th1 cytokines (IFN- γ) compared with those from group A or B (Fig. 2, a). IL-4 secretion in cells of mice immunized with *rEg.myophilin* significant increased before PSCs challenge (2 weeks after the 3rd immunization). IL-10 showed a decreasing tendency after infection. However, IL-10 was significantly higher in group A and B compared with group C after infection (Fig. 2, c).

Discussion

The present study explored the effect of immunization of mice with *rEg.myophilin* on the outcome of subsequent secondary hydatidosis and the associated immune response. In this

Fig. 2 O.D. value bar of IFN- γ , IL-4 and IL-10 in immunized mice before and after infection. Different stages were selected for cytokine examination: 0 week (before immunization), 6th week (2 weeks after 3rd immunization), 7th week (1 week after infection), 31st week (25 weeks after infection). * or Δ means significantly different between time points or groups ($P < 0.05$)



way, the present approach referenced and improved not only previous studies that investigated the immune response generated by inoculation of mice with live or dead PSC (Zhang et al. 2003; Hernandez-Pomi et al. 1997; Severi et al. 1997; Dematteis et al. 1999; Al-Qaoud and Abdel-Hafez 2005) but also the immunoprotection studies of recombinant protein in recent years (Li and Wang 2007; Shi et al. 2009; Lin and Ding 2004). Evidently, immunization with *rEg.myophilin* leads to effective immunity protection compared with the other two control groups, between which there is no significant difference in cyst load reduction. That will provide a foundation for *rEg.myophilin* to be a valuable vaccine.

REg.myophilin specific IgG, IgG1, IgG2a and IgE were significantly elevated in mice immunized with *rEg.myophilin* compared with the control groups which showed a delayed increase of these antibodies, indicating that all or some of these antibodies are relative with immunoprotection. IgG2a was induced to a higher level before infection, but it decreased after the first week of infection. Explanation for the decline in IgG2a is the neutralization of this antibody subclass by parasitic antigens (Al-Qaoud and Abdel-Hafez 2005). There is no significant difference in IgG2b and IgG3 between *rEg.mophilin* group and the other two groups, one possible explanation is either *rEg.myophilin* could not induce higher level of specific IgG2b and IgG3 or *rEg.myophilin*-induced-specific IgG2b and IgG3 didn't show a high affinity to native myophilin antigen. The other explanation is these antibodies don't distribute mainly in serum, but in body cavities (Baz et al. 1999).

Cytokines study in patients indicates that the comparison of Th1/Th2 production shows that the induction of these mediators is related to the cystic localization, the clinical stage and clinical evolution. Generally speaking Th1 cytokines are related to the protective immunity; in contrast Th2 cytokines are responsible to the susceptibility to disease and associated with chronic stage, clinical complications and secondary locations (Mezioug and Touil-Boukoffa 2009). In our study, both IFN- γ and IL-4 showed a peak at the 7th week (1 week after infection) in *rEg.myophilin* group, but IL-4 showed a significant increase at the 6th week (2 weeks after the 3rd immunization and before infection) and IFN- γ appeared this sign at 7th week (1 week after infection). In addition, IFN- γ dominated at the first week of post-infection in *rEg.myophilin*-immunized mice, and this can be seen as an indicator of greater Th1 than Th2 response. IL-4 level was significantly elevated before infection, and it suggests that IL-4 is mainly induced by *rEg.myophilin* but not pathogens PSCs in this study. This is inconsistent with observations made before which showed a significant increase in early post-infection (Li and Wang 2007). The elevated IL-4 may be related to stimulating the proliferation and differentiation of mature B cell and assists to drive class switching to IgE which plays a key role in parasite immunity (He 2005a, b). IL-10 increasing, a general feature of chronic infections by helminthes, can polarize the host immune response toward Th2. In our research IL-10 didn't show an increasing before or after infection in *rEg.myophilin* group compared with control groups which appeared significantly increasing from early infection to experiment termination. The similar results are reported by earlier research about effects of IL-10 in studies of *Eg.* infection or other recombinant vaccine candidate tests (Rigano et al. 2007; Baz et al. 2006; Fraize et al. 2005; Ortona et al. 2003). It indicated cell immunity is further polarized into Th1 after vaccination of *rEg.myophilin*, but how *rEg.myophilin* inhibits secretion of IL-10 in PSCs challenged mice, need further studies.

In summary, effective protection rate was reached in mice immunized with *rEg.myophilin*. *rEg.myophilin* has been selected as a vaccine candidate. Humoral and cytokine response played an important role in this protection. However, this immunoprotection is due to suppression of the immune escape induced by *Eg* or direct role played by specific antibodies or both need further investigation.

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