

# Mechanism of protective immunity by vaccination with recombinant *Echinococcus granulosus* glutathione S-transferase (Chinese strain) in mice

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**Abstract.** The aim of this study was to investigate the immunoprotective effects of recombinant *Echinococcus granulosus* glutathione S-transferase (rEgGST) against the development of protoscolices (PSCs), and to determine the mechanisms underlying this protection. ICR mice were subcutaneously immunized three times with rEgGST at weeks 0, 2 and 4, followed by the intraperitoneal administration of *E. granulosus* PSCs at week 10. Six mice in each group were sacrificed at 0, 2, 4, 6, 10, 18 and 30 weeks following the initial vaccination in order to observe the macroscopic and microscopic effects of parasite development. Various analyses were subsequently conducted, including determination of the levels of immunoglobulins (Igs) and cytokines. Significant differences were observed a number of indices of immune response following immunization with rEgGST. These included reduced cyst formation and elevated levels of IgG1, IgG2a, IgG3, IL-2, IL-4, IL-10 and IFN- $\gamma$ , which indicated an increased percentage of immune helper cells. The results of the present study suggest that immunization with rEgGST in mice is able to successfully reduce the PSC-induced formation of cysts and to stimulate an immune response, suggesting that rEgGST possesses potential value as a candidate vaccine for PSC infection.

## Introduction

Human cystic echinococcosis, also known as cystic hydatid disease (CHD), affects humans and livestock and is caused by

infection with the larval stage of *Echinococcus granulosus*. CHD can be seriously harmful to human health and occurs worldwide (1,2). In China, the prevalence of CHD is more extensive than that of alveolar echinococcosis, with 600,000-1,300,000 individuals suffering from CHD (3). At present, the primary methods of treating CHD include early prevention via annual check-ups, medication and surgical operation. However, these approaches are frequently prohibitively expensive, particularly in undeveloped countries and remote areas. Therefore, there is an urgent requirement for highly effective CHD treatments that are relatively inexpensive.

Vaccination of livestock may provide a novel approach for the control of CHD. Previous studies have reported the use of vaccines to effectively protect certain animals, including sheep, goats and bovines, against CHD induced by the cysts of *E. granulosus* (4-9).

In our previous study, a Chinese strain of *E. granulosus* glutathione S-transferase (EgGST) was cloned and sequenced (10), and the capacity of EgGST to induce an immune response and immunoprotection was tested in an experimental model of hydatidosis in mice. In the present study, recombinant EgGST (rEgGST) was expressed in *Escherichia coli* and purified for antigen preparation (11). Following the vaccination of mice with rEgGST, the resulting immunoprotection was analyzed and the protective mechanisms were investigated to assess the potential of rEgGST as a novel molecular vaccine.

## Materials and methods

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from freshly isolated *E. granulosus* protoscolices (PSCs) using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The *E. granulosus* protoscolices were extracted aseptically from fertile *E. granulosus* cysts from the livers and lungs of infected sheep. The EgGST gene was amplified by RT-PCR (Promega Corporation, Madison, WI, USA) using two primers according to the sequence of *E. granulosus*. Primer I: *Eco*RI recognition site, 5'-ATGAATTCATGGCTCCCACTCTGGCTT-3'; Primer II: *Not*I recognition site, 5'-GTGCGGCCGCGTCACCTAACA

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GTCACCAC-3'. Each primer contained *EcoRI/NotI* restriction enzyme sites and was synthesized by Beijing SBS Genetech, Co., Ltd. (Beijing, China). RT-PCR was performed in a 50- $\mu$ l reaction mixture, containing 10  $\mu$ l 5X buffer (Promega Corporation), 2  $\mu$ l Mg<sup>2+</sup> (25 mmol/l), 1  $\mu$ l dNTP (10 mmol/l), 1  $\mu$ l avian myeloblastosis virus reverse transcriptase (Promega Corporation), 1  $\mu$ l *Thermus flavus* DNA polymerase (Promega Corporation), 5  $\mu$ l of each primer, 5  $\mu$ l RNA and 20  $\mu$ l diethylpyrocarbonate-treated water. The reaction protocol for RT-PCR was as follows: 48°C for 45 min, 94°C for 2 min, followed by 40 cycles of 30 sec at 94°C, 60 sec at 60°C and 2 min at 68°C, with a final extension for 7 min at 68°C. RT-PCR products were identified using 1% agarose gel electrophoresis (Liuyi Instrument Factory, Beijing, China).

*Subcloning of EgGST gene into expression plasmid vector.* The target fragment was purified using a gel cleanup kit (SBS Genetech, Co., Ltd.) and inserted between the *EcoRI* and *NotI* sites of the expression vector pET-28a (Novagen; Merck KGaA, Darmstadt, Germany). The recombinant vector EgGST/pET-28a was identified by restriction digestion using *EcoRI* and *NotI* and the EgGST insert was verified by sequencing, performed by Sangon Biotech Co., Ltd. (Shanghai, China). *E. coli* BL21 (DE3) pLysS, provided by Dr Xiao Wei (University of Saskatchewan, Saskatoon, Canada) was transformed for induced expression of His6-tagged EgGST protein.

*Expression and purification of rEgGST.* Protein expression was induced at 25°C by cultivation of the transformed *E. coli* BL21 overnight in the presence of isopropyl- $\beta$ -D-thiogalactoside (IPTG; Promega Corporation) at a final concentration of 0.6 mmol/l. The recombinant His6-tagged rEgGST was purified from the extract of transformed *E. coli* BL21 (DE3) by Ni<sup>2+</sup> chelate affinity chromatography (Novagen) according to the manufacturer's instructions. Purified His6-tagged protein was analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Protein concentrations were determined using the Bradford method (12).

*Immunization.* A total of 84 male 6-week old ICR mice were obtained from the Experimental Animal Centre of Ningxia Medical University (Yinchuan, China). Mice were allocated at random into two groups containing 42 mice each. Mice in group A received three subcutaneous immunizations with 10  $\mu$ g rEgGST in 100  $\mu$ l phosphate-buffered saline (PBS) emulsified in Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The three immunizations were delivered at 2-week intervals, starting at week 0 in Freund's complete adjuvant and followed by two booster immunizations in Freund's incomplete adjuvant at weeks 2 and 4. Mice in the control group B were injected with the corresponding adjuvant and PBS. This study was approved by the Ningxia Medical University Ethical Committee.

*Challenge infection and protective immunity.* Six weeks after the final vaccination, on week 10, a challenge infection was induced in the mice via the intraperitoneal injection of 1,500 PSCs. Six mice in each group were sacrificed at different 0, 2, 4, 6, 10, 18 and 30 weeks following the initial vaccination,

in order to obtain sera and spleen cells. Mice were sacrificed by cervical vertebra dislocation. Subsequently, the carcasses were dissected and examined superficially for visible hydatid cysts. The percentage of protection was determined according to the method and formula described by Dempster *et al.* (13): Protective immunity in vaccinated mice (%) = (average number of cysts in the test group/average number of cysts in the control group) x 100.

*Serum collection.* Six mice in each group were sacrificed at 0, 2, 4, 6, 10, 18 and 30 weeks following the initial vaccination in order to observe the macroscopic and microscopic effects of parasite development. Serum samples were collected and stored individually at -84°C.

*Cytokine measurements using ELISA.* The optical density values of a number of cytokines were determined using ELISA. Spleens were isolated from mice and splenocytes were harvested. A suspension of single splenocytes was prepared after removing erythrocytes via hypotonic lysis and resuspending the samples in RPMI 1640 (Gibco Life Technologies, Carlsbad, CA, USA) by vigorous pipetting. Viable cells counted by trypan blue exclusion (5x10<sup>6</sup> cells/ml) were exposed to medium, 5  $\mu$ g/ml concanavalin A (Sigma-Aldrich) and 10  $\mu$ g/ml rEgGST and incubated for 72 h. Supernatants of lymphocyte cultures were added to a pre-coated microplate ELISA kit (Jingmei Biotech Co., Ltd., Shenzhen, China) and maintained at 37°C for 2 h. After washing with PBS and 0.05% Tween 20 (PBST), 25-50 ng goat biotin-conjugated antibodies against mouse IL-2 (F10092-A), IL-4 (F7695-A), IL-10 (F7701-A) and IFN- $\gamma$  (F10077-A; 1:1000; Jingmei Biotech Co. Ltd.) were added per well and the plates were incubated for 2 h at 37°C. Following four washes in PBST, peroxidase-labeled streptavidin was added for 1.5 h at 37°C. Plates were washed and incubated with substrate for 0.5 h at 37°C. Finally, the reaction was stopped with 100 ml sulfuric acid (2 M). Optical density was measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Antibody measurement using ELISA.* Serum antibody levels were quantified using ELISA at 0, 2, 4, 6, 10, 18 and 30 weeks after the first of the three immunizations (14). In this assay, 96-well microtiter plates (Sino-American Biotechnology Co., San Diego, CA, USA) were coated with rEgGST (10  $\mu$ g/100  $\mu$ l/well) and incubated overnight in 0.1 M carbonate buffer (pH 9.6) at 4°C. Serum samples were diluted 1:100 in PBST and tested in duplicate. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (ab97265) and IgG subclass antibodies against IgG1 (ab97240), IgG2a (ab97245) and IgG3 (ab97260; Abcam, Cambridge, UK) at a 1:1,000 dilution in PBST. Antibody titers were measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Inc.).

*Statistical analysis.* All data comparisons were tested for significance using one-way analysis of variance. SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

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

Group	Mice (n)	Cysts (n)	Immunity (%)
Control	6	3.67±3.14	-
rEgGST	6	0.33±0.51 <sup>a</sup>	89.39 <sup>a</sup>

Data are expressed as mean ± standard deviation. <sup>a</sup>P<0.01 vs. the control group. rEgGST, recombinant *Echinococcus granulosus* glutathione S-transferase.

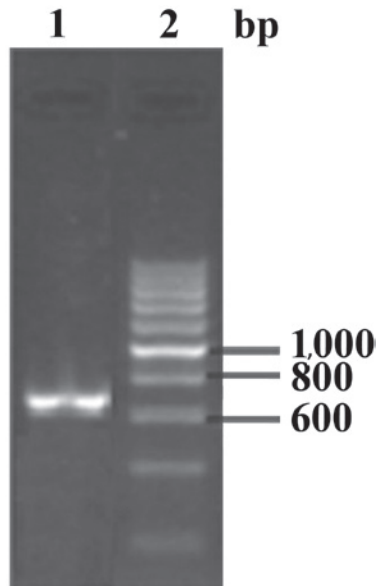


Figure 1. Amplified product of the glutathione S-transferase gene. Lane 1, polymerase chain reaction product; lane 2, DNA marker.

## Results

**Expression and purification of rEgGST.** The EgGST gene was amplified using RT-PCR, generating an amplified product of ~660 bp (Fig. 1). The recombinant expression plasmid EgGST/pET-28a was constructed and transformed into *E. coli* BL21 (DE3). The plasmids extracted from transformed *E. coli* BL21 (DE3) were digested using the restriction enzymes *EcoRI* and *NotI* (Fig. 2). The insert sequence was analyzed using DNA sequencing and confirmed to be the EgGST gene, suggesting that the recombinant plasmid EgGST/pET-28a had been constructed successfully. Following IPTG induction, the His6-labeled recombinant protein was purified using a Ni<sup>2+</sup>-chelating column. SDS-PAGE staining results demonstrated that the His6-tagged EgGST protein had been successfully expressed in *E. coli* BL21 (DE3) and purified efficiently from the *E. coli* lysate, with a molecular weight of ~28 kD (Fig. 3).

**Antibody measurement using ELISA.** Sera from animals treated with PBS or rEgGST were tested using the ELISA method. The levels of IgG were increased following rEgGST immunization, typically from about week 4 after the initial immunization until week 10. The animals were challenge-infected at

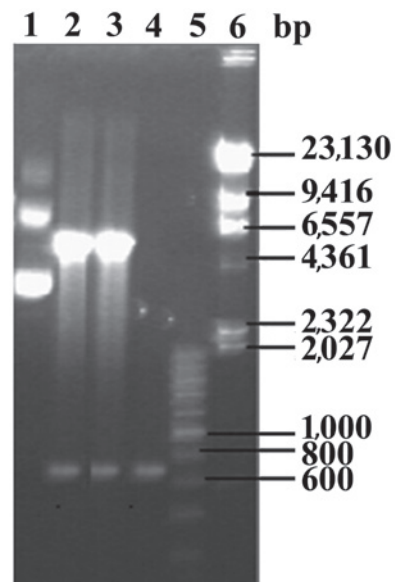


Figure 2. Restriction enzyme digestion of recombinant expressed plasmid *Echinococcus granulosus* glutathione S-transferase (GST)/pET-28a by *EcoRI* and *NotI*. Lane 1, recombinant plasmid; lane 2-3, GST/pET-28a digested by *EcoRI* and *NotI*; lane 4, polymerase chain reaction-amplified product; lane 5, 200-bp DNA marker; lane 6,  $\lambda$ DNA/*HindIII* marker.

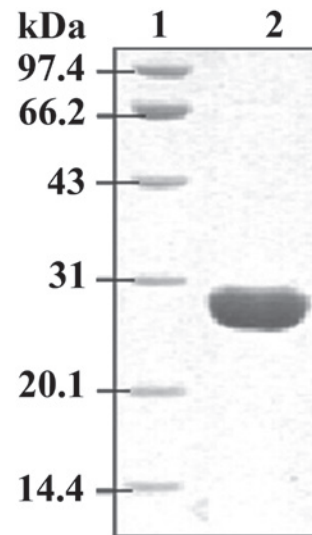


Figure 3. SDS-PAGE analysis purification of recombinant *Echinococcus granulosus* glutathione S-transferase (rEgGST). Lane 1, molecular weight standards marker; lane 2, purified rEgGST protein.

week 10. Following the challenge infection, the levels of total IgG remained elevated (Fig 4A), the levels of IgG2a and IgG3 declined gradually (Fig. 4C and D), and the levels of IgG1 continued to increase until week 30 (Fig. 4B).

**Analysis of cytokine levels in mice immunized with rEgGST by ELISA.** The animals' reaction to rEgGST was further evaluated by measuring the levels of a number of cytokines, specifically interferon (IFN)- $\gamma$ , interleukin (IL)-2, IL-4 and IL-10. The levels of IFN- $\gamma$  and IL-2 in the immunized group were significantly higher compared with those in the control group at week 6 after the first immunization, while the levels of IL-4 and IL-10 remained unaltered during the period of observa-

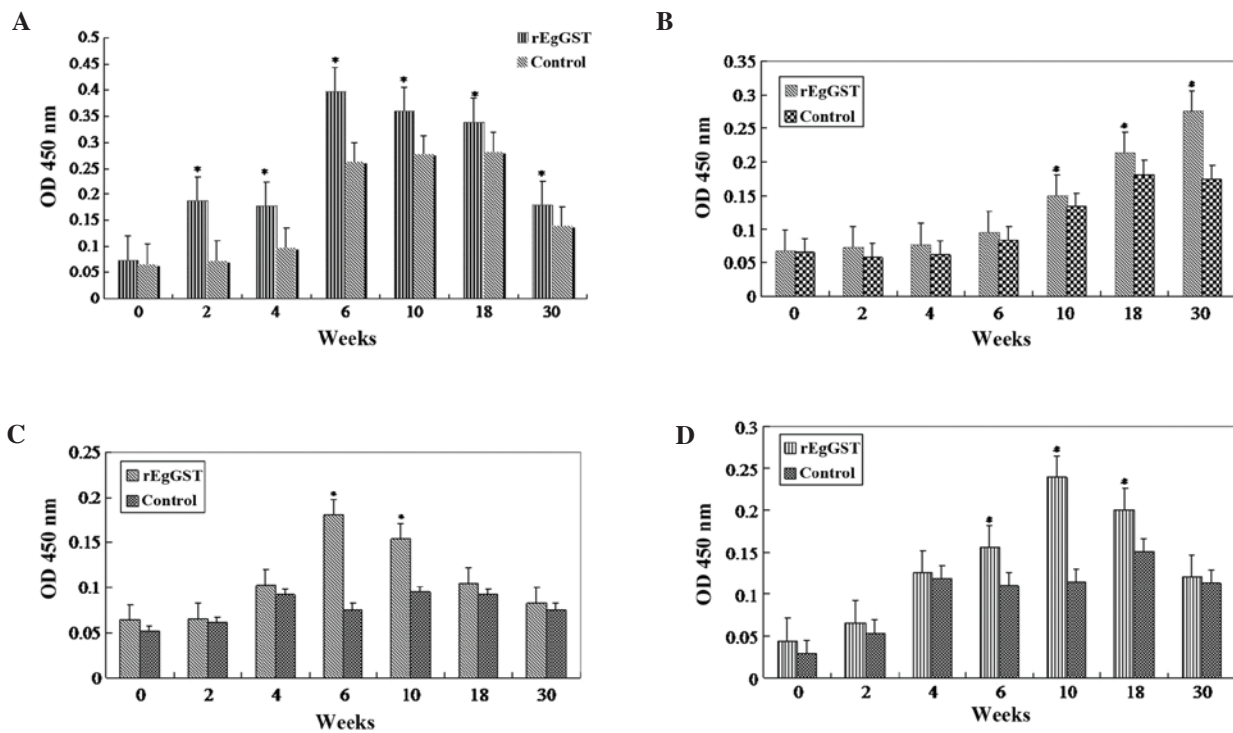


Figure 4. Total IgG antibody and IgG subclass response as detected by ELISA analysis of sera. (A) IgG, (B) IgG1, (C) IgG2a and (D) IgG3 response. Different stages were selected for antibody examination: Week 0 (before the first immunization), week 2 (second immunization), week 4 (third immunization), week 6, week 10 (challenge infection), week 18 and week 30. Data are presented as the mean  $\pm$  standard error of the mean of 6 mice per group. \*P<0.05 vs. control. rEgGST, recombinant *Echinococcus granulosus* glutathione S-transferase; OD, optical density.

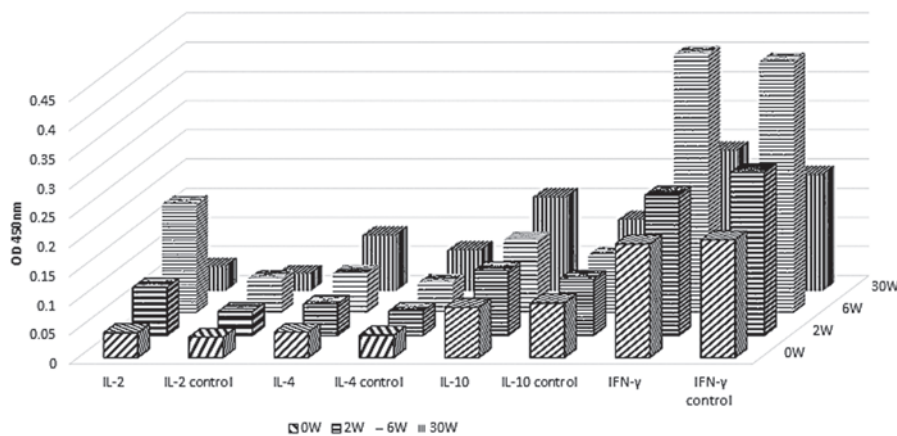


Figure 5. Detection of cytokines in mice at various time points following vaccination. OD, optical density; IL, interleukin; IFN, interferon.

tion. After the challenge infection at week 10, the levels of IFN- $\gamma$  and IL-2 decreased to normal levels by week 30. The levels of IL-4 and IL-10 did not change significantly following the challenge infection in the immunization group (Fig. 5). These results indicate that type 1 T helper (Th1) cells mediate the primary response following immunization.

**Protective immunity in mice.** Mice in each group were sacrificed at 5 months (30 weeks) after the initial immunization. The internal organs of the mice were examined for the presence of hydatid cysts. Mice vaccinated with rEgGST exhibited significantly reduced numbers of hydatid cysts compared with the control group (Table I; P<0.01). The

protective immunity induced by rEgGST was calculated to be 89.39%.

## Discussion

Previous studies have reported that the 14-3-3 protein (15), myophilin (16), ferritin (17) and P29 (18) that are secreted by *E. granulosus* exhibit marked immunogenic properties. The aim of the present study was to determine whether immunization with rEgGST was able to induce effective protective immunity when compared with a control group. A positive result may be used to provide an experimental basis for the potential use of rEgGST as a vaccine.

Glutathione S-transferases (GSTs) are a family of detoxification enzymes (19), and *Schistosoma mansoni* 28 kDa GST (Sj28GST) has been recognized as an effective protective antigen against *S. mansoni* (20). For *E. granulosus*, the observation of GST protein expression at various stages of parasite development provided an experimental basis for the investigation of the potential of EgGST as a vaccine candidate against echinococcosis (21). Therefore, GST is considered to be a potential treatment for the prevention of shistosomiasis infection (22).

In the present study, immunization with rEgGST stimulated humoral immunity in mice following the third immunization. Increased serum levels of total IgG and the IgG subtypes IgG2a, IgG1 and IgG3 were observed in mice following rEgGST immunization and challenge infection compared with the control group. These results indicate that an immune response may be effectively activated by treatment with rEgGST. In addition, IgG, IgG2a, IgG1 and IgG3 may be involved in the early protective immune response. As the time following the induction of the challenge infection increased, IgG2a and IgG3 levels decreased gradually while the levels of IgG1 increased continuously. This observation may be related to the parasite escaping from host immune surveillance. rEgGST was able to induce the production of high levels of antibody following immunization, which remained elevated following early infection. These results indicate that rEgGST possesses notable immunogenicity and immunoreactivity.

Cytokines are commonly produced by T helper cells in humoral and cellular immunity in order to mediate the regulation of parasitic diseases. Th1 cells produce interleukin (IL)-2 and interferon (IFN)- $\gamma$  to promote IgG2a and IgG3 production. Th2 cells produce IL-4 and IL-10 and promote a humoral response, in particular via the induction of IgG1. The Th1 protective response mediates protective immunity and helps the host to eliminate hydatid disease, while the Th2 response promotes the humoral immune response and mitigates parasitism (23-25). In the present study, four cytokines were selected (IFN- $\gamma$ , IL-2, IL-10 and IL-4) for examination of their dynamic changes in mice following treatment with rEgGST and the induction of a challenge infection using PSCs. Following rEgGST immunization, the levels of Th1-type cytokines (IFN- $\gamma$  and IL-2) significantly increased; however, the levels of Th2-type cytokines (IL-4 and IL-10) remained unaltered. Levels of Th1-type cytokines decreased following the induction of the PSC challenge infection, and the levels of Th2-type cytokines remained the same. These results suggest that rEgGST induces the Th1-type immune response, which gradually transitions into the Th2-type response at later stages of infection. The changes in cytokines levels are typically associated with the changes in the levels of specific antibodies. It was observed that following rEgGST immunization, the Th1-dependent increase of IgG2a and IgG3 levels was coordinated with the increase of Th1-type cytokines. However, the increase of Th2-dependent IgG1 was not associated with an increase of Th2 type cytokines. These results suggest that rEgGST immunization specifically stimulates Th1 cells, which subsequently induce the production of Th1-dependent antibodies and Th1-type cytokines. However, the mechanism by which rEgGST stimulates the observed immune response in animals remains unclear.

In conclusion, the results of the present study demonstrate that 89.39% protection may be induced by administering rEgGST in a murine model of echinococcosis. Therefore, rEgGST is potentially competent to be used as the anti-hydatid component of a novel vaccine candidate, with the aim of effectively preventing and controlling of the development of hydatid disease. However, the mechanism underlying the observed rEgGST-induced protection remains unclear and requires further study.

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