Original Article

Echinococcus Granulosus 14-3-3 Protein: A Potential Vaccine Candidate Against Challenge with *Echinococcus Granulosus* in Mice*

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Abstract

Objective To investigate the protective immunity against *Echinococcus granulosus* in mice immunized with rEg14-3-3.

Methods ICR mice were subcutaneously immunized three times with rEg14-3-3, followed by the challenge with *Echinococcus granulosus* protoscoleces intraperitoneally and then sacrificed after six months of post-challenge to detect the proliferation of splenocytes by MTT assay, and to measure the secretion of IL-2, IL-4 , IL-10, and IFN -γ by ELISA. The rate of reduced hydatid cyst and the levels of IgE, IgG and IgG subclasses in sera were examined.

Results Mice vaccinated with rEg14-3-3 and challenged with protoscoleces revealed significant protective immunity of 84.47%. ELISA analysis indicated that the immunized mice generated specific high levels of IgG and the prevailing isotypes of IgG were IgG1 and IgG2a. Splenocytes from mice immunized with rEg14-3-3 showed a significant proliferation response. The secretion of IFN-γ and IL-2 increased significantly in the vaccinated mice whereas there was no significant difference in IL-4 and IL-10 levels between vaccinated and control mice.

Conclusion The results indicate that the rEg14-3-3 vaccine could induce a high level of protective immunity as a promising vaccine candidate to prevent cystic echinococcosis.

Key words: Eg14-3-3; *Echinococcus granulosus*; Vaccine; Immunoprotection

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INTRODUCTION

Echinococcosis is a cosmopolitan zoonosis that is distributed widely around the world such as in sub-Saharan Africa, Central Asia, South America and the Mediterranean region, with a considerable impact on both human and animal health and with important socio-economic consequences in endemic areas[1-2]. This disease is caused cestodes belonging to the genus *Echinococcus* in their larval stages, which is increasingly concerned in recent decades. The larval infection (hydatid disease, hydatidosis) is characterized by long-term growth of metacestode cysts in the intermediate hosts mainly including human and livestock. The two major species of medical and public health importance are *Echinococcus granulosus* and *Echinococcus multilocularis,* which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE) respectively. In China, the prevalence of cystic echinococcosis is more extensive than that of alveolar echinococcosis.

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It is reported that about 600 000 to 1 300 000 people are suffering from cystic echinococcosis, among which 1/3 are children[3]. In spite of great advances in helminths research, no effective therapy has been developed to control or eradicate this disease. To a certain extent, the traditional chemotherapy and surgery play an important role in treating the disease but is not applicable for its prevention. Therefore, the development of highly effective antiechinococcal drugs or effective vaccines is urgently needed, which would completely prevent the infection or arrest the development of the parasite at a certain stage of its life cycle to block the transmission. In cystic echinococcosis, sheep, cattle, pigs and humans have been infected as intermediate hosts by *E. granulosus* in the metacestode stage . To find vaccines in intermediate hosts could be a very effective and convenient approach to reduce the incidence and transmission of the hydatid disease due to the definitive host not eating organs with protoscoleces. It is known that *E. granulosus* undergoes many development stages in its life cycle, including oncosphere, protoscoleces, adult worm, and egg. Therefore, single antigens may be limited to induce effective protective responses or single antigen molecules cannot completely prevent different development stages of *Echinococcus granulosus* in some cases. It is therefore imperative to find new potential vaccine candidates or drug action targets for infection or transmission prevention. The cocktail vaccine combined with several immunoprotective antigens could be considered for this purpose, as several vaccine candidates against *Echinococcus granulosus* have been cloned, expressed and characterized, such as EG95 host-protective antigen from *Echinococcus granulosus* oncospheres[4-5], P-29[6] and ferritin from *Echinococcus granulosus* protoscoleces[7].

In our study, we chose Eg14-3-3 (*Echinococcus granulosus* 14-3-3) proteins as the research target because of theirpotential use in treating and/or preventing hydatidosis. This assumption is based, mainly on their role as major regulative molecules in the growth and development of parasites. It is proven that the 14-3-3 exist in different development stages of *Echinococcus* species, including the egg, onchosphere, protoscolex and adult periods[8-10]. 14-3-3 proteins are a family of highly conserved proteins found to be expressed in all eukaryotic organisms studied so far. As basically intracellular proteins, they play a key role in basic cellular events related to cellular proliferation and development, such as signal transduction, cell-cycle control, cell differentiation and cell survival[11]. The 14-3-3 proteins have been described, characterized, and isolated in studies of several types of parasites, including *Schistosoma japonicum*[12], *Schistosoma mansoni*[13], *Plasmodium*[14], and *Toxoplasma gondii*[15]. The 14-3-3 proteins have been proposed as a vaccine candidate, as they showed immunoprotection in some helminths as schistosomiasis. However, limited information is available on their protective potential against cystic echinococcosis and the immune response they induce. In our previous study, we cloned and sequenced Eg14-3-3 of the Chinese strain collected in our laboratory[16], and the immune response and induced protection were tested in our experimental model of hydatidosis in mice.

MATERIALS AND METHODS

Collection of Protoscoleces

Brood capsules were collected aseptically from fertile E. granulosus cysts in the livers of an infected patient, who was hospitalized in the Department of Liver Surgery, the Affiliated Hospital of Ningxia Medical University. The collected protoscoleces 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 U/mL of streptomycin sulfate. The viability of protoscoleces was determined by Trypan blue exclusion assay. Only those batches containing more than 90% viable protoscoleces were used for mice infection.

Expression and Purification of rEg14-3-3

Plasmid of Eg14-3-3/pGEM-T (made in our laboratory) was digested with EcoRI and XhoLI. Then the purified fragment with the expected length of Eg14-3-3 (744bp) was ligated into expression plasmid vector pET28a (Novagen). The resulting recombinant expression plasmid Eg14-3-3/pET28a was transformed into *E.coli* BL21 (DE3) pLysS (The gift of Dr. Xiao Wei, the Saskatchewan University of Canada). Protein expression was induced at 37 °C for 5 hours in the presence of isopropyl-b-D-thiogalactoside (IPTG, Promega) at a final concentration of 0.4 mmol/L. The recombinant His6-tagged Eg14-3-3 was purified from the extract of transformed E.coli BL21(DE3)pLysS by nickel

chelate affinity chromatography (Novagen) according to the manufacturer's instructions. The purified His6-tagged protein was analyzed for purity on a 12% SDS–PAGE gel. Protein concentration was determined by Bradford assay[17].

Animal Immunization Protocols

40 male ICR mice of 6 to 8 weeks (Experimental Animal Center of Ningxia Medical University, Yinchuan, China) were randomly allocated into two groups with each group of 20 mice. Mice in Group 1 was immunized subcutaneously in the back with 50 μg of rEg14-3-3 in 100 mL PBS emulsified in Freund's adjuvant for a total of three times (first immunization in Freund's complete adjuvant at week 0 and followed by two booster immunizations in Freund's incomplete adjuvant at weeks 2 and 4). The mice in Group 2 (control group) were injected with corresponding adjuvant plus PBS as control. The mice were bled via the tail vein at 2-week interval before immunization (Wk 0) and thereafter at Wk 2, 4, 6, 8, and 10. Sera were stored individually at -20 °C before being used for an enzyme-linked immunosorbent assay (ELISA) and the Western blot analysis.

Enzyme-Linked Immunosorbent Assay

The serum antibodies including IgG, IgE and their subclasses after immunization with rEg14-3-3 or PBS were quantified by ELISA as described previously[18]. Briefly, ELISA-plates (Nunc, Roskilde, Denmark) were coated with rEg14-3-3 5 μg/100 μL per well. The above-mentioned Eg14-3-3-immunized mouse sera were tested at 1:100 dilution. The serum from PBS -immunized mice was used as a negative control. Peroxidase-labelled goat anti-mouse conjugate IgE, IgG, IgG1, IgG2a, IgG2b, and IgG3 (Sino-American Biotechnology Company, Beijing, China) was used at 1: 1000 dilution. Optical densities were measured at 490 nm.

Western Blot Analysis

rEg14-3-3 and protoscoleces natural antigen were separated electrophoretically in a 12% polyacrylamide gel and transferred onto nitrocellulose membranes (Millipore, Tokyo, Japan). Membranes were then cut into strips and blocked in 5% skim milk at 37 °C for 2 h. After that, the strips were washed with PBS-T for three times and incubated overnight at 4 °C with immunized or control mice sera (1:100 dilution). After washing, membranes were further incubated with HRP-conjugated goat anti-mouse IgG (1:1000 dilution) for 2 h at 37 °C. Finally, membranes were visualized by the addition of PBS containing 0.5 mg/mL of 4-chloro-1-naphtol (Sino-American Biotechnology Company), 0.015% H_2O_2, and 16% methanol.

Preparation and Cultivation of Splenocytes

Six weeks after the last immunization, 10 mice from each group were killed and their spleens were removed under aseptic conditions. The suspension of single splenocytes was prepared after removing erythrocytes by hypotonic lysis and being resuspended in RPMI 1640 (Gibco) by vigorous pipetting. The cell suspension was added into the 96-well flat-bottomed tissue culture plates (Sigma) at 200 μL/well for 72 h, and then cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Splenocytes Proliferation Assays and in vitro Cytokine Assay in Mice

Splenocytes suspensions from immunized and control mice were prepared in RPMI 1640 supplemented with 10% FCS. Viable cells counted by trypan blue exclusion (5×10^5 cells/mL) were exposed to medium, 5 μg/mL ConA (Sigma) and 10μg/mL rEg14-3-3 and incubated in triplicate for 72 h. Proliferation was assessed by MTT (Sigma, USA) assay. The absorption at 570 nm for each well was measured by using a microtiter plate reader (BioRad). Culture supernatants from the proliferation assay were collected at 72 h for the assessment of both Th1 (IFN-γ, IL-2) and Th2 (IL-4, and IL-10) cytokine production. Cytokine profile was analyzed by sandwich ELISA with a Quantikine M kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Challenge Infection and Protective Study

At Wk 6 after the final vaccination, the rest 10 mice from each group were challenged with 2000 viable protoscoleces intraperitoneally (Sylvia Dematteis.2001). Six months post-challenge, the carcasses were dressed and examined for the visible hydatid cysts. The percentage of protection in mice was determined according to the method of Dempster[19]. Protective immunity is calculated as the vaccinated mice (%) = (1-average of cysts in test group/average of cysts in control group)× 100%.

Statistical Analysis

All data comparisons were tested for

significance by using one-way analysis of variance (ANOVA). *P*-value<0.05 was considered as significant.

RESULTS

Expression and Purification of rEg14-3-3

The Eg14-3-3 proteins are composed of 247 amino acids, with a molecular weight of 27.9 kD. With a goal of testing a novel molecular vaccine for Echinococcosis, we digested the Eg14-3-3 gene with EcoRI and XhoLI, producing a fragment of 744 bp. This fragment was inserted into pET28a.The DNA sequence of the insert and the reading frame of the recombinant Eg14-3-3/pET28a were confirmed by a sequencing analysis. Plasmid rEg14-3-3/pET28a was then transformed into E. coli BL21(DE3) plysS. Following IPTG induction, the His6- tagged recombinant protein was purified with Ni2+-chelating column. The purity and the size of the recombinant protein was checked by SDS-PAGE staining, which showed a high purity of the extracted protein and a molecular weight of 31 kD as expected (Figure 1).

ELISA and Western Blot Analysis

We monitored the changes of the IgG level in mouse sera with ELISA. As shown in Figure 2, the level of the total serum IgG in treated mice after the first immunization increased marginally at Wk 2, significantly at Wk 4, and to the highest level at Wk 6, followed by slow decline of the IgG level up to Wk 10. As expected, the IgG level in control mice did not change. Then there arose the question which specific subclasses of IgG were induced by rEg14-3-3 treatment. As shown in Figure 3, the levels of IgG1 and IgG2a isotypes were significantly higher in treated mice than in the control mice. In contrast, the levels of IgG2b, IgG3, and IgE did not differ. Control mice (adjuvant plus PBS) did not generate detectable anti-rEg14-3-3 immune responses.

To determine whether immunization with rEg14-3-3 in mice can stimulate immune response to natural antigens, we tested the immune reaction of antiserum from treated mice to rEg14-3-3 and native protoscoleces antigen from human hydatid cysts by Western blot. The antiserum prepared from mice vaccinated with rEg14-3-3 can react with both rEg14-3-3 and protoscoleces natural antigen, with the specific band migrating to 31 KD (Figure 4). In contrast, there was no observable reaction between serum prepared from control mice and rEg14-3-3 or protoscoleces natural antigen .

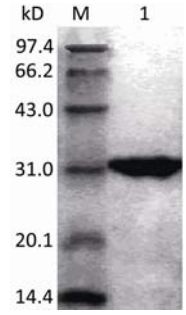

Figure 1. SDS-PAGE analysis of the purified rEg14-3-3. The gel was stained by coomassie brilliant blue. M, molecular mass markers; lane 1, purified rEg14-3-3.

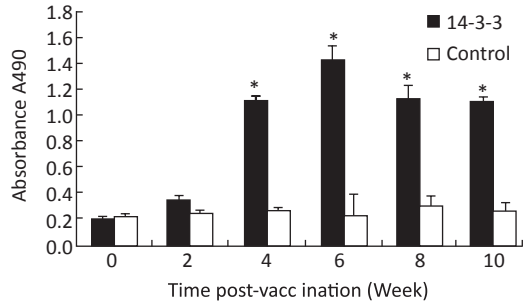

Figure 2. Serum IgG antibody levels in mice immunized with rEg14-3-3 and adjuvant plus PBS.(*IgG Antibody levels between the 13-3-3 group and the control group, *P*<0.05).

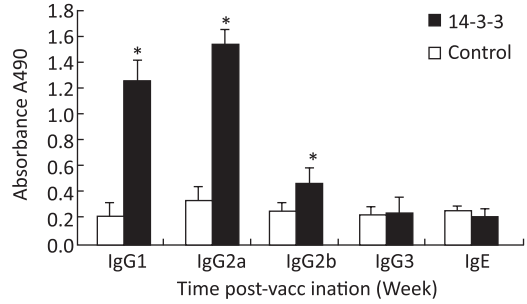

Figure 3. IgG isotype distribution and IgE of the antibody response against rEg14-3-3 as detected by ELISA in serum samples taken from ICR mice 6 weeks after first immunization. (*Antibody levels differ significantly between the 13-3-3 group and the control group, *P*<0.05).

Protective Immunity in Mice

Then we studied the protective immunity of the recombinant vaccine in mice. Mice injected with either rEg14-3-3 (treated group) or PBS (control gro up) were sacrificed 6 months after the challenge of protoscoleces of *E. granulosus* and their internal organs were examined carefully for the visible hydatid cysts. First, we counted the number of visible cysts in all mice. As shown in Table 1, the average number of visible cysts decreased significantly in the treated group (0.6 per mouse), compared with the number in the control group (3.86 per mouse). These results indicated that the protective immunity induced by rEg14-3-3 was 84.47%. Second, we measured the sizes of individual cysts in both groups and found a significant decrease from an average of 2.25 mm in the control group to 0.27 mm in the treated group (Table 1). These results indicated that rEg14-3-3 injection could reduce the number as well as the size of the formed cysts.

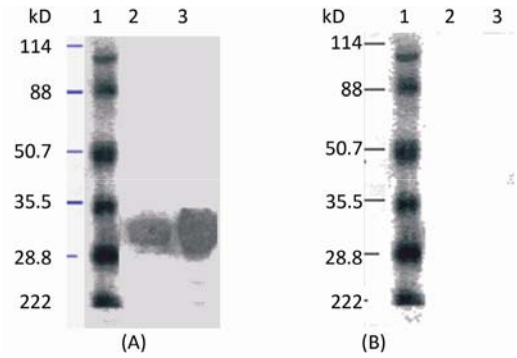

Figure 4. Western blot analysis of rEg14-3-3. A shows recognition of the rEg14-3-3 by the rEg14-3-3 vaccinated mice antiserum with Lane M being protein marker with low molecular weigh, Lane 1 the rEg14-3-3 and Lane 2 the protoscoleces natural antigen; B shows recognition of the Eg14-3-3 by the control mice antiserum with Lane M being protein marker with low molecular weigh, Lane 1 the rEg14-3-3 and Lane 2 the protoscoleces natural antigen.

Splenocytes Proliferation Assays and Cytokine Assay in Mice

Splenocytes from mice vaccinated with rEg14-3-3 proliferated in the presence of rEg14-3-3, while splenocytes from mice immunized with adjuvant plus PBS did not proliferate. The mean stimulation index for the vaccinated mice was 2.14 times higher than the index for the negative control mice (P<0.05) (Table 2). Splenocytes from both groups were stimulated equally with ConA, indicating that the spleen leukocytes were functional and responsive to nonspecific mitogens. Splenocytes from mice vaccinated with rEg14-3-3 produced a significantly higher level of IFN-γ and IL-2 after antigen-specific stimulation compared to control mice (PBS plus adjuvant). In contrast, there were no significant differences in the production of IL-4 and IL-10 induced by rEg14-3-3(Figure 5).

Table 1. Numbers of Hydatid Cysts and Protective Immunity in Vaccinated and Control Mice

Group	No. of Mice	No. of Mice with Cysts	No. of Cysts (Mean±SD)	Size of Hydat Cysts (Mean±SD) In mm	Protection(%)
14-3-3	10	2	0.60±0.787	0.27± 0.15	84.47%
Control	10	10	3.86±3.141	2.25± 0.63	-

Table 2. Splenocytes Proliferation Level Induced by Different Stimuli.(The results are shown as the stimulation index)

Group	The proliferation level of spleen leukocytes induced by different antigens stimuli (Mean±SD)		
	ConA	rEg14-3-3	medium
rEg14-3-3	0.5894±0.1203	0.4775±0.0860*	0.2198±0.0726
Control	0.5645±0.1940	0.2230±0.0336	0.2144±0.0515

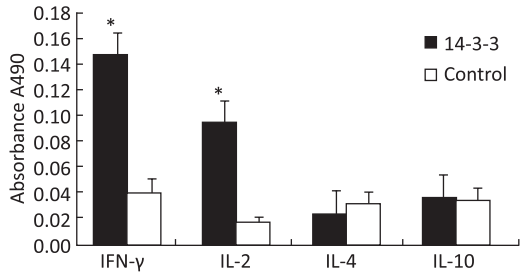

Figure 5. Cytokine IFN-γ ,IL-2,IL-4 and IL-10 responses of splenocytes from ICR mice. (*Cytokine levels differ significantly between the 13-3-3 group and the control group, P<0.05)

DISCUSSION

In this study, we report the protective immunity of promising vaccine candidates, 14-3-3 proteins from the *E.granulosus*. The putative importance of 14-3-3s in the Helminth development makes it a potential drug and vaccine target. More extensive characterization of the 14-3-3 proteins has been performed in *Schistosoma* spp and *Echinococcus* spp. Using a cocktail including *S. japonicum* 14-3-3 vaccinated mice and upon cercarial challenge, Zhang and etc reported that the protective immunity could reach up to 45%[20]. The 14-3-3 from *S.mansoni* have also been used for vaccination against the homologous infection in mice. The use of the recombinant Sm14-3-3-1 protein alone or as a fusion protein with *S. japonicum* glutathione-Stransferase (GST) can induce protective immunity more than 46% upon challenge infection. For *Echinococcus*[21], the fact that 14-3-3 protein expression has been identified at different parasite stages provided a solid basis to investigate their potential as vaccine candidates against echinococcosis. The Em14-3-3 recombinant protein was used to vaccinate mice subsequently challenged with *E. multilocularis* egg infections. Liver lesion counts in vaccinated animals were reduced from 43 mean lesions per animal to 1 mean lesion per animal[22]. This high reduction was similar to that obtained in Em95-vaccinated mice (78.5%-82.9% protection against parasite infection)[23-24]. In this study, we expressed a 31 KD recombinant 14-3-3 protein of Chinese strain. The recombinant 14-3-3 protein was verified to possess antigenicity and immunogenicity, as it can induce a specific antigen response in Wk 2 vaccinated ICR mice. Additionally, the sera from the recombinant 14-3-3 -vaccinated mice can recognize specifically the natural antigen from protoscoleces, suggesting that the rEg14-3-3 has the same epitope with natural antigen and could potentially act as a vaccine to immunize mice. Thus, it might induce the host to produce a specific immunity response against *E. granulosus*. Treatment with r14-3-3 protein might stimulate the host to produce a specific immune response against E. granulosus. Our results of the challenge infection confirmed the presumption that a significant reduction in the parasite burden was obtained by rEg14-3-3 immunization. Finally, rEg14-3-3 protein reduced both the number and the size of cysts in mice. In summary, these results suggest that rEg14-3-3 have the potential to be applied as a vaccine against *E. granulosus*.

We found that the rEg14-3-3 vaccine could induce significant cellular and humoral immune response. High levels of rEg14-3-3 IgG were detected in the serum from mice vaccinated with rEg14-3-3 after Wk 6. Thus, rEg14-3-3 vaccination can induce the production of specific rEg14-3-3 antibodies in mice. Simultaneously, T lymphocytes from mice immunized with rEg14-3-3 showed a significant proliferation response to rEg14-3-3.

Numerous studies have demonstrated that humoral immunity plays a crucial role in the protection against *E. granulosus*. Specific antibodies against rEg14-3-3 can mediate the protective immunity[25]. As shown in this study, rEg14-3-3 induced production of specific antibodies in mice. The protective efficacy of humoral immunity in *E. granulosus* was correlated not only with the level of IgG, but also with the isotype of IgG. The prevailing isotypes of IgG induced by rEg14-3-3 in mice were IgG1 and IgG2a, implying that specific antibodies induced by rEg14-3-3 antigen can induce protective immunity.

Little is known about the cellular responses generated by the *E. granulosus* and *E. multilocularis* recombinant vaccines. The secondary *E. granulosus* hydatid cysts in mice immunized with the BCG- EG95 were reduced significantly, and this was associated with elevated levels of IL-2, IFN-γ, and decreased IL-4, suggesting that Th1 responses may play a major role against challenge infection in this vaccine mode[26]. Furthermore, mice immunized with *E. multilocularis* BCG-Em14-3-3 vaccines also induced significant Th1 responses in mice challenged with *E. multilocularis* protoscoleces[27]. Protection conferred by rEg14–3-3 vaccination against *E. granulosus* protoscoleces-challenged in ICR mice appears to include both specific cellular and humoral responses, as the immunity response of sj14-3-3 and sm14-3-3 vaccine protected mice against challenge infection separately[28-29]. The *E.granulosus* vaccine trials reported here indicate that rEg14-3-3 is highly immunogenic and induces a strong Th1-like cellular response, as shown by the production of a high level of IFN-γ, as well as IgG2 antibody response. It is known that Th1 cells producing IL-2 and IFN-γ can activate macrophages, mediate delayed–type hypersensitivity and induce the production of IgG2a[30]. In contrast, Th2 cells induce the production of IL-4, IL-5, IL-10, and IL-13[31]. We showed that treatment with rEg14-3-3 preferentially produced IgG2a. The levels of IFN-γ in the splenocytes supernatants were significantly induced in mice

treated with rEg14-3-3, whereas the IL-4 levels did not change. Thus, both the antibody response and the cytokine production pattern reveal that Th1 response is the predominant cell immunity response to the vaccine antigens.

In conclusion, we have demonstrated that rEg14-3-3 vaccine is capable of inducing strong and effective protection against *E.granulosus* protoscoleces-challenged infection. Since both cellular and humoral responses were detected in Eg14-3-3-immunized mice, combined effects of the two types of responses could account for the protection. Further studies are therefore necessary to evaluate whether modification of immunization protocols and use of different adjuvant will lead to protection against *E.granulosus* in all its development stages.

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