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Hamid Reza Rahimi, Bahador Sarkari, Tahereh Mohammadzadeh, Seyed Mahmoud Sadjjadi

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Immune Responses to Antigens of *in vitro* Reared *Echinococcus granulosus* Adult Worms in Balb/c Mice

Hamid Reza Rahimi[1], Bahador Sarkari[1,2], Tahereh Mohammadzadeh[1], Seyed Mahmoud Sadjjadi[1,2*]

[1]Department of Parasitology and Mycology, School of Medicine, [2]Center of Basic Researches in Infectious Diseases, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Cystic echinococcosis (CE), also known as echinococcosis/hydatidosis, is one of the most important parasitic diseases in the world. It enhances both humoral and cellular (Th1 and Th2) responses in infected host. Different antigens of the worm may favor the Th1 or Th2 immune responses in CE patients. **Objective:** To evaluate the humoral and cellular immune responses of Balb/c mice against the crude and excretory/secretory (E/S) antigens of *in vitro* reared *Echinococcus granulosus* adult worms. **Methods:** A total of 20 Balb/c mice divided into 5 groups of 4 mice each. Three groups of mice (n=4) were immunized with crude, E/S and an immunodominant antigen of *in vitro* reared *Echinococcus granulosus* adult worms on day 1 and 28. The fourth and the fifth groups were negative control groups and received PBS plus adjuvant, or nothing, respectively. Two weeks after the second injection, the mice were killed and their blood was collected for determining antibody responses, and their spleens were employed for proliferation assay. Total IgG were measured by indirect ELISA. Spleen cells of immunized mice were cultivated and exposed to different antigens of adult worms including E/S and crude antigens. Level of IFN-γ, IL-12, IL-4 and IL-10 were measured in the recovered cell culture supernatants by capture ELISA. **Results:** Total IgG assay showed the highest level of antibody produced in mice immunized with crude antigens. Proliferation assay showed a statistically significant production of cytokines in the mice immunized with crude antigens ($p<0.05$). The highest levels of IFN-γ, IL12 and IL-4 were produced in mice immunized with crude antigen of the *in vitro* reared *Echinococcus granulosus* adult worms followed by E/S antigens. Immunodomonant antigen induced the lowest levels of cytokines (IL-12, IFN-γ, IL-4 and IL-10) in immunized mice. **Conclusion:** A significant levels of Th1 related cytokines (IFN-γ and IL-12) were produced in Balb/c mice immunized with crude antigen of the *in vitro* reared *Echinococcus granulosus* adult worms.

Keywords: Antigen, *Echinococcus granulosus*, Excretory, Mice

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*Corresponding author: Prof. Seyed Mahmoud Sadjjadi, Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, Tel/Fax: (+) 98 711 2305291, e-mail: smsadjjadi@sums.ac.ir

INTRODUCTION

Echinococcosis/hydatidosis, is one of the most important zoonotic parasitic diseases in the world (1,2). It is caused by the larval stage (metacestode stage) of the tape worm *Echinococcus granulosus* (3). Accidental rupture of cysts or rupture during surgery of cysts may result in an important medical problem (4). This is due to the ability of protoscoleces (PSC) to develop into new cysts (3). In this regard, it is very important to kill the protoscoleces before the removal of the cyst from human body (4,5). This parasite enhances both humoral and cellular responses in its intermediate host (6,7). Humoral responses will result in the production of immunoglobulins which are important for the diagnosis of patients (8-11). However, cellular responses also will take place in the infected host which are important criteria for the prevention of the disease (12,13).

Prominent quantities of IFN-γ, IL-4, IL-5, IL-6 and IL-10 observed in the majority of patients with hydatidosis supports Th1 and Th2 cell activation in CE patients. In particular, Th1 cell activation seems to be more related to protective immunity, while Th2 cell activation is linked to the susceptibility to the disease (14).

Infection of Balb/c mice with protoscoleces of *Echinococcus granulosus* has been used as a model for the study of secondary hydatidosis as well as associated immune responses in immunization and infection trials (15-19). It has been demonstrated that injection of live PSC of *Echinococcus granulosus* in peritoneal cavity of animal models is associated with the induction of a Th2 response, which appears to be important for parasite survival, whereas dead PSC induces a dominant Th1 response (19). A dominant Th1 response is mainly associated with the killing of injected PSC while the cyst development is related to Th2 responses (20).

Mice immunized with antigen B of *Echinococcus granulosus* secrete a high level of interferon gamma (IFN-γ) while a marked increase in interleukin-4 (IL-4) production has been seen in the mice immunized with crude sheep hydatid fluid (CSHF) or protoscoleces homogenate (17).

Different antigens from different stages of the worm life cycle has been used in favor of the immune responses in definitive and intermediate hosts (17,20,21); however, no study has been carried out using antigens of *in vitro* reared adult worms, so far.

In the present study immune responses against the excretory/secretory (E/S), crude and immuno-domonant antigens of *in vitro* reared *Echinococcus granulosus* adult worms were evaluated in Balb/c mice.

MATERIALS AND METHODS

Production of *in vitro* Reared Worms by Cultivation of Protoscoleces. Cultivation of the protoscoleces to adult worms were carried out as stated by Mohammadzadeh et al. (22). Briefly, sheep liver and lung hydatid cysts were collected from Shiraz abattoirs in Fars province, south of Iran. A total of 10,000 protoscoleces (PSCs) with viability over 85% were chosen, using eosin staining method for each culture. *In vitro* cultivation of protoscoleces was performed as originally described by Smyth et al. (23) with some modifications. The larvae were cultivated in a biphasic medium, containing coagulated bovine serum as the solid phase and the CMRL

1066 culture media (Biosera) as the liquid phase. Other supplements such as serum was added to the medium as described by Smyth et al. (23) and modified by Mohammadzadeh et al. (22).

Preparation of Excretory/Secretory and Crude Antigens. For preparation of excretory/secretory (E/S) antigens; the *in vitro* reared adult worms were harvested from the culture flasks and transferred to the protein free media (CMRL 1066, pH=7.4) supplemented with glucose (4.0 g/l) and gentamicin (200 µg/ml), and stored at 37°C in CO_2 (5%) incubator. The supernatant was collected every 12 hours (up to 48 hours) and dialyzed overnight against PBS and stored at -20°C until use. Freezing, thawing and homogenization were applied on all adult worms (n=1000) followed by centrifugation at 7500 × g for 30 min at 4°C. The supernatants were collected and stored at -20°C until used as crude antigens.

Preparation of Immunodomonant Antigens of *in vitro* Reared Adult Worms. The immunodomonant antigens of *in vitro* reared adult worm antigens were detected by SDS-PAGE and Western blotting by the method described by Sarkari et al. (23). In this regard, crude antigens of *in vitro* reared worms were separated by SDS-PAGE and screened with a panel of human sera from CE patients (n=40) and healthy subjects (n=40). The band which was present in immunoblotting of all of the CE patients and not in any of the healthy subjects was considered as immunodominant antigen. The antigen was extracted from polyacrylamide gels as follows: A 12.5% polyacrylamide gel electrophoresis with crude antigen (20 µg) was used. A strip was on the right or the left side of the gels, stained with Coomassie blue, aligned with the unstained gel. The area in the unstained gel corresponding to the stained band was cut, and its protein content was extracted using the elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH=7.5) followed by incubation in a rotary shaker at 30°C overnight and centrifugation at 7500 g for 10 minutes. Supernatants were stored at -20°C until use. (Tech Tip N.51, Thermo Scientific, www.thermo.com/pierce, Accessed on 10, Sept, 2011).

Immunization of Balb/c Mice. Three groups of female Balb/c mice (n=4 in each group) were used for immunization purposes. In addition, two groups of mice were considered as negative controls (receiving PBS plus adjuvant or left without injection).

Mice were subcutaneously injected with 1µg/µl of each antigen on day 1 and 28. The first injection was with an equal volume of Freund's complete adjuvant (Sigma, USA) while the second injection contained Freund's incomplete adjuvant (Sigma, USA). Two weeks after the second injection the mice were killed and their blood collected for the evaluation of humoral and cellular responses.

Analysis of Antibody Responses by Enzyme-Linked Immunosorbent Assay (ELISA). Total IgG antibody responses to each antigen were measured by indirect ELISA against the negative control group (without any injection) and those which had received PBS and adjuvant. Microplates were coated with either 5µg/ml (100 µl/well) of crude, E/S or immunedominant antigens and incubated at 4°C overnight. Each antigen was tested with the sera of mice which were immunized with the same antigen. The sera of negative control and PBS plus adjuvant groups were tested with the three different antigens mentioned above. Excess antigen was removed by washing the plates five times in phosphate buffered saline-Tween 20 (PBST 0.05%, pH=7.4). Blocking was done with 3% skimmed milk in PBST for 1.5 hours. The wells were washed and 100 µl of serum samples from each mouse (1/100 dilution in PBST) was added to each microplate well. Anti-mouse IgG conjugated with horseradish peroxidase was used as a

secondary antibody. ELISA plates were developed with O-phenylenediamine substrate, and the optical density (OD) was measured at 495 nm. Serum antibody titers were determined by measuring the last dilution that resulted in three standard deviations above the negative controls.

Proliferation and Cytokine Assay. Mice spleens were removed from different groups, homogenized and their lymphocytes were isolated using Ficoll (25). The cell suspension was washed with complete RPMI medium, followed by resuspension in RPMI medium containing FCS (10%) and gentamycin (100µg/ml). Cell suspensions were added to 96-well plates (1.5×10^5 cells/well) and were supplemented either with the medium alone, Concanavalin A (Sigma, USA), or with either 20 µg of crude, E/S or immunodomonant antigens. The treated cells were incubated at 37°C for 3 days.

The supernatants were collected 72 hours post cultivation and stored at -70°C for cytokine assay. Sandwich ELISA kits were used to determine the concentrations of IFN-γ, IL-12, IL-4 and IL-10 in the recovered supernatants as recommended by the manufacturer (Mabtech, Sweden). Cytokine levels of spleen cells of non-stimulated mice group (negative control) and the mice stimulated with PBS and adjuvant were compared.

Statistical Analysis. The data were statistically analyzed using SPSS version 16 software. Kruskal-Wallis test (non-parametric independent group comparisons) was used to compare the mean values of cytokines.

RESULTS

Selection of the Immunodominant Antigen. Screening of sera from pathologically confirmed CE patients along with those from healthy subjects was performed using Western blotting. A band close to 28 KD was detected in all of positive cases but not in healthy subjects (Figure 1).

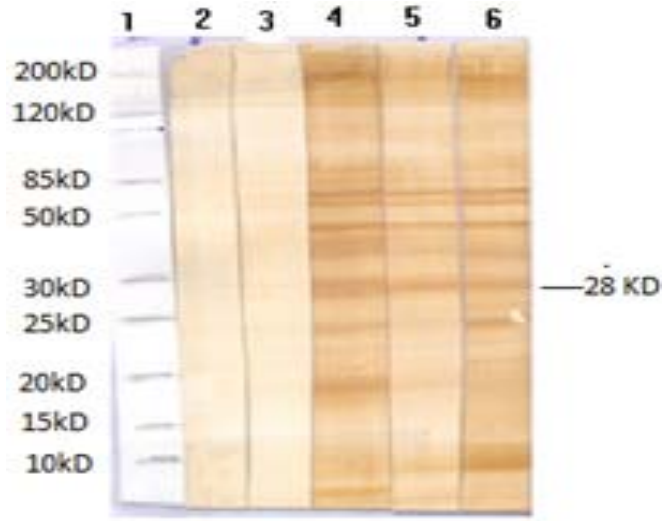

Figure 1. A nearly 28 kDa antigen of reared adult worms detected in the sera of cystic echinococcosis patients. 1: molecular marker, 2 and 3: sera from healthy subjects; 4- 6: sera from confirmed echinococcosis patients.

Serum Antibody Responses Following Immunization with ES, Crude and Immunodominant Antigens. Total IgG assay showed the highest level of antibody production in mice immunized with crude antigens followed by those immunized with ES and the immunodominant antigens. The mean optical densities (OD) of the aforementioned groups were 1.450, 0.797 and 0.384 at 495 nm, respectively. The mean OD of the negative control group which had no injections and the group which had received adjuvant and PBS were 0.114 and 0.147 at 495 nm, respectively.

Cytokine Assay. Results of proliferation assay showed a significant production of cytokines in the mice immunized with different antigens (Figure 2). The differences between controls and antigen-immunized mice in the production of cytokines were statistically significant ($p<0.05$).

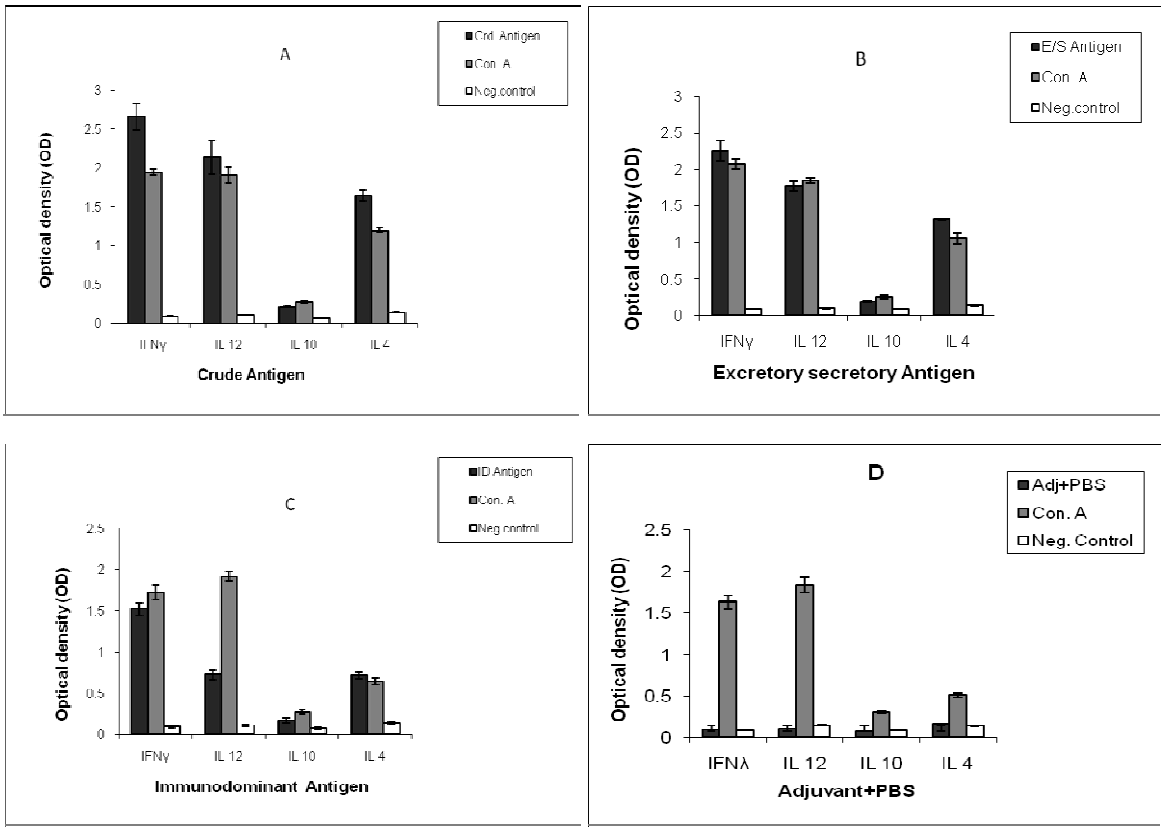

Figure 2. Cytokine production in mice immunized with crude (A), E/S (B) and immune-dominant (C) antigens and adjuvant plus PBS (D). Crd: Crude; Con A: concanavalin A; Neg: negative; ID: immunedominant antigen.

The highest levels of IFN-γ and IL-12 were produced in mice immunized with crude antigens of the *in vitro* reared *E. granulosus* adult worms (Figure 2A) while a high level of IL-4 was also produced by those mice immunized with the crude antigens (Figure 2A). Furthermore, a significant level of IL-4 was produced in mice immunized by the crude antigen followed by the ES antigen (Figure 2B). The lowest levels of all four cytokines (IL-12, IFN-γ, IL-4 and IL-10) were observed in mice immunized by the immunodominant antigen (Figure 2C).

Production of Th1 related cytokines (IFN-γ and IL-12) was prominent in the immune cells of Balb/c mice following immunization with crude antigens of *in vitro* reared adult worms, although the level of one of Th2 related cytokines (IL-4) were also high in mice immunized with this antigen. Cytokines production by the group receiving only PBS and adjuvant is shown in Figure 2D.

DISCUSSION

The aim of the present study was to explore the immune responses of the antigens of the *in vitro* reared adult worms of *Echinococcus granulosus* in Balb/c mice which is a suitable experimental model for CE. Many studies have been done on immunological responses against cystic echinococcosis in humans and animals (14,17,18,20,26). However, in such studies, researchers have been using different antigens such as hydatid cyst fluid, homogenated psc, eggs and oncosphere, etc. from *Echinococcus granulosus*. These antigens were obtained either from adult worms isolated from dog intestine, or from hydatid cyst fluid of the intermediate hosts. In the present study, antigens from *in vitro* reared *Echinococcus granulosus* adult worms were used and their immune responses in Balb/c mice were evaluated.

The level of IL-4, IL-5 and IL-10 in patients with hydatidosis have been evaluated and a highly significant level of these cytokines have been observed in hydatidosis patients in comparison with the healthy controls (26). This means that infection of the intermediate hosts with one of the live stages of *E. granulosus* induces a dominant Th2 immune response. Dead antigens from different stages of *E. granulosus* induce Th1 immune responses that are effective in protection against the disease (19). In our study, following immunization of Balb/c mice with antigens of the dead reared *E. granulosus* induction of dominant Th1 immune response was observed.

Production of Th1 and Th2 related cytokines and the cyst load in different groups of 3 Balb/c mice per group using intra-peritoneal injection of live protoscoleces at different time intervals were assessed by Rogan, who demonstrated a Th2 dominant activity with elevated IL 4 and IL 10, three months post infection. This is an indication of the establishment of the cysts in such mice (20).

Antibody and cytokine responses have been studied in Balb/c mice immunized with *Echinococcus granulosus* crude sheep hydatid fluid (CSHF), antigen B (AgB) and protoscoleces homogenate (PSH) in Al-Qaoud et al. study (18). In their work, they demonstrated that the intramuscular administration of CSHF induces a Th1 response in immunized mice, while the intra-peritoneal immunization with PSH induced both Th1 and Th2 responses. Intra-peritoneal immunization of mice with AgB resulted in a dominant Th2 response. In addition, immunization of Balb/c mice using the subcutaneous route with the above mentioned antigens induced the production of a highly significant level of total IgG (especially IgG1) compared with other routes of immunization such as intramuscular and intraperitoneal. In our study, a high level of total IgG was produced after subcutaneous immunization of Balb/c mice with reared *E. granulosus* adult worm's antigens. In another study conducted by Hashemitabar et al. (27), lambs immunized with formalin fixed *E. granulosus* adult worms crude antigens from dog intestine showed a higher level of antibody production in comparison with those which were immunized with the hydatid fluid. They also reported that the level of antibody produc-

tion between lambs immunized with hydatid fluid and the protoscoleces was not statistically different.

Moreno et al. studied total IgG antibody production in 6 dogs and showed a significant increase of total IgG at days 14 and 28 post infection (21). In our study a significant level of total IgG were also obtained on day 14 after the second immunization of Balb/c mice.

Increased antibody response (IgG and IgM) was seen after infection of Balb/c mice with viable PSCs (28). The level of IgG, especially IgG1 against oncospheral antigens increased from week four post infection (29). It means that the antigens prepared from the whole *E.granulosus* organism might be a suitable candidate for research in immunization and diagnosis of hydatidosis in intermediated hosts.

Inoculation of dead PSC induced a dominant Th1 response that was found to be associated with the killing and clearance of injected PSCs (20), while the Th2 activity with elevated IL-4 and IL-10 were obtained in Balb/c mice spleen cell culture supernatant after challenging with live PSCs (19). However such data are not available with crude and ES antigens of *in vitro* reared adult worms of *E. granulosus* and this has been focused in our study. In the present study reared adult worms of *E.granulosus* crude, E/S and one of the immunedominant antigens were used to immunize Balb/ c mice. Since working with adult worms of *E.granulosus* isolated from dogs has a potential risk of contamination for persons who are handling the worms, use of *in vitro* reared adult worms seems to be more suitable for immunization purposes including vaccine development or diagnosis oriented studies especially in the definitive host. These worms do not produce fertile eggs (22).

Our results demonstrated a high level of IFN-γ followed by IL-12 (Th1 responses) and the secretion of IL 10 and IL4 (Th2 responses) in response to the crude antigens followed by the E/S and the immunodominant antigens, respectively. However, simultaneous elevated levels of IFN-γ and IL-4 were seen in mice immunized with the crude antigens.

Although a high level of total IgG was produced following the immunization of the mice with different antigens, such immune responses were more prominent in mice which were immunized with the crude antigens. Simultaneous elevated levels of IFN-γ, IL-4, IL-5, IL-6 and IL-10 were detected in the majority of CE patients is an indication for the activation of Th1 and Th2 cells in CE (14). In particular, Th1 cell activation seems to be more related to protective immunity, whereas Th2 cell activation is related to the susceptibility to disease.

Considering a high level of Th1 related cytokines (IFN-γ and IL-12) produced by the immune cells of Balb/c mice, following immunization with antigens of *in vitro* reared adult worms of *E. granulosus,* it can be suggested that these antigens might be a suitable candidate for vaccination trials in experimental animal models (15,16,30). Mass *in vitro* cultivation of protoscoleces and converting them to adult worms will produce enough of such antigens for future studies.

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