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Determination of Hydatid Cyst Fluid and Protoscolex Peptides Protective Effect in Mice

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Abstract: The aim of this study was to compare immunization of mice against hydatid cyst with two less than 30 kD antigens of fluid and protoscolex. Hydatid cyst fluid was aseptically aspirated from individual fertile cysts, clarified by centrifugation at 5000 × g for 15 min at 4 °C and concentrated and dialyzed with PBS using an Amicon filter (USA). Protoscolices were washed 3-times with PBS and sonicated with ultrasonic disintegrator, for 3 ×15 seconds on ice. The prepared solution was centrifuged for 30 min at 5000 × g and then filtered by Amicon Ultra. After purification of less than 30 kD proteins by Amicon filter, protein measurement was done by Bradford method. Then with the help of freeze drying device it was transformed to powder form, prepared for injection. Twenty seven mice were randomly divided into 3 groups of 9 mice (two immunized and one control group). Results showed that the level of antibody in case groups was significantly higher ($P< 0.05$) than control group. The mice immunized with hydatid cyst fluid less than 30 kD proteins produced higher antibody than mice immunized with protoscolex less than 30 kD proteins. Findings of this study indicated that the protective immunity in vaccinated mice was 100%. A mouse model has been developed to evaluate potential protective antigens which could render intermediate hosts resistant to a challenge infection with *Echinococcus granulosus* protoscolex in experimental infection.

Key words: Hydatid cyst · Taenia · Antigen · Protoscolex · Immunization

INTRODUCTION

Cystic echinococcosis, caused by the larval stage of *Echinococcus granulosus*, is a global public health problem. Whilst in a few localities such as New Zealand, the parasite has been effectively controlled or even eradicated; in most endemic regions it remains a persistent problem [1]. In spite of successful control programs in some countries or regions, the parasite still has a very wide geographical distribution. There is clear evidence for the emergence or re-emergence of human cystic *Echinococcosis* in parts of China, central Asia, Eastern Europe and Israel [2]. However, there is a clear need for new advances in the prevention of echinococcosis [3].

Hydatid cysts of *E. granulosus* develop in internal organs (mainly the liver and lungs) of humans and intermediate hosts such as sheep, horses, cattle, pigs, goats and camels [4] as unilocular fluid filled bladders [5]. Taenia (cestoda) antigens (derived from adult worms, metacestod stages or oncospheres) interact with the immune system of the host and may lead to the production of specific antibodies. It is generally accepted that *Echinoccocus* spp is unaffected by the immune response during the developing stage [6]. Enzyme-linked immunosorbent assay (ELISA) based on detection of these antibodies has been developed for diagnosis of *Echinococcus* infection in definitive hosts. Over the past 30 years the use of human infections has been very evident, largely through the detection of parasite specific antibody in the serum. In the last decade more advanced approaches such as circulating antigen detection, lymphocyte proliferation responses and cytokine analyses and molecular techniques have been applied to provide useful information on these infections [7].

However, it is still antibody detection which is most widely used for confirmation of clinical diagnosis and in epidemiological surveys [8]. Furthermore, experimental infections of mice with eggs or oncospheres of *E. granulosus* showed that susceptibility varies with different strains of mice. *In vitro* experiments have also shown that neutrophils, in association with antibodies, can bring about the killing of *E. granulosus* oncospheres, suggesting a possible role for antibody-dependent cell-mediated cytotoxicity reactions. At the early stages of disease, there is a marked activation of cell mediated immunity to the parasite [9]. The purpose of this study

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was to determine whether immunization of mice with hydatid fluid and protoscolices antigens might induce the humoral immune response and antibody production against the two antigens.

MATERIALS AND METHODS

E. granulosus hydatid cysts were obtained from the livers of sheep (2–3 years old animals) slaughtered at abattoirs in Amol, Mazandaran, Iran. Hydatid cyst fluid was aseptically aspirated from fertile cysts, centrifuged at 5000 × g for 15 min at 4 °C and concentrated and dialyzed against PBS using an Amicon Ultra-30, 30,000 MWCO centrifugal filter device (Millipore,USA).

Protoscolices were washed 3-times with PBS and Hank's salt solution (Sigma, St. Louis, USA) containing 100 U/ml of penicillin G and 100 mg/ml of streptomycin sulfate. Samples were freeze-thawed 3 times and mixed with four volumes of PBS, pH 7.4, containing sodium azide at 0.1 mg/ml. Samples were then sonicated at 110 V, 170 W ultrasonic disintegrator (Hielscher, Germany), for 3×15 seconds on ice. The prepared solution was then left on ice for one hour and centrifuged for 30 min at 5000×g and then filtered by Amicon Ultra. Protein concentrations were measured by Bradford method and kept at -20°C until used.

Immunization and Challenge: Twenty seven mice (Wistar strain, male, 2-3 month old) were randomly divided into 3 groups of 9 mice (two immunized and one control group). The mice in groups one and two received 100 μg (100 μl) of less than 30 kDa *E. granulosus* antigens of hydatid cyst fluid and Protoscolex plus 100 μl of Freund's complete adjuvant, respectively. Mice in control group were immunized with adjuvant in PBS. For second immunization after two weeks mice were treated with the same solution plus Freund's incomplete adjuvant. Two weeks after the second immunization, each mouse was challenged with 1000 protoscolices intraperitoneally as described previously. Mice were sacrificed by CO2 five months post challenge. Blood samples were collected before each immunization from mice and sera were separated by centrifugation at 5000×g for 3 min after placing the samples at 4°C overnight. Sera were stored at -20°C until used.

ELISA: To screen the activity of antibody against two types of protein antigens (hydatid fluid and protoscolices antigens), ELISA was carried out as described by [10]. Assays were carried out in 96-well micro-titer plates. 100 μl of all the solutions (less than 30 kDa proteins) were used per well, except the blocking solution (BSA 3%) which 300 μl of it was used. The wells were washed four times with PBS (pH 7.2) containing 0.1% v/v of Tween-20. Appropriate wells were coated with 5 μg of hydatid fluid and Protoscolex of *E. granulosus* proteins in 100 μl of 0.1 M $NaHCO_3$ and the plates were then left exposed to air overnight at room temperature to allow the solution to dry. The following day, after washing, wells were blocked with 300 μl of 3% (w/v) bovine serum albumin (BSA) in PBS and then incubated for 2 h at room temperature to block any remaining unblocked attachment sites on the wells. After washing the wells, diluted sera of mice were added and then the plates were incubated 1 h at room temperature. The plate was washed and then the second antibody, sheep anti-mouse IgG HRP phosphatase (Sigma, USA) was added at 1:5000 dilutions into all the wells and was incubated for 1 h at room temperature. The plate was washed as described above to remove the excess conjugates. For color development, 100 μl of TMB was added to each well as a substrate and the reaction was terminated after 15 min by adding 100 μl of 1M HCL solution to each well. The absorbance at 490 nm was measured in ELISA reader (Bio-Rad, California, USA).

Dot Blot Protocol: Have nitrocellulose membrane ready, grid was drawn by pencil to indicate the region going to blot. Then using narrow-mouth pipet tip, 1 μl of samples spoted on to the nitrocellulose membrane at the center of the grid. The membrane was allowed to dry, then non-specific sites were blocked by soaking in 5% BSA in TBS-T (0.5-1 hr in Room Temperature). 10cm Petri Dish was used for reaction chamber. Incubated with primary antibody (0.1-10 μg/ml for purified antibody, 1:100 dilutions for Anti sera) dissolved in BSA/TBS-T for 30 min at RT. Washed three times with TBS-T (3×5 min). Then Incubated with secondary antibody conjugated with HRP (for optimum dilution, follow the manufacturer's recommendation) for 30 min at RT. After that were washd three times with TBS-T (15 min×1, 5 min×2), then once with TBS (5 min). Then Incubated with ECL reagent for 1 min, covered with Saran-wrap (excessive solution from the surface were removed) and were exposed to X-ray film in the dark room.. Finally the signal from unknown sample was compared to that of standard and the concentrations were estimated.

Statistical Analysis: The results of these experiments were analyzed by Non parametric t-test and Repeat measure using SPSS version 10. Significant association was identified when P-value of less than 0.05 was observed.

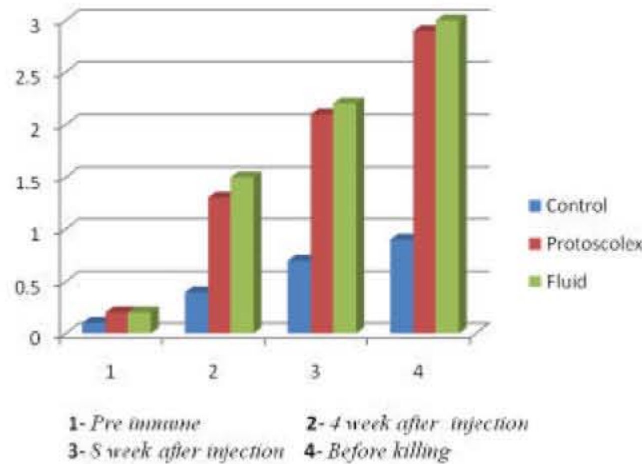

Fig. 1: Mean absorbance of test and control groups antibody detection again hydatid cyst on day 0, 28 and 56 and before killing

Fig. 1: Dot blot of hydatid cyst infected mice and comparison with control group

RESULTS

Results showed that the level of antibody production by using these two antigens was different and in all of these groups (mice which were immunized with hydatid cyst fluid and protoscolex of *E. granulosus* less than 30 kDa proteins) level of antibody was significantly higher ($P< 0.05$) than control group. The mice immunized with hydatid cyst fluid produced higher antibody ($P< 0.05$) than mice immunized with Protoscolex on day 28 (four weeks after the first immunization). Level of antibody on day 56 (8 weeks after the second immunization) in mice immunized with hydatid cyst fluid was higher than in Protoscolex group ($P< 0.05$).

Results of dot blot test also showed that 1/400 diluents of serum in comparison with other dilutions was the best titer for causing immunization (Figure 1).

DISCUSSION

Findings of this study showed that the protective immunity in vaccinated mice was 100%. A mouse model has been developed to evaluate potential protective antigens which could render intermediate hosts resistant to a challenge infection with *Echinococcus granulosus* protoscolex in experimental study [11]. Hashemi tabar in Mashhad showed that none of the vaccinated mice with the whole body of *E. granulosus* had cysts which indicated 100% protective immunity and also showed that in all of the control mice there were a lot of cysts in internal organs after 8 months [12]. Also he indicated that the level of antibody in mice which were immunized with adult worms of *E. granulosus* on day 28 was seven times higher than before immunization and was higher than hydatid cyst fluid and protoscolex. Level of antibody in mice immunized with adult worms of *E. granulosus* was also higher than in hydatid cyst fluid and protoscolex groups at day 49 [13].

In our study, the levels of antibody production against two groups of less than 30 kDa antigens (fluid and protoscolex) were evaluated in mice. Mice immunized with Protoscolex did not produce high level of antibody four weeks after the first immunization in comparison to those immunized by fluid. Antibody produced by hydatid cyst fluid of *E. granulosus* was 4 to 5 times more than before immunization. Lin et al., reported that the specific IgG was induced during the 3rd week and continued to increase until week 10 [14]. Although the level of antibody on day 56 and before killing was very higher than controls, but the level of antibody in mice immunized with fluid antigen of *E. granulosus* was only slightly higher than protoscolex group.

It is suggested that this antigen is a good candidate for protection against hydatid cyst. Infection of BALB/c mouse with protoscolices of *E. granulosus* develops a model for the study of secondary hydatidosis and the associated immune response in immunization and infection trials [15].

Immunogenicity of two *Echinococcus granulosus* antigens EgA31 and EgTrp in mice were investigated by ELISA and these antigens elicited high antibody titer and so use of both antigens separately or in combination as candidate vaccine proteins is discussed [16]. It has been shown that rEgP-29 induced high levels of specific antibodies in mice after the third immunization. The protective efficacy of humoral immunity in *E. granulosus* was not only correlated with the level of IgG, but also associated with the isotype of IgG [17].

Zhang showed that mice produced lower levels of antibodies than of a secondary challenge infection given three weeks later by a different route (intraperitoneal, subcutaneous or intravenous injection). Most mice did not evoke significant antibody responses against oncospheral antigens until five weeks post-infection [18]. Antigens derived from cyst fluid [19] and protoscolices [20] have been used against *E. granulosus* and mice produced a higher level of antibody against both antigens. Although two type of peptide antigens described in this paper can be used for ELISA test, but it is necessary to study and compare the specificity and sensitivity of these antigens in diagnosis of hydatid cyst.

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