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Short Paper

Antibody response against hydatid fluid, protoscolex and whole body of *Echinococcus granulosus* antigens in lambs

Hashemi Tabar, G. R.[1, 2*] **and Razmi, G. R.**[1]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2]Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

[*]**Correspondence:** G. R. Hashemi Tabar, Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: hashemit@um.ac.ir

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Summary

In this study, the immunogenicity of three types of antigens (hydatid fluid, protoscolices and whole body of *E. granulosus*) was investigated in lambs by ELISA. Sixteen 4–6-month-old lambs of mixed sexes were divided into 4 groups of 4 lambs (three immunized and one control group). Twelve lambs as immunized groups received 2 mg of hydatid fluid, protoscolices and whole body of *E. granulosus* antigens dissolved in 1 ml of PBS per immunization for each lamb, respectively. As an adjuvant, Freund's complete adjuvant (FCA) was mixed with antigens to form a water-in-oil emulsion which was inoculated subcutaneously on day 1 of the trial. Each control lamb was inoculated with a total of 2 ml of PBS emulsified in equal volumes of FCA. Lambs were boosted on day 28 with the same preparation as described above except that FCA was replaced by Freund's incomplete adjuvant (FIA). Three weeks after the second immunization, each lamb received a challenge infection with 2000 protoscolices intraperitoneally and also 10 gravid proglotid of *E. granulosus* orally. Sera were collected before and after immunization and serum antibodies were tested by ELISA. The results showed that the production of antibody had a significant difference between the test groups and the control ($P<0.05$). Lambs immunized with whole body of *E. granulosus* showed the highest antibody production. The level of antibody production between the lambs immunized with hydatid fluid and the protoscolices was not different significantly ($P>0.05$), whereas, the level of antibody production between the lambs immunized with hydatid fluid and whole body of *E. granulosus* was different significantly ($P<0.05$). The results of this study showed that the antigens of whole body of *E. granulosus* might be a good candidate for immunization and diagnosis of hydatid cyst in the intermediate hosts of *E. granulosus*.

Key words: *Echinococcus granulosus*, Antibody, ELISA, Lambs

Introduction

Hydatid cysts of *Echinococcus granulosus* (*E. granulosus*) develop in internal organs (mainly liver and lungs) of humans and intermediate hosts (such as sheep, horses, cattle, pigs, goats and camels) as unilocular fluidfilled bladders (Zhang *et al*., 2003). Cystic echinococcosis (CE) is a major public health problem in sheep-raising regions of the World (Moro *et al*., 2005). Cystic echinococcosis in farm animals causes considerable economic problems due to the loss of edible liver. Significant loss of meat and milk production, as well as the value of the fleece from infected sheep may also occur. These losses are of special significance in countries of low economic output where sheep production is of particular importance (Torgerson *et al*., 2001). There are no reliable methods for the routine diagnosis of infection in living animals, but in rare cases cysts have been identified by ultrasonography alone or in conjunction with serum antibody detection (Eckert *et al*., 2001). It is generally accepted that *Echinoccocus* is unaffected by the immune response during the developing

stage. However, natural infections in sheep indicate that some cysts can be destroyed during the latter stages of development, with the relatively frequent occurrence of dead, calcified metacestodes or necrotic cysts (Eckert and Deplazes, 2004). These are due to the degeneration of the primary cyst, leaving the cavity full of host leukocytes and protoscolex-derived daughter cysts. There is no direct evidence that the death of such cysts is due to an immunological phenomenon, but it is a likely possibility. If a progression in cyst degeneration does take place, then the immune response may play a role in the death of the parasite. This may signify increased immunological stimulation with cyst progression. Unfortunately, there are no detailed studies of immunological events associated with the degeneration of different types of cyst (Zhang *et al*., 2003). In the present research, we investigated whether immunization of lambs with three antigens could produce specific antibody responses against challenge with protoscolices and adult *E. granulosus* worms.

Materials and Methods

Preparation of samples

Sheep cystic livers and lungs and positive blood (blood from infected sheep) samples were prepared. Hydatid fluid (HF) aspirated from cysts in the liver and lungs was centrifuged at 5000 g for 30 min (4°C) to remove protoscolices (Pr) and stored at -20°C until used. Protoscolices were washed 3-times with Hank's solution. The sample was freeze-thawed three times and mixed with four volumes of PBS, pH = 7.4, containing sodium azide at 0.1 mg/ml. The sample was then sonicated in an 170 W ultrasonic disintegrator (Hielscher, Germany), 2 × 15 sec on ice until no intact protoscolices were visible. The preparation was then left on ice for one h, centrifuged for 30 min at 10,000 g and then filtered (0.22 µm) (Ahmad *et al*., 2001). Also, a soluble protein of mature *E. granulosus* was prepared (Hashemi Tabar *et al*., 2005). Briefly, two hundred of the mature *E. granulosus* which were kept in 10% formaline for 8 months in the Department of Parasitology were washed three times with Hanks solution and after washing with PBS [pH = 7.3], the sample was freezed-thawed three times in liquid nitrogen at 42ºC and homogenized in a blender. The sample was sonicated at 110 V, 170 W ultrasonic disintegrator (Hielscher, Germany) for 3 × 15 sec on ice and then centrifugated for 15 min at 10000 g. Finally, the sample was filtrated with a 0.22 µm filter. Protein concentrations were measured by Bradford (1976) method and the amount of each antigen (hydatid fluid, protoscolices and whole body of *E. granulosus*) was roughly 1 mg/ml. Antigens were kept at -20ºC until used.

Immunization and challenge

Sixteen 4–6-month-old lambs of mixed sexes were divided into 4 groups of 4 (three immunized and one control group). Twelve immunized lambs received 2 mg of HF, protoscolices and whole body of *E. granulosus* antigens dissolved in 1 ml of PBS per immunization for each lamb, respectively. As an adjuvant, Freund's complete adjuvant (FCA) was mixed with antigens to form a water-in-oil emulsion giving a dose of 2 ml which was inoculated subcutaneously on day 1 of the trial. Each control lamb was inoculated with a total of 2 ml of PBS emulsified in equal volumes of FCA. Lambs were boosted on day 28 with the same preparation as described above except that FCA was replaced by Freund's incomplete adjuvant (FIA). Three weeks after the second immunization, each lamb was challenged with 2000 protoscolices intraperitoneally and 10 adult *E. granulosus* worms as described by Hashemi Tabar *et al*. (2005).

ELISA

ELISA was performed in 96-well microtitration plates (Polysorb, PISHTAZ TEB). The plates were coated with secreted antigens of *E. granulosus* (diluted in 10 mM carbonate buffer [pH = 9.6] in order to give protein concentrations of 2.5 µg/ml to detect *E. granulosus*-specific antibody. All the solutions were used at 300 µl per well. 100 µl of sera samples at a 1:10 dilution in PBS containing 1% v/v of bovine serum albumin

(PH = 7.4) was loaded into duplicate wells and incubated for 1 h at room temperature (RT). Duplicate positive and negative control sera were used. The wells were washed by ELISA washer five times with PBS-Tween 20 (0.05%). Then, 100 µl of HRP-labeled polyclonal antibodies against sheep IgG at a 1:2,000 dilution in PBS + 1% BSA was loaded into all the wells and incubated for 1 h RT. The plate was washed as described above to remove the excess conjugate. For colour development, 100 µl of TMB was added to each well as a substrate and the reaction was terminated after 15 min by the addition of 100 µl of 1 M of HCL solution to each well. The absorbance at 490 nm was monitored in ELISA reader.

Statistical test

The data of these experiments were analyzed by Proc mixed using SAS software version 9.1 under repeated measurement method. The effect of treatment, time and also the interaction between treatment and time were analyzed. The model of our design is:

$$X_{ijkm} = \mu + \nu_i + \varphi_{k(i)} + \tau_j + \nu\tau_{ij} + \varepsilon_{m(ijk)}$$

μ - Mean
ν_i - Effect of treatment on response variable
$\Phi_{k(i)}$ - Subject effect nested within treatment
τ_j - Time effect
$\nu\tau_{ij}$ - Treatment * Time interaction
ε - Error term

Results

The results of the analyses of repeated measure showed that the production of antibody was significant between treatments in different times and was also significant between the test groups and the control ($P<0.05$). Lambs immunized with whole body of *E. granulosus* showed the highest antibody production. The level of antibody production between lambs immunized with hydatid fluid and protoscolices was not different significantly ($P>0.05$), whereas the level of antibody production between lambs immunized with hydatid fluid and whole body of *E. granulosus* was different significantly ($P<0.05$) (Fig. 1). The effect of time was also significant, and the production of antibody between day 0 and 49 was changed by quadratic trend. The interaction between times and treatments was significant ($P<0.05$; Fig. 2).

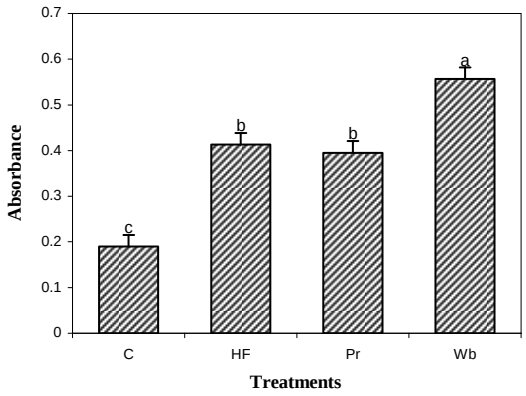

Fig. 1: Titer of antibody against hydatid fluid, protoscolices and whole body of *E. granulosus* antigens in test and control groups during immunization by ELISA. Letters C, HF, Pr and Wb represented control, hydatid fluid, protoscolices and whole body of *E. granulosus*, respectively

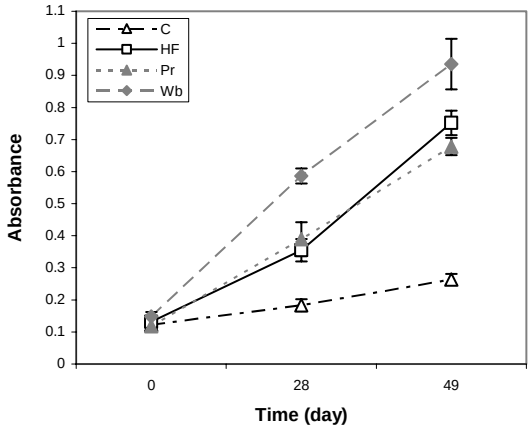

Fig. 2: The trend of change in antibody production in different times and in all treatments. Letters C, HF, Pr and Wb represent control, hydatid fluid, protoscolices and whole body of *E. granulosus*, respectively

Discussion

Much progress has been made from the characterization of the EG95 vaccine which can be used to prevent hydatid infection in intermediate animal hosts of *E. granulosus*. The vaccine comprises a single recombinant oncosphere antigen and the adjuvant Quil A and induces complement-fixing antibodies

that kill the invading oncosphere early in an infection. In the majority of vaccinated animals, no hydatid cysts occur following a challenge infection. However, a small number of viable cysts may develop in some vaccinated animals (Lightowlers and Heath, 2004). It has been reported that a primary infection with oncospheres can induce total or a high degree of protection against a subsequent challenge and confirms that natural immunity can be stimulated in the intermediate host as the result of a primary infection (Zhang *et al*., 2001). By using oncosphere antigen, Heath and Lawrence (1996) reported that a higher level of immunity was achieved in sheep against *E. granulosus*.

In the present study, the levels of antibody were different with the three antigens. Lambs which were immunized against HF and Pr did not produced much antibody 4 weeks after the first immunization, whereas the level of antibody produced by whole body of *E. granulosus* on day 28 was roughly 4 times that of pre-immunization. The level of antibody 3 weeks after the second immunization was considerably high in all three groups, and in lambs immunized with whole body of *E. granulosus*, was much higher than in the HF and the Pr groups. In experimentally infected sheep, antibodies to hydatid antigens can be detected as early as 4 to 6 weeks post-infection (Yong *et al*., 1984) that persist for at least 4 years (Jenkins and Rickard, 1984). The degree of protection did not show a simple relationship to the titre of the antibody, as determined by an ELISA using a solubilised antigen (Dempster *et al*., 1995). Sheep were immunized with either free peptide or peptide conjugated to diphtheria toxoid and challenge infected with *E. granulosus* eggs, and all of the peptides elicited a specific antibody (Woollard *et al*., 2001). It is also reported that natural intermediate host animals such as sheep produce very poor antibody responses to infection as compared with relatively high levels of specific antibody seen in human infections (Lightowlers *et al*., 1999).

Little research has been reported on humoral response against hydatidosis in sheep, and most studies have been conducted on mice. In the study reported by Lin *et al*. (2004), the specific IgG was induced during the third week after vaccination with Eg95 DNA vaccine and continued to increase until week 10. Severi *et al*. (1997) showed that antibody response was followed over 68 weeks in 17 Balb/c mice intraperitoneally infected with *E. granulosus* protoscolices (PSC) and in three mice immunized with dead PSC. The infected mice showed similar profiles of specific IgG and IgM with maximum titres from week 38 to 53. Immunized mice did not show significant levels of specific IgM and, after week 15, showed IgG titres lower than the infected mice. Heath *et al*. (1992) and Hernandez and Nieto (1994) have used antigens derived from cyst fluid and protoscolices against *E. granulosus*; respectively, and the mice showed a high level of antibody against both antigens. In a previous study, we showed that protective immunity induced by whole body antigens of *E. granulosus* in both mice and sheep was higher than protoscolex and hydatid fluid antigens (Hashemi Tabar *et al*., 2005). Haghpanah *et al*. (2003) reported a strong response to protoscolices and protective immunity in mice following immunization with a surface protein of protoscolices. In another experiment by Navidpour *et al*. (2003) the protective immunity in buffaloes induced by egg and oncosphere of *E. granulosus* antigens were 76.7 and 83.5%, respectively.

In the present study, all the immunized lambs showed a strong response to the three antigens on day 49, as compared to the control group, but there was a marked increase in production of the antibody against whole body of *E. granulosus* on day 49. Thus, the antigens of whole body of *E. granulosus* might be a good candidate for immunization and diagnosis of hydatid cyst in an intermediated host.

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