Iranian J Parasitol: Vol. 3, No. 3, 2008, pp. 18-23

Original Article

Immunoregulatory Cytokine (TGF-ß And IL-10) Responses in Mice Inoculated With Protoscoleces and Major Hydatid Fluid Antigens of Cystic Echinococcosis

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(Received 21 Feb 2008; Accepted 9 July 2008)

Abstract

Background: Our objectives were to investigate whether immunomodulatory cytokines, TGF-β and IL-10, are stimulated in response to cystic echinococcosis (CE) components in mice model, and whether major hydatid fluid antigens or live protoscoleces could equally contribute to such cytokines.
Methods: Protoscoleces were obtained by aseptic puncture of fertile sheep hydatid cysts. Hydatid fluid antigens (HFAgs) and Antigen B (AgB) were prepared by partial purification and electroelution, respectively. Of the 25 Balb/c mice assigned in four groups, the first group was inoculated ip with 2000 live protoscoleces; the second and the third groups were injected ip with 50 µg HFAgs and 50 µg AgB in 200 µl of PBS, respectively. Control group was only injected with PBS. The sera concentration of TGF-ß and IL-10 were determined by ELISA. Data were analyzed using One-Way ANOVA and Tukey-HSD tests to compare differences between means.
Results: The mean concentration of TGF-ß in those groups injected with protoscoleces, HFAgs and AgB were significantly higher than control group. However, in the case of IL-10 such differences were only detected in mice that were inoculated with protoscoleces (356±11 pg/ml) compared to control (207±9 pg/ml), HFAgs and AgB groups.
Conclusion: TGF-ß and IL-10, two important immunomudulatory cytokines are induced by different molecules or components of CE, so that AgB could induce TGF-ß and components of protoscolex, other than AgB and Ag5, could induce IL-10.

Keywords: Echinococcus granulosus, *Protoscolex, AgB, TGF-β, IL-10*

Introduction

Cystic echinococcosis (CE) or cystic hydatid disease, caused by infection with the larval stage of the dog tapeworm (*Echinococcus granulosus*), is a major zoonosis of worldwide distribution (1). The disease is characterized by long-term growth of fluid-filled cysts in different organs of human and domestic
animals, commonly in liver and lungs. The main feature of the disease is the existence of the chronic infection with detectable humoral and cellular responses against the parasite. Parasite survival *in vivo* depends on efficient evasion mechanisms starting to operate as the parasite develops toward a hydatid cyst.
A general feature of chronic infections by helminthes is that they polarize the host immune response toward a type-2 cytokine pattern, i.e. secretion of interleukin-4 (IL-4), IL-5 and IL-10, but this response does not correlate with resistance in all cases (2). There is increasing evi-

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dence that these infections also stimulate regulatory T cells and/or regulatory B cells. These immunosuppressive cells produce immunomodulatory cytokines such as transforming growth factor-ß (TGF-ß) and IL-10 that switch off inflammatory and protective immune responses (3, 4). In the recent years many parasite-derived molecules able to exert immunomodulatory activities have been reported (2, 4).

In the case of CE, studies have elucidated that many immunomodulator activities are related to hydatid fluid and its antigenic components as well as protoscoleces, playing an important role in the chronic establishment of disease (5-10). These experiments commonly focused on Th1/Th2 cytokine polarization by CE and its putative role in the host-parasite interaction. However, no direct evidence exists to demonstrate induction of TGF-ß and IL-10, tow major immunomodulatory cytokines, by AgB and protoscoleces. The characterization of parasite-derived immunoregulatory components is highly important to identify the evasion strategies by the parasite from host immune system to permit the establishment of chronic CE. Thus, the objectives of this study were to investigate whether immunomodulatory cytokines, TGF-ß and IL-10, are stimulated in response to CE components in mice model, and whether live protoscoleces or AgB could equally contribute to such cytokines. For this purpose, sera cytokines were evaluated in the mice, inoculated intraperitonealy (ip) with live protoscoleces, AgB and hydatid fluid antigens.

Materials and Methods

***Mice and protoscoleces*:** Adult Balb/c mice originated from Razi Vaccine and Serum Research Institute (Karaj, Iran) and bred at the animal house in Zanjan Faculty of Medicine were used in this experiment. The Ethical Committee of the university approved the study. Protoscoleces were obtained by aseptic puncture of fertile sheep hydatid cyst, then were washed three times with phosphate buffered saline (PBS) pH 7·2, containing 40 µg gentamycin/ml (5). Parasite viability was determined by eosin exclusion test (11). Only those batches with viability over 90 percent were used.

***Antigens*:**

Hydatid fluid was obtained by aseptic aspiration from sheep hydatid cysts, and was clarified by centrifugation at 10,000 *g* at 4°C for 60 min (12). Hydatid fluid antigens (HFAgs) were obtained by dialyzing and precipitating 100 ml hydatid fluid in 0.005 M acetate buffer (pH 5.0, overnight) (13). The precipitate containing the two major antigens of B and 5, was completely dissolved in 10 ml of 0.2 M PBS, pH 8. Then the HFAgs was filtered through a 0.45 µm pore size membrane filter (Millipore S.A., Molsheim, France), lyophilized and stored until subsequent use.

Purified AgB preparation was obtained by electroelution as described by Ioppolo *et al*. (12) with some modifications. In brief, the HFAgs solution (1 mg protein/ml) was separated in the discontinuous buffer system of Laemmli, using 12.5% single well gel under reducing SDS-PAGE (13). The part of the gel below 20 kDa marker was removed and protein was eluted from polyacrylamide strips with a model AE-6580 Max Yield NP electroeluter (ATTO Corporation, Japan) at 2 mA/tube for 60 min at 4°C as recommended by the manufacturer. SDS was removed through a sephadex G-10 column. The eluted antigen was filtered through a 0.2 µm pore size disposable syringe filter and aliquots of 1 ml vials stored at -20°C as an AgB until subsequent use. The total protein content was determined by the Brad-ford protein assay (14).

***Experimental design*:**

A group of mice (n=6, protoscolex group) was inoculated ip with 2000 live protoscoleces suspended in 200 µL of PBS (9). The second (n=6, HFAgs group) and the third groups (n=7, AgB group) of mice were injected ip with 50 µg HFAgs and 50 µg AgB in 200 µL of PBS, respectively. The other group of mice (n=6) was only injected with 200 µL of PBS as the control group (7). In HFAgs, AgB and control groups,

injections were repeated twice on the 14th and 28th days with the same doses of preparations. Finally on the 35th day, all mice were anaesthetized and blood samples obtained by cardiac puncture. To analyze the cytokines, sera were immediately prepared and stored in double aliquots at -20°C.

***Cytokine assays*:**

The cytokine concentrations were determined in all mice sera with commercially capture-ELISA kits (BMS608/2 mTGF-ß and BMS614 mIL-10 ELISA, Bender MedSystem, Austria) according to the manufacturer's instructions. The optical densities of final reaction were read at 450 nm using an automatic microplate reader (State Fax® 2100, Awareness, USA).

***Statistical analysis*:**

Results of the cytokine concentrations were expressed as arithmetic mean±SEM and ranges. The One-Way ANOVA and Tukey-HSD tests were used to compare differences between means. Differences were regarded as significant with $P < 0.05$ in all experiment groups.

Results

The eluted AgB showed two bands at 8 and 16 kDa upon 12.5% gel under reducing SDS–PAGE.

Results of serum cytokine analysis were shown in Table 1 for all experiment groups, according to mean and range of TGF-ß and IL-10 concentrations. The mean concentrations of TGF-ß in those groups injected with protoscoleces, HFAgs and AgB were significantly higher than control group (in all cases; $P < 0.05$) (Fig. 1). Conversely, in the case of IL-10 significant difference was only detected in mice inoculated with protoscoleces (356±11 pg/ml) compared to control group (207±9 pg/ml) ($P < 0.05$), and such differences were not observed in mice injected with HFAgs or AgB from control (Fig. 2). In other words, only protoscoleces could induce rising of serum IL-10 and two other antigenic preparations could not show this effect.

Table 1: Concentration of serum cytokines in Balb/c mice injected with protoscoleces, hydatid fluid antigens (HFAgs) and antigen B (AgB) Asterisks Indicate differences compared to control group. † Indicates differences compared to HFAgs, AgB and control (PBS) groups (in all cases; $P < 0.05$).

Experiment groups (No.)	TGF-ß conc. (ng/ml)		IL-10 conc. (pg/ml)	
	Mean (±SEM)	Range	Mean (±SEM)	Range
Protoscolex (6)	189 (3)*	181-197	356 (11)†	323-386
HFAgs (6)	162 (8)*	137-185	207 (9)	168-228
AgB (7)	154 (11)*	97-181	222 (38)	154-446
PBS (6)	26 (11)	9-60	207 (9)	181-241

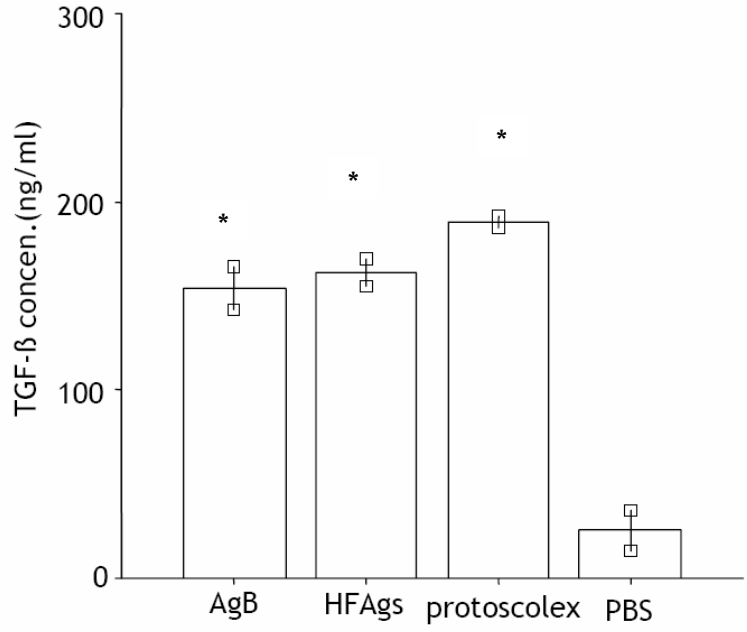

Fig. 1: Concentration (mean±SEM) of serum TGF-ß in Balb/c mice injected with protoscoleces, hydatid fluid antigens (HFAgs), antigen B (AgB) and PBS. Asterisks Indicate differences compared to control (PBS) group ($P < 0.05$).

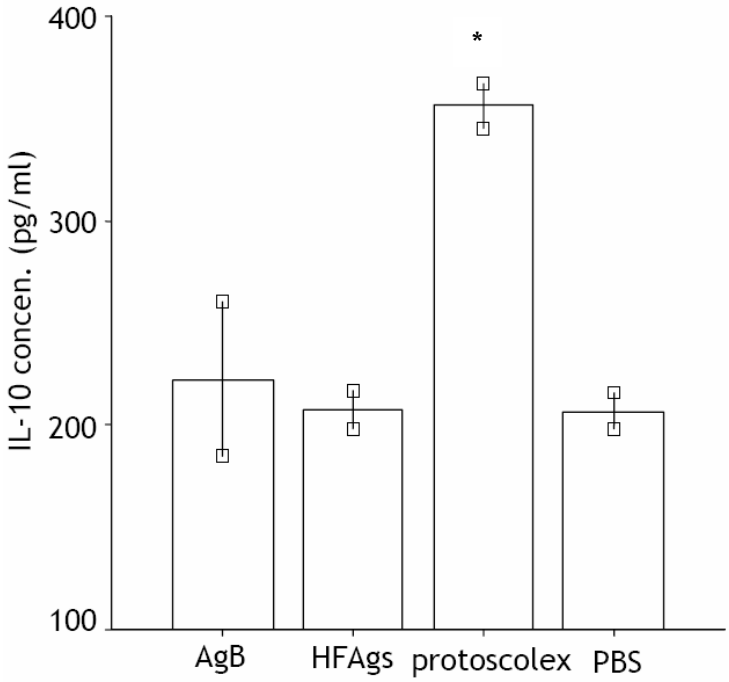

Fig. 2: Concentration (mean±SEM) of serum IL-10 in Balb/c mice injected with protoscoleces, hydatid fluid antigens (HFAgs), antigen B (AgB) and PBS. Asterisk indicates differences compared to HFAgs, AgB and control (PBS) groups (P<0.05).

Discussion

Increasing evidence shows that some parasitic components play an important role in modulation of host immune system, by suppressing the function of certain immune cells as well as stimulating other cells (2, 4, 15). These infections can stimulate regulatory T cells and/or regulatory B cells, which produce immunomodulatory cytokines such as TGF-ß and IL-10 that switch off inflammatory and protective immune responses; reviewed in (3, 4).
Our results showed that all three preparations, i.e. AgB, HFAgs and protoscoleces could increase serum level of TGF-ß in mice. However, protoscoleces could only induce IL-10 response and the two other antigenic preparations did not show such effect.
In recent years, studies have elucidated that many immunomodulator activities are related to hydatid fluid and its antigenic components as well as protoscoleces, playing an important role in the chronic establishment of disease (5-10). Dematteis *et al*. (5) show that, a type-2 cytokine pattern (IL-10, IL-4 and IL-5) was secreted by splenocytes from mice infected with live protoscoleces, as early as the first week of infection. Rigano *et al*. (8) showed that antigen B (AgB) of hydatid fluid plays a role in the escape from early immunity by inhibiting polymorphonuclear cell chemotaxis and eliciting a non protective Th2 cell response. The experiment of Mondragon and co-workers (16) is the only available study that shows protoscoleces could progressively increase *in situ* expression of IL-10 and TGF-ß along with decrease in IL-6 and TNF-α in Balb/c mice. They conclude that the parasite induces a local immunosuppression probably mediated by IL-10 and TGF-ß; therefore it seems that such mechanism would assist the parasite in escaping the harmful host cell-mediated responses. In addition, here we showed that AgB and HFAgs, similar to protoscoleces, could induce TGF-ß response in mice model. Therefore, it is tempting to speculate that AgB present in HFAgs, and hence in protoscoleces, could target special cells inducing the immunomodulatory TGF-ß cytokine. However, an important point is why these two antigenic preparations (HFAgs and AgB) could not induce IL-10 response, while the protoscoleces could. We suggest that different parasite-derived molecules or components may be involved in the TGF-ß or IL-10 responses. Since major contents of partial purified HFAgs are AgB and Ag5, which are lipoproteic molecules (17) and unable to induce IL-10 secretion, we envisage that IL-10 inducing fraction of protoscolex is other than AgB and Ag5, and may be non-lipoproteic molecules. Dematteis *et al*. (7) showed that a purified carbohydrate rich fraction from protoscoleces, namely E4+, may contribute to the induction and sustaining of the type-2 cytokine response by stimulating IL-10 secretion in early experimental hydatid infection in the Balb/c mice. Furthermore, Cardozo *et al.* (18) demonstrated that E4+ is immunogenic in humans. Interestingly, the stimulation of PBMC cultures from humans with E4+ evoked higher levels of IL-10 response than with crude extracts.
In conclusion, TGF-ß and IL-10, two important immunomudulatory cytokines are induced by different molecules or components of CE, so that AgB could induce TGF-ß and components of protoscolex, other than AgB and Ag5, could induce IL-10.

Acknowledgments

We would like to thank Dr. Nouralddin Mousavinasab and Mr. Jamshid Mohammadi for their kind cooperation. We are grateful to the Research Deputy of Zanjan University of Medical Sciences for financial support. The authors declare that they have no Conflict of Interests

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