Association between Serum IL-4 Levels and Cyst Relapsing in Cystic Echinococcosis

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Summary

IFN-γ and IL-4 levels of peripheral blood mononuclear cell (PBMC) cultures and sera of the cystic echinococcosis patients with primary and relapsed infections were compared. In this regard, PBMC of the patients and controls were cultured in RPMI and stimulated with different hydatid fluid antigens of (SHF, pH5PPT, B-Ag, F1 and F2). IFN-γ and IL-4 of the culture supernatants as well as the sera of echinococcosis patients were evaluated by ELISA. The results indicate that the level of IL-4 produced by PBMC of the patients was higher than that of healthy group and the difference was significant. The levels of IL-4 in PBMC culture and sera of the patients with relapsed hydatidosis were significantly higher than those with primary infection means the immune response pattern of the patients, who have not received any antiparasitic drug after surgery, may shift toward Th2 activation and susceptibility to the infection. Contrary, the levels of IFN-γ produced by PBMC and in sera of the patients treated surgically once do not differ significantly with those treated two times or more.

Keywords: *Echinococcus granulosus*, cytokine, IFN-γ, IL-4, cyst relapsing

Introduction

In cystic echinococcosis both T and B cells are active however, the immunophysiopathology research indicates T cell is more important (Scott *et al* 1989). In addition, both Th1 and Th2 are active in echinococcosis and concurrent

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intervention of their cytokines is observed (Rigano *et al* 1996). Th1, which is characterized by secretion of IFN-γ and TNF-α adapt at macrophage activation and immunoglobulin selection for isotypes that mediate antibody-dependent cellular cytotoxicity and complement activation (Sinigaglia *et al* 1999). Th2 is characterized by secretion of IL-4 and IL-10 and is an important factor in promoting humoral immune responses. IL-4 and IFN-γ regulate IgE and IgG4 responses (Ishizaka *et al* 1990, King & Nutman, 1993). Cytokines produced by peripheral blood mononuclear cell (PBMC) as well as in sera of surgically treated echinococcosis patients have been studied previously (Rigano *et al* 1995, 1999, Torcal *et al* 1996, Touil-Boukoffa *et al* 1997) but the cytokine profile of hydatidosis patients with primary and relapsed infection have never compared. According to the study of Rigano *et al* (1997) the variability and severity of the clinical expression of this parasitosis probably reflects not only the duration and intensity of infection, but also the variety of human immunological responses to parasite antigens.

The objectives of the present study were first, to compare different hydatid antigens capability in stimulating PBMC of echinococcosis patients to produce IFN-γ and IL-4, second to compare IFN-γ and IL-4 levels of PBMC cultures and sera of the patients with primary and relapsed infections.

Materials and Methods

Antigen. Different antigens of hydatid fluid were prepared as follow:

1) CSHF. Hydatid cysts of liver and lungs of infected sheep collected at slaughterhouse. Sheep hydatid fluid (SHF) was aspirated from the cysts aseptically and the fluid centrifuged at 1500*g* for 15min. 2) pH5PPT. Albumin and globulin of the CSHF removed according to Oriol *et al* (1971) method. In brief, the CSHF dialyzed against low ionic strength acetate buffer (0.005 M) then the globulin removed with ammonium sulfate to 40% saturation. 3) B-antigen. The pH5PPT was boiled at 100°C for 15min in water bath and centrifuged at 5000*g* for 60min. The

supernatant was used as partially purified B-antigen. 4) F1 and F2 antigens. The lipid extracted from pH5PPT by the method of Day and Levy (1968) then the solution eluted through Sephadex G-200 column. Fractions 1 and 2 of the eluted solution were used in PBMC culture assay.

Protein concentration of the antigens was determined by Bradford (1976) method. All the antigens sterilized with 0.2μ pore size filter before use.

Blood and serum samples. Blood and serum samples were obtained from 10 healthy controls and 20 patients with clinically diagnosed either primary (n=14) or relapsed hydatidosis (n=6) confirmed by surgery. All the patients had not received any antiparasitic drug for a long period. The sera were frozen at–20°C until use.

PBMC isolation and cell culture experiments. Under sterile condition 10ml of peripheral blood was collected and added to Ficoll, centrifuged at 400*g* for 30min then the buffy coat collected and washed three times with RPMI-1640 medium (Sigma, USA) containing 25mM Hepes, 2mM L-glutamine, 100IU/ml penicillin and 100μg/ml streptomycin. After third washing, the PBMC was resuspended in RPMI contain 10% FBS (Fetal bovine serum) inactivated at 56°C for 30min. PBMCs were counted and each 5×10^6cells/ml were placed in a 24 well flat-bottomed plate (Nunc, Denmark). Finally, 10μg/ml mitogen Phytohaemagglotinin (PHA) (Sigma, USA) as positive control, the prepared antigens each of 100μg/ml and, medium and PBMC alone for spontaneous assay were added into separated wells. The plates were incubated at 5%CO_2 and 37°C incubator for 72h, and then the supernatents were collected and frozen at –20°C until use.

Cytokine assay. IL-4 and IFN-γ of cell culture supernatant and sera were evaluated by ELISA (IL-4 and IFN-γ Enzyme immunoassay kits, Immunotech®, France). The ranges of IL-4 detection were 15.6-1000 pg/ml in supernatant and 0.24-15.6 pg/ml in sera. The ranges of IFN-γ detection were 0.39-25 IU/ml in supernatant and 0.097-1.56 IU/ml in sera. Each sample was tested in triplicate.

Statistical analysis. Results were expressed as geometric mean and ranges. For comparison of cytokine production Wilcoxon signed rank test was used for non-parametric data.

Results

Table 1 shows that the IL-4 and IFN-γ produced by PBMC of echinococcosis patients stimulated with different antigens and mitogen were higher than that of healthy group. As depicted in table 2, although IFN-γ produced by PBMC of the patients with relapsed cystic echinococcosis was higher than those with primary infection, but the difference was not significant statistically. Whereas the difference between IL4 produced by PBMC of the patients with primary and relapsed cystic echinococcosis was statistically significant ($P<0.05$).

Table 2. *IL-4 and IFN-γ produced by PBMC (Peripheral Blood Mononuclear Cells) of the patients with primary and relapsed cystic hydatidosis stimulated by different antigens*

Antigen	IL-4				IFN-γ			
	Patients with primary infection		Patients with relapsed infection		Patients with primary infection		Patients with relapsed infection	
	Positive culture/examined culture (%)	Mean conc. in pg/ml (Range)	Positive culture/examined culture (%)	Mean conc. in pg/ml (Range)	Positive culture/examined culture (%)	Mean conc. in IU/ml (Range)	Positive culture/examined culture (%)	Mean conc. in IU/ml (Range)
CSHF	11/14 (78)	65.37 (<15-117)	6/6 (100)	133.66 (87-225)	14/14 (100)	1.88 (0.5-3.5)	5/6 (83)	2.08 (<0.4-3.7)
pH5PPT	11/14 (78)	43.00 (<15-81)	6/6 (100)	76.33 (40-110)	14/14 (100)	1.24 (0.4-2.2)	5/6 (83)	1.70 (<0.4-2.8)
B-Ag	8/14 (57)	35.28 (<15-46)	6/6 (100)	43.80 (15-80)	13/14 (92)	0.86 (<0.4-1.6)	4/6 (66)	1.10 (<0.4-1.6)

Table 1. *IL-4 and IFN-γ produced by PBMC (Peripheral Blood Mononuclear Cells) of the hydatidosis patients and healthy controls stimulated by different antigens*

Stimulator	IL-4				IFN-γ			
	Hydatidosis patients PBMC		Healthy Individual PBMC		Hydatidosis patients PBMC		Healthy Individual PBMC	
	Positive culture/examined culture (%)	Mean conc. in pg/ml (Range)	Positive culture/examined culture (%)	Mean conc. in pg/ml (Range)	Positive culture/examined culture (%)	Mean conc. in IU/ml (Range)	Positive culture/examined culture (%)	Mean conc. in IU/ml (Range)
CSHF	17/20 (85)	89.29 (<15-225)	2/10 (20)	21 (<15-27)	19/20 (95)	1.69 (<0.4-3.7)	7/10 (70)	1.3 (<0.4-2.8)
pH5PPT	17/20 (85)	55.80 (<15-110)	2/10 (20)	19.25 (<15-20)	19/20 (95)	1.38 (<0.4-2.8)	5/10 (50)	1.14 (<0.4-2.16)
B-Ag	14/20 (75)	39.26 (<15-80)	0/10 (0)	<15	18/20 (90)	0.90 (<0.4-1.6)	4/10 (40)	1.06 (<0.4-1.2)
F1	5/20 (25)	23.11 (<15-42)	0/10 (0)	<15	7/20 (35)	0.59 (<0.4-0.8)	3/10 (30)	0.4 (<0.4-0.6)
F2	9/20 (45)	30 (<15-61)	0/10 (0)	<15	8/20 (40)	0.67 (<0.4-1.2)	3/10 (30)	0.48 (<0.4-0.9)
Mitogen	19/20 (95)	341 (<15-1623)	5/10 (50)	183 (<15-217	20/20 (100)	9.29 (2.5-33.7)	10/10 (100)	13.3 (7-30.7)
Spontaneous	4/20 (20)	25 (<15-30)	0/10 (0)	<15	4/20 (20)	0.40 (<0.4-3)	4/10 (40)	0.6 (<0.4-1.7)

IL-4 was measurable in sera of 70% of echinococcosis patients and 30% of control group. The mean of IL-4 in sera of the patients was 6.70pg/ml ranging from <0.97 to 15.70pg/ml and in control group was 1.25pg/ml ranging from <0.98 to 2.50pg/ml. The difference between two groups was statistically significant ($P<0.01$) (Table 3). The mean amount of IL-4 in sera of the patients treated surgically once differed significantly from those treated two times or more. The production of IL-4

in patients with relapsed hydatid was significantly higher than those with primary echinococosis. IFN-γ was measurable in sera of 100% of echinococcosis patients and control group. The mean of IFN-γ in sera of the patients was 0.28IU/ml ranging from 0.20 to 0.39IU/ml and in control group was 0.33IU/ml ranging from 0.20 to 0.40IU/ml (Table 3). The mean amount of IFN-γ in sera of the hydatidosis patients and control group as well as patients treated once surgically did not differ significantly from those treated more than one time.

Table 3. *Cytokines levels in sera of hydatidosis patients and control group*

Cytokine		Hydatidosis Patients			Healthy Individuals
		primary infection	relapsed infection	Total	
IL-4	Positive/examined (%)	8/14(57)	6/6(100)	14/20(70)	3/10 (30)
	Mean conc. (pg/ml) (Rang)	5.60 (0.97-8.00)	8.27 (4.20-15.70)	6.70 (<0.97-15.70)	1.25 (<0.98-2.50)
IFN-γ	Positive/examined (%)	14/14 (100)	6/6(100)	20/20 (100)	10/10 (100)
	Mean conc. (IU/ml) (Rang)	0.28 (0.20-0.39)	0.29 (0.20-0.38)	0.28 (0.20-0.39)	0.33 (0.20-0.40)

Discussion

The present work examined *in vitro* production of cytokines by PBMC of echinococcosis patients, which were stimulated with different hydatid fluid antigens. *In vitro* cytokine production has been previously demonstrated using whole hydatid fluid or pH5PPT (Profumoet *et al* 1994, Rigano *et al* 1995). Although all the antigens used in the present work induced IL-4 and IFN-γ levels, but CSHF was shown to be the best stimulator of PBMC for cytokines production. Ag-driven cytokine in the patients appeared to be significantly higher than spontaneous cytokine production. In healthy controls, CSHF-driven cytokine production was also

much higher than that produced by other antigens and in addition, spontaneous stimulation indicated the cross reactivity of CSHF with other non-specific antigens in cytokine production. Indeed CSHF may contain a set of antigens that can induce both Th1 and Th2 subsets. In this stimulation assay, the production of IL-4 in echinococcosis patients was much higher than that of healthy controls.

Twenty percent of echinococcosis patients produced measurable amounts of IL-4 in unstimulated culture supernatants as well as IFN-γ production. In the study of Rigano *et al* (1995) 73% and 33% of the patients had produced measurable amounts of IL-4 and IFN-γ respectively. Stimulation of PBMC from echinococcosis patients with PHA, produced larger quantities of IL-4 and smaller amount of IFN-γ comparing with healthy individuals. This data indicate even in nonspecific stimulation the direction of the cytokine production of the patients moved toward IL-4 production. However, both IL-4 and IFN-γ were produced at comparatively smaller amounts in echinococcosis patients (Rigano *et al* 1995).

Results obtained from the present study support potential regulatory role of IL-4 and IFN-γ in *Echinococcosis granulosus* infections. Our results showed that the amount of IL4 produced by PBMC of echinococcosis patients stimulated with different antigens was significantly higher than that of healthy individuals ($P<0.005$). This indicates the activation of Th2 in echinococcosis patients, which have not received any antiparasitic drug for a long term. Meanwhile the high amount of IFN-γ in echinococcosis patients suggesting that Th1 contribute in the immune response regulation concurrently, but the role of Th2 response seem to be more evident. In parasitic infections a strong Th2 response correlates with susceptibility to the disease, whereas a Th1 response correlates with protective immunity (Grau & Modlin 1991, Scott *et al* 1989).

The mean amounts of IL-4 produced by PBMC of hydatidosis patients stimulated with crude antigen in Rigano *et al* (1995) and our studies were measured 73pg/ml and 89.29pg/ml, respectively. In the present work 85% of PBMC of the patients

produced measurable amounts of IL-4 while it was reported 75% by Rigano *et al* (1995). The differences between these data may be due to the difference in baseline level measured by two different kits. The baseline level was 45pg/ml in Rigano and 15pg/ml in our studies.The mean IFN-γ produced by PBMC of the patients stimulated with CSHF was 1.96IU/ml while 85% of the patients produced measurable amount of IFN-γ. In study of Rigano *et al* (1995) 41% of the patients produced measurable amount of IFN-γ and the mean amount of IFN-γ was 60pg/ml (1.8IU/ml). This variation also can be due to the baseline differences of the two kits. The amount of IL-4 in the sera of echinococcosis patients was also higher than that in control group. IL-4 was measurable in sera of 70% of the patients with mean concentration of 6.70pg/ml. While, according to the study of Rigano *et al* (1999) 13 of 15 serum samples (87%) of echinococcosis patients contained measurable amounts of IL-4 with mean concentration of 1.45pg/ml. The difference in concentration was due to effects of pharmacological treatment on cytokines pattern in patients with cystic echinococcosis. The results of their study showed a clear association between IL-4/IL-10 cytokines level in patients with CE and outcome of albendazole therapy. The role of drug treatment on strengthening protection against human cystic echinococcosis by means of alteration of the direction of immune response towards fortifying of Th1 activation was suggested (Rigano *et al* 1999). Our data indicate that the concentrations of IL-4 in PBMCs culture and sera of the patients with relapsed hydatidosis were significantly higher than those with primary infection. This means, the pattern of immune response of the patients who have not received any antiparasitic drug after surgery, may shift toward Th2 activation and susceptibility to the disease. In conclusion, serum IL-4 raising following hydatid cyst surgery may suggest an evidence of hydatidosis relapsing. More studies need to evaluate this change in a large population. Alteration of immune response from Th2 toward Th1 due to drug treatment or cytokine therapy in the patients with hydatid cyst infection can be a good topic for study in future.

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