Clin Exp Immunol 1996; **105**:456–459

Production of IL-5 and IL-6 by peripheral blood mononuclear cells (PBMC) from patients with *Echinococcus granulosus* infection

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(Accepted for publication 3 June 1996)

SUMMARY

The role of Th2 cytokines in human hydatidosis was evaluated in ELISA determining IL-5 and IL-6 production in PBMC cultures from 27 pharmacologically treated hydatid patients and from 13 uninfected controls. PBMC from patients produced large amounts of parasite antigen-driven IL-5, whereas PBMC from uninfected individuals produced none. In contrast, PBMC from patients and from uninfected controls produced large amounts of parasite antigen-driven IL-6. Immunoglobulin isotype analysis revealed that IL-5 production correlated significantly with IgE and IgG4 expression (IL-5/IgE $r = 0{\cdot}5$, $P < 0{\cdot}05$; IL-5/IgG4 $r = 0{\cdot}6$, $P < 0{\cdot}05$). The high IL-5 levels in supernatants from patients' PBMC did not correspond to an increase in eosinophils. Neither IL-5 nor IL-6 production showed an association with the outcome of therapy. Overall, these findings confirm that the lymphocytes of individuals with *Echinococcus granulosus* infection contain Th2-like subpopulations.

Keywords *Echinococcus granulosus* hydatid disease IL-5 IL-6 immunoglobulin isotypes

INTRODUCTION

By activating positive and negative regulatory signals cytokines influence the activities of T lymphocytes, B lymphocytes and other cells that mediate immune responses. Th2 cytokines regulate IgE and IgG4 production and eosinophil expression in human helminth infections. *Echinococcus granulosus* in humans triggers a humoral and cellular response characterized by elevated serum IgE/IgG4 and by concurrent intervention of Th1 and Th2 cytokines [1]. We have recently provided evidence that Th1 cell activation is associated with protective immunity, and that Th2 cell activation is associated with susceptibility to hydatid disease [2].

Continuing our programme of research into the immunology of hydatidosis, to evaluate further the role of cytokines in human *E. granulosus* infection we assessed IL-5 and IL-6 Th2 cytokine production in PBMC cultures from pharmacologically treated hydatid patients. We also investigated a possible correlation between IL-5 and IL-6 cytokine production and immunoglobulin isotype expression.

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PATIENTS AND METHODS

Blood samples

Blood samples were obtained from 27 patients with clinically diagnosed hydatidosis (18 with cysts in the liver, two with cysts in the lung and seven with cysts in multiple sites) and from 13 sex- and age-matched uninfected controls. In three serologically negative patients surgery after therapy confirmed the diagnosis of hydatidosis. None of the subjects studied had a history of atopic manifestations. All procedures were approved by the local Ethical Committee and all subjects gave their informed consent to the study. Patients had been pharmacologically treated about 1 year before our study with 3-month cycles of albendazole (10–12 mg/kg per day), sometimes repeated. Patients were divided into four groups according to the effectiveness of treatment determined by objective criteria mainly based on imaging methods: (i) full responders—disappearance or evident decrease in the size of cyst, or distinct degenerative changes in cyst morphology, or both; (ii) partial responders—successful treatment of cyst(s) in one organ but not of others, or of some cysts but not of others; (iii) low responders—slight changes in cyst morphology or clinical improvement, or both; and (iv) non-responders—no change in cyst size/morphology or occurrence of a relapse (volumetric increase of cysts, appearance of new cysts or of new daughter cysts). Haemocromocytometric analysis showed that only three patients

had peripheral blood eosinophilia (values > 5% were considered abnormal).

Cell cultures and cytokine assays

PBMC from hydatid patients and healthy controls were cultured as previously described by Riganò *et al.* [1] in the presence of 10 μg/ml or 100 μg/ml of crude concentrated sheep hydatid fluid (SHF), 10 μg/ml of non-parasite antigen (PPD), 50 ng/ml of mitogen phorbol 12-myristate 13-acetate plus 1 μg/ml ionomycin (PMA/ion) and 10 μg/ml of mitogen phytohaemagglutinin (PHA), amounts proved effective in our previous experiments (unpublished). Supernatants from non-stimulated cultures and from mitogen-stimulated cultures were collected after 48 h of culture, those from unstimulated, SHF- and PPD-stimulated cultures after 72 h and 120 h of culture. Preliminary kinetic studies (unpublished) showed these incubation times as optimum for inducing cytokine production. IL-5 and IL-6 production in the supernatants was quantified by ELISA (Quantikine; R&D Systems, Minneapolis, MN) as recommended by the manufacturer. IL-5 assays had a detection limit of 7·8 pg/ml and IL-6 of 3·13 pg/ml. Supernatants were diluted 1:100 and 1:200 for use in IL-6 ELISA.

ELISA assay for specific immunoglobulin isotypes

Immunoglobulin isotypes were determined by ELISA as described by Riganò *et al.* [2]. Values higher than 0·3 optical density (OD) for IgE, 0·6 OD for IgG, 0·1 OD for IgG1, IgG2, IgG3 and 0·2 OD for IgG4 (mean $\pm$ 2 s.d. of the absorbance readings of the uninfected controls) were considered positive.

Statistical analysis

Wilcoxon signed-rank test for non-parametric data was used to compare cytokine production in patients and in controls. Differences with a confidence interval of 95% or higher were considered statistically significant ($P < 0{\cdot}05$). Spearman's rank correlation was used to determine cor-relation between cytokine production and immunoglobulin isotype responses.

RESULTS

Echinococcus granulosus antigen induced high IL-5 production in 24 of the 27 pharmacologically treated hydatid patients (89%) (Table 1). IL-5 concentrations in supernatants of PBMC obtained from patients corresponded to $\approx$ 22–500 pg/ml during antigenic exposure, and production reached maximum at 120 h in cultures with 10 pg/ml of SHF. SHF induced no detectable IL-5 in uninfected controls. To assess the specificity of SHF-driven IL-5 production we analysed PPD-driven cytokine production. PPD-stimulated PBMC cultures from 12 of 23 patients (60%) and three of 12 uninfected controls (25%) contained small amounts of IL-5, the mean IL-5 value being higher in patients than in

Table 1. IL-5 production in PBMC cultures from 27 pharmacologically treated hydatid patients and 13 uninfected controls

			Patients		Controls		
Stimulus	Time of culture (h)	Dose of stimulus (mg/ml)	Positive cultures/ examined cultures (%)	Mean concentrations, pg/ml (range)	Positive cultures/ examined cultures (%)	Mean concentrations, pg/ml (range)	Patients *versus* controls (P)
		10	17/20	225*	0/5	<7·8	2×10^{-3}
	72		(85)	(<7·8–500)			
		100	10/16	143	0/11	<7·8	1×10^{-2}
SHF			(62)	(<7·8–500)			
		10	21/24	374†	0/9	<7·8	5×10^{-6}
	120		(87)	(<7·8–500)			
		100	17/20	329**	0/10	<7·8	1×10^{-6}
			(85)	(<7·8–500)			
Total number of positive subjects/ examined subjects			24/27 (89)		0/13		
	72		2/17	20*	1/2	31·4	
PPD		10	(12)	(<7·8–60)	(50)		
	120		11/18	47†**	2/10	8·7	2×10^{-2}
			(61)	(<7·8–170)	(18)	(<7·8–14)	
Total number of positive subjects/ examined subjects			12/23 (60)		3/12 (25)		
PMA/ion	48	0·05/1	24/24	349	7/11	207	
			(100)	(48–500)	(64)	(<7·8–500)	
PHA	48	10	13/13	220	7/8	163	
			(100)	(24–500)	(87)	(<7·8–440)	
	48	—	0/25	<7·8	0/5	<7·8	
None	72	—	0/20	<7·8	0/11	<7·8	
	120	—	0/25	<7·8	0/6	<7·8	

SHF, Sheep hydatid fluid; PPD, mycobacterial protein purified antigen; PMA/ion, phorbol myristate acetate/ionomycin; PHA, phytohaemagglutinin. Statistically significant differences: *P=2×10^{-5}; †$P = 2 \times 10^{-6}$; **$P = 2 \times 10^{-5}$.

Table 2. IL-6 production in PBMC cultures from 20 pharmacologically treated hydatid patients and 10 uninfected controls

			Patients		Controls	
Stimulus	Time of culture (h)	Dose of stimulus (mg/ml)	Positive cultures/ examined cultures (%)	Mean concentrations, pg/ml (range)	Positive cultures/ examined cultures (%)	Mean concentrations, pg/ml (range)
		10	7/7	26 485	NT	NT
	72		(100)	(6400–70 000)		
		100	6/6	101 667	4/4	82 500
			(100)	(20 000–180 000)	(100)	(30 000–220 000)
SHF		10	12/12	20 602	NT	NT
	120		(100)	(2820–75 000)		
		100	5/5	60 000	6/6	54 000
			(100)	(4000–130 000)	(100)	(3000–180 000)
	72		7/7	29 429	3/3	46 733
PPD		10	(100)	(12 500–90 000)	(100)	(5200–100 000)
	120		6/6	54 267	4/4	62 375
			(100)	(1600–190 000)	(100)	(9500–150 000)
PMA/ion	48	0·05/1·0	11/11	21 776	2/2	23 500
			(100)	(2035–70 000)	(100)	(22 000–25 000)
PHA	48	10	6/6	51 717	3/3	70 500
			(100)	(18 300–75 000)	(100)	(1500–150 000)
None	120	—	7/8	25	3/3	48
			(88)	(<3·13–40)	(100)	(12–110)

SHF, Sheep hydatid fluid; PPD, mycobacterial protein purified antigen; PMA/ion, phorbol myrisate acetate/ionomycin; PHA, phytohaemagglutinin; NT, not tested.

controls. SHF-stimulated cultures from patients' PBMC produced significantly higher mean IL-5 concentrations than did PPD-stimulated supernatants. Although mitogen-driven IL-5 production was higher in PBMC supernatants from patients than from uninfected controls, the difference did not reach statistical significance. Unstimulated cultures from patients and controls contained no measurable IL-5. Of note, even though 89% of the patients produced high levels of IL-5, only three of them had peripheral blood eosinophilia. SHF antigen induced high IL-6 production in all 20 hydatid patients and all 10 uninfected controls (Table 2). PBMC supernatants from patients and controls contained similar mean IL-6 concentrations. IL-6 production reached maximum at 72 h of culture with 100 pg/ml of SHF. In patients' and controls' PBMC cultures, PPD and mitogen both induced high IL-6 production. Most patients and controls spontaneously produced IL-6 at 5 days of culture.

Immunoglobulin isotype analysis revealed a significant correlation between SHF-driven IL-5 production and IgE, IgG4 expression (IL-5/IgE $r = 0{\cdot}5$, $P < 0{\cdot}05$; IL-5/IgG4 $r = 0{\cdot}6$, $P < 0{\cdot}05$), but no correlation between IL-5 and the other immunoglobulin isotypes (Table 3). No association was found between SHF-driven IL-6 cytokine production and immunoglobulin subclass expression. Analysis of cytokine/antibody profiles revealed no differences between patients according to their response to chemotherapy.

DISCUSSION

Confirming participation of the Th2 cell subpopulation in human hydatid disease, in the current study of 27 pharmacologically treated hydatid patients we observed high production of cytokines IL-5 and IL-6. Our finding that SHF induced high IL-5 production in hydatid patients but none in uninfected controls explicitly shows the determinant role of IL-5 in human hydatid disease. The increased IL-5 production observed in patients could result from increased IL-5 secretion by each cell, or from an expanding IL-5-secreting cell population. Current evidence suggests that IL-5 exerts its effect primarily on the development and maturation of eosinophils [3]. Despite the high percentage of patients in our study who produced IL-5 (89%), only three patients had peripheral blood eosinophilia. These data and recent findings by Sturm *et al.* [4] showing that patients with alveolar echinococcosis but without eosinophilia secrete large amounts of IL-5 suggest that in human cystic and alveolar hydatidosis increased IL-5 production does not relate to increased numbers of eosinophils.

In this study, regardless of the stimulus, PBMC from patients and controls invariably produced elevated amounts of IL-6. Again, this finding agrees with recent evidence from Sturm *et al.* [4] of comparable levels of IL-6 in patients with *E. multilocularis* infection and normal donors. It therefore argues against a close link between IL-6 production and *E. granulosus* infection. IL-6 is normally induced by viral infections, autoimmune diseases, chronic inflammation, lymphoid malignancy or chemical substances such as lipopolysaccharides [5]. The high production of IL-6 we noted in normal cells presumably depended on the chemical composition of the *Echinococcus* antigen used to stimulate PBMC in our cultures. Yet once induced, IL-6 could intervene in IL-4-dependent induction of IgE synthesis by providing a late amplification signal [6]. IL-6 is a pleiotropic cytokine produced by various cell types other than T lymphocytes (macrophages/monocytes and B lymphocytes). It regulates immune responses, acute-phase reactions, and haematopoiesis, and may play a central role in host defence mechanisms, intervening in cellular interactions during parasitic infections [7].

Table 3. Sheep hydatid fluid (SHF)-driven IL-5 production and immunoglobulin isotype expression in 27 pharmacologically treated patients

		Immunoglobulin isotype analysis					
Patients*	IL-5† (pg/ml)	IgE	IgG	IgG1	IgG2	IgG3	IgG4
FR	500	0·20	3·00	3·00	0·13	1·10	0·30
FR	500	1·70	3·00	2·00	0·20	0·90	3·00
FR	500	0·20	3·00	1·80	0·05	0·60	2·40
PR	<7·8	0·15	0·48	0·13	0·00	0·00	0·00
PR	500	0·28	3·00	3·00	0·15	1·00	2·10
PR	500	0·80	3·00	1·90	0·10	0·50	0·20
PR	500	1·02	1·90	1·80	0·40	0·50	1·97
PR	<7·8	0·12	0·77	0·10	0·08	0·08	0·11
PR	320	1·60	1·60	0·70	0·12	0·20	1·50
LR	200	0·30	2·20	0·50	0·03	0·12	0·21
LR	280	0·47	2·20	1·10	0·02	0·35	1·90
LR	500	0·20	0·65	0·13	0·05	0·12	0·06
LR	500	0·37	1·60	1·60	0·17	0·14	1·00
LR	400	0·40	1·20	1·20	0·19	0·96	1·70
LR	300	0·50	3·00	3·00	0·24	0·30	3·00
LR	200	0·17	3·00	0·99	0·01	0·02	0·08
LR	500	1·20	1·70	1·30	0·13	0·35	1·30
LR	500	0·60	1·50	0·70	0·07	0·12	1·60
LR	500	0·20	1·70	0·40	0·02	0·19	0·09
LR	500	0·85	3·00	3·00	0·20	1·10	3·00
NR	500	3·00	2·10	0·60	0·03	0·06	0·97
NR	110	2·60	3·00	0·90	0·01	0·10	1·60
NR	500	1·20	3·00	3·00	0·60	1·90	2·30
NR	<7·8	0·34	0·60	0·03	0·00	0·07	0·22
NR	240	0·50	1·60	0·17	0·02	0·06	2·17
NR	240	0·37	3·00	3·00	0·20	0·50	1·70
NR	280	0·40	1·60	0·30	0·08	0·08	0·08

* Patients were classified into full responders (FR), partial responders (PR), low responders (LR) and non-responders (NR) according to the effectiveness of pharmacological treatment.

† The highest value found in response to SHF.

IL-5/IgE, r=0·5, P<0·05; IL-5/IgG4, r =0·6, P<0·05 by Spearman's rank correlation.

The major role of IL-6 is to induce the terminal differentiation of B cells into plasma cells, thus contributing to the development of antigen-specific humoral responses [5]. The effect of IL-5 on human B cells remains controversial [8]. We previously observed a significant correlation between IgE and IgG4 production in hydatid patient sera. The simultaneous development of elevated IgE and IgG4 suggests that in human hydatidosis, as in other parasitoses, these two responses come under the regulatory control of the cytokine system [1]. Patients who produced high levels of IgE and IgG4 showed a parallel increase in concentrations of IL-4 and IL-10 Th2 cytokines [2]. In this study immunoglobulin isotype analysis revealed a significant correlation between IL-5 production and IgE/IgG4 expression. The similarly high IL-6 levels found in all patients excluded an association between IL-6 cytokine production and immunoglobulin subclass expression.

Interestingly, although the three serologically negative patients in the present study produced high levels of IL-6 and two of them also produced IL-4 and IL-10 (data not shown), they were also the only three IL-5-negative patients. We therefore conclude that rather than being involved in eosinophilia, IL-5 probably participates in the development of humoral responses to human hydatid disease.

Finally, in contrast to our previous observation for the Th2 cytokines IL-4 and IL-10 [2], in this study we found no association between IL-5 and IL-6 production and the outcome of therapy.

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