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Analysis of cytokine and specific antibody profiles in hydatid patients with primary infection and relapse of disease

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SUMMARY

We studied in vitro *cytokine production by peripheral blood mononuclear cells (PBMC) from patients with primary and recurrent hydatid disease when cells were incubated with mitogen (PHA) and antigen from hydatid cyst fluid (HCFAg); levels of specific IgE, IgG4 and eosinophil counts were also measured in sera. When specifically stimulated, PBMC from patients produced higher levels of IL-2 ($P < 0{\cdot}02$), IFN-γ ($P < 0{\cdot}0028$) and IL-5 ($P < 0{\cdot}01$) than those from uninfected donors, whereas IL-10 levels were comparable. Notably, IL-5 was also produced in higher levels ($P < 0{\cdot}01$) by PBMC from patients when incubated with PHA. The IL-5:IFN-γ ratio was significantly greater ($P < 0{\cdot}02$) when measured in response to specific stimulation than it was for PHA-stimulated cultures. These cytokine data suggest a bias towards a Th2-response which is in agreement with the high levels of IgG4 and IgE observed. The polarized response appears to be related to clinical status, as differences between patients with primary infection and those with relapse of disease were demonstrated, with significantly higher levels of IgE ($P < 0{\cdot}003$), IgG4 ($P < 0{\cdot}04$) titres and eosinophil counts ($P < 0{\cdot}04$) in the latter; in addition a tendency to an increased production of IL-5 buy lower IFN-γ was also observed in this group. These results merit further study as they are suggestive of a putative role of Th2-like responses in susceptibility to reinfection by* E. granulosus.

Keywords *hydatid disease*, E. granulosus, *cytokines, IgE, IgG4*

INTRODUCTION

Human cystic hydatidosis, due to the larval growth of *E. granulosus* is a chronic condition with persistent antigenic stimulation evoking both cellular and humoral specific responses (Lightowlers & Gottstein 1995). Production of IgG4 and IgE specific for parasite antigens are elicited to high levels during infection (Aceti *et al*. 1993, Riganò *et al*. 1995a).

A link between humoral response and cytokines has recently been reported. Riganò *et al*. (1996) found positive correlation between *in vitro* IL-5 production and levels of specific IgG_4 and IgE. Torcal *et al* (1996) demonstrated a relationship between total IgG production and serum IL-4 levels. Taken together, these results indicate a potential role for Th2 cells in the upregulation of antibody expression in hydatid disease. Nevertheless, Th1-like cells have also been demonstrated to be activated during infection (Riganò *et al*. 1995a) and may also be involved in regulation.

It has been demonstrated in several parasitic infections that the balance between Th1- and Th2- like cytokines during infection may have profound implications for susceptibility/resistance to disease (Scott *et al.* 1989, Sher & Coffman 1992).

In human *E. granulosus* infection, a positive correlation has been reported between resistance to pharmacological treatment and Th2 response -in terms of IL-4, IL-10 but not IL-5, IL-6 production-while IFN-γ production was correlated with successful treatment (Riganò *et al*. 1995b, 1996).

Nevertheless, the role of Th1 and Th2 populations in susceptibility to the establishment of cysts as well as to the outcome of disease in untreated patients remains unclear.

Relapses of hydatid disease—appearance of new cysts in patients who had been surgically treated—is a severe problem often associated with accidental spillage of protoscoleces (PSC) during surgery or after traumatic rupture of cysts; recurrence rates of hydatid disease as high as 22% have been reported in the literature (Little *et al*. 1988).

The development of new cysts concomitantly with

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specific immune responses evolved by the host indicate a lack of protective immunity at least in some patients. Several putative mechanisms have been postulated which might be used by *E. granulosus* to interfere with potentially effective host immune responses (Nieto *et al*. 1993). Modulation by the parasite of the type of Th response may be relevant to controlling the nature of humoral and cellular effector mechanisms during infection, though any correlation between these mechanisms and susceptibility to infection is still unknown.

There is now evidence that the emerging Th cell phenotype may be controlled during infection (e.g. by administration of appropriate counter-regulatory cytokines or blocking cytokines with a role in pathogenesis) leading to the possibility of inducing an effective response with minimal pathology to the host (Modlin & Nutman 1993, Masihi 1994).

In this context, the understanding of the role of particular cytokines in immunoregulation in patients with hydatid disease necessary for development of strategies of immune intervention in order to avoid relapses of the disease.

The aim of this study was to assess lymphocyte function (proliferation, cytokine production and antibody response) to further assess the involvement of Th1- and Th2-like cytokines in regulation of the immune response to crude antigens from *E. granulosus* in hydatid patients with primary or recurrent hydatid disease.

MATERIALS AND METHODS

Subjects

Twenty-five patients (15 women and ten men) with ages from 31 to 75 years were included in this study. All patients had clinical diagnosis of hepatic hydatid disease but two of them also presented abdominal cysts. None of the patients had been pharmacologically treated for at least one last year previous to this study. No atopic manifestations nor malignant disease were reported among the subjects studied. Diagnosis was later surgically confirmed.

Relapse of disease (previous surgeries) and activities of cysts were taken into account as clinical features. Twelve patients were referred with known previous surgery between two and eight years before. The remaining 13 patients presented primary hydatidosis.

The stages of the cysts were judged by ultrasound and/or CT scanning patterns according to Gharbi (1981). Seventeen patients had cysts with clear fluid with or without protoscoleces (Type I–III) and the remaining eight patients presented heterogeneous patterns with calcified cysts in some cases (Type IV–V). These categories are referred to as active and inactive cysts respectively.

Twenty-three healthy volunteers were included as the control group. These had neither personal nor familiar history of hydatid disease and negative specific serology (antibody titres < 1/16) was confirmed by indirect haemagglutination using a commercial kit ('Cellognost Echinococcosis', Behring, Marburg, Germany).

Informed consent was obtained from all patients before study and blood withdrawal in accordance with the guidelines of the Spanish Department of Health.

Blood samples

Twenty ml of venous blood samples were collected from patients and healthy donors in Vaicutainer tubes (Becton Dickinson, Mountain View, CA, USA) containing preservative-free heparin.

Serum samples were also obtained in each case and were cryopreserved at −20°C until use.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from patients and healthy donors by density gradient centrifugation on lymphocyte separation solution (Lymphoprep, Nycomed, AS, Oslo, Norway) as described by Böyum (1968). PBMC were resuspended at the concentration of 1×10^6 viable cells/ml in culture medium (AIM V Serum- free Medium; Gibco BRL, Grand Island, NY, USA) supplemented with 2 mM L-glutamine (Sigma). Viability tests were performed with trypan blue 0·25% in PBS (Sigma) and viability was routinely greater than 90%.

Freshly isolated PBMC were immediately used for proliferation and cytokine production assays.

Antigens and mitogen

Hydatid cyst fluid antigen (HCFAg) obtained from bovine cysts as previously described (Carol *et al*. 1989) was kindly provided by the Immunology Laboratory, University of the Republic (Salto, Uruguay).

For PBMC cultures 120 mg of lyophilized HCFAg was dissolved in 2·3 ml of sterile PBS and exhaustively dialysed against PBS. Following sterilization by passage through 0·22 μm membrane filters, protein content resulted to be 1·75 mg/ml according to the method of Bradford (BioRad Laboratories, Richmond, CA, USA) using Bovine Serum Albumin (BSA) as standard.

Sterile solutions of Bovine Serum (Sigma) and Bovine Serum Albumin-BSA (Sigma) were prepared in PBS at 500 μg/ml and 250 μg/ml respectively.

Phytohaemagglutinin (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in sterile PBS and conserved at −20°C at a concentration of 5 mg/ml until use.

Proliferation assay

For lymphoproliferation assay microcultures of 2×10^5 PBMC in a final volume of 200 μl of medium were performed in 96-well flat-bottom microplates (Nunclon^{TM}, NUNC, Denmark). For each sample triplicate wells were simultaneously incubated in two plates (at 37°C and humidified atmosphere of 5% CO_2 in air) during three days with PHA at a final concentration of 5 μg/ml and for seven days with HCFAg selected according to previous kinetic assay. Optimal dose of HCFAg for PBMC stimulation was previously evaluated within the range of 1·6–50·0 μg/ml and used for the rest of the study.

Triplicate wells with PHA (1 μg/ml) were incorporated in plates incubated during seven days as a positive control of proliferation.

Depending on the availability of cells, in some cultures Bovine Serum and BSA were also included as control antigens at final concentrations of 25 and 50 mg/ml respectively.

Mitogenic activity of HCFAg was evaluated by incubating PBMC from healthy donors ($n=6$) with optimal concentration of antigen during three days, in parallel with PHA-driven proliferation.

In all cases triplicate unstimulated cultures were included.

Cultures were pulsed with 0·5 μCi/well of methyl-^{3}H-thymidine (specific activity = 5·0 Ci/mmol, TRA.120, Amersham, UK) during the last 18 h of culture.

Cells were harvested onto glass-fibre filters (GF/A, Whatman) and the uptake of ^{3}H-thymidine into DNA was measured by liquid scintillation β-counting.

Proliferative responses were measured as cpm and geometric means of triplicate values for each antigen or mitogen was divided by the geometric mean cpm of unstimulated control wells to obtain a stimulation index (SI).

Production of PBMC culture supernatants and quantitation of cytokines

In parallel with proliferation assays PBMC cultures at a concentration of 1×10^6 cells/well were performed in 24-wells flat-bottom microplates (Costar, Cambridge, MA, USA) at 1 ml/well to obtain supernatants. For each sample, triplicate wells were incubated with optimal dose of HCGAg, PHA (5 μg/ml) and culture medium alone as described before. After 48, 72 and 96 h supernatants were collected and cryopreserved at −70°C in sterile conditions until use.

Because of different cell recoveries from blood samples the number of individuals tested varied for some determinations.

IL-10 and IFN-γ were assayed using ELISA kits (Quantikine^{TM}; R&D System Inc., Minneapolis, MA, USA). The minimum thresholds of these assays were 1·5 and 3 pg/ml respectively.

IL-2 production was quantified by ELISA kit (Interleukin-2 Citoquine, Predicta^{TM}; Genzyme, Cambridge, MA, USA) as recommended by the manufacturer. The detection limit of these assays was 4 pg/ml.

IL-5 was assayed by a two-site ELISA employing an immobilized MoAb on the microplate (Maxisorb^{TM}, Nunc), purified rat anti-mouse/human IL-5 (clone TRFK5, Pharmingen, Cambridge, UK) at 2 μg/ml according to manufacturer's recommendations.

Binding of biotinylated antibodies was detected using Streptavidin Peroxidase conjugate (Sigma) at 2·5 μg/ml in PBS. Colour was developed with ABTS (2,2′-azino-di-(3-ethyl-benzthiazoline sulphonate)) solution (0·3 mg/ml) in citrate buffer (1 mg/ml) and H_2O_2 (0·03%). Optical density (OD) was measured at 405 nm.

The concentration of IL-5 was calculated by reference to standard curves constructed with known amounts of recombinant human IL-5 (Pharmingen). The sensitivity of this test was determined to be approximately 31 pg/ml.

In all assays results were expressed as the mean of duplicate determinations.

Specific antibody titres

HCFAg-specific IgG_4 was quantified by ELISA using microtitre plates (Maxisorb, Nunc, Denmark) coated with 20 μg/ml of HCFAg in PBS, overnight at 4°C. Plates were blocked with 1% bovine serum albumin in PBS (BSA-PBS) for 2 h (37°C) and washed three times with 0·05% Tween 20 in PBS (Tw-PBS).

Serum samples from patients and healthy donors (diluted from 1 : 100 to 1 : 1600) were incubated for two h (37°C). The plates were washed as before and 100 μl/plate of biotinylated anti-IgG_4 monoclonal antibody (Sigma) diluted 1 : 2500 was added for two h (37°C). After the plates were washed as above 100 μl/well of streptavidin-peroxidase (Sigma) 2·5 μg/ml was added for 45 min at room temperature.

Washes were performed as before and plates were developed using 0·3 mg/ml in citric acid solution, pH = 4·35 and H_2O_2 (0·03%). Optical density (OD) was measured at 405 nm.

A reference serum was used on each plate for quantification. Values of OD from serum samples were compared to those from a standard curve and expressed as arbitrary units per ml (AU/ml).

The lower limit for positives (cut off value) was determined as the mean of normal control +2 SD.

Concentrations of specific IgE were assayed by FEIA using a commercial kit (Pharmacia CAP System p2 Echinococcus, Kabi-Pharmacia Diagnostics, Sweden) according to the manufacturer's instructions. Fluorescence was measured

by means of Fluoro Count FC 96 (Kabi-Pharmacia Diagnostics). Data from patients samples were compared directly with fluorescence from standard run in parallel. Results were expressed as kU/l and values were considered to be positive when $>0{\cdot}35$ kU/l.

Eosinophil counts

Lymphocytes, monocytes and granuloctye subpopulations were counted from fresh blood samples by using an automated autoanalyser (Coulter). Eosinophil counts were expressed as a percentage of total leucocytes.

Statistical analysis

The Mann-Whitney rank test for non-parametric data was used to compare immunological parameters between groups of patients and non-infected individuals. Differences with a confidence level of 95% or higher were considered statistically significant ($P<0{\cdot}05$).

Spearman's rank test was used to assess correlation between immunological parameters.

RESULTS

Proliferative response of PBMC from patients and healthy donors

When PBMC were incubated with a final concentration of $5\,\mu$g/ml of PHA (three days), strong proliferative responses were obtained in samples from both patients and healthy donors with no statistically significant differences between them (geometric means SI = 92 and 86 respectively).

Stimulation indices were measured for a range of 1·6–$50{\cdot}0\,\mu$g/ml of HCFAg in cultures of PBMC (seven days) from patients and healthy donors and significant differences between groups were obtained with doses of 6·0–$50{\cdot}0\,\mu$g/ml; the intermediate dose ($12{\cdot}5\,\mu$g/ml) was selected for use throughout this study (Figure 1).

HCFAg-stimulated cultures from hydatid patients, exhibited significantly higher SI than those from healthy donors (geometric means SI = 13·8 and 3·8 respectively, $P=0{\cdot}0002$) and an arbitrary threshold was defined as the mean SI from control group + SD. According to this criterion 67% of hydatid patients showed significant specific proliferative responses (Figure 2). Non-responder patients were observed in every group and no correlation was obtained between proliferative response and clinical feature. For non-responder patients the high thymidine uptake values in response to PHA ($1\,\mu$g/ml, seven days) indicated good viability of the cultured PBMC (data not shown).

No proliferative response was obtained when PBMC from five patients were in parallel incubated with BSA ($25\,\mu$g/ml) and Bovine Serum ($50\,\mu$g/ml) as controls (mean SI = 1·1 and 1·9 respectively).

When PBMC from six healthy donors were incubated

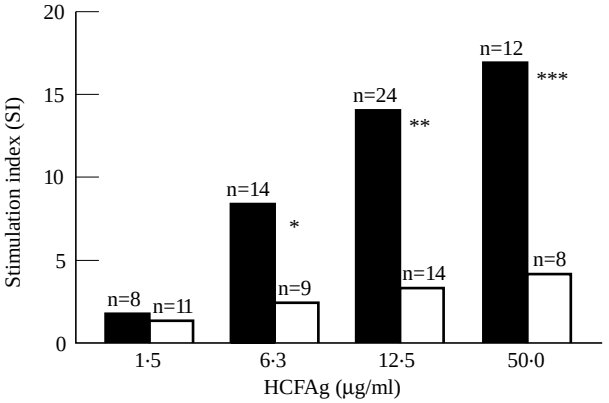

Figure 1 Distribution of HCFAg-driven proliferation (g. means) when PBMC from hydatid patients (solid bars) and healthy donors (open bars) were stimulated with different concentrations of HCFAg in cultures. n = number of individual assayed. Statistically significant differences between groups: $*P=0{\cdot}009$, $**P=0{\cdot}0002$ and $***P=0{\cdot}009$.

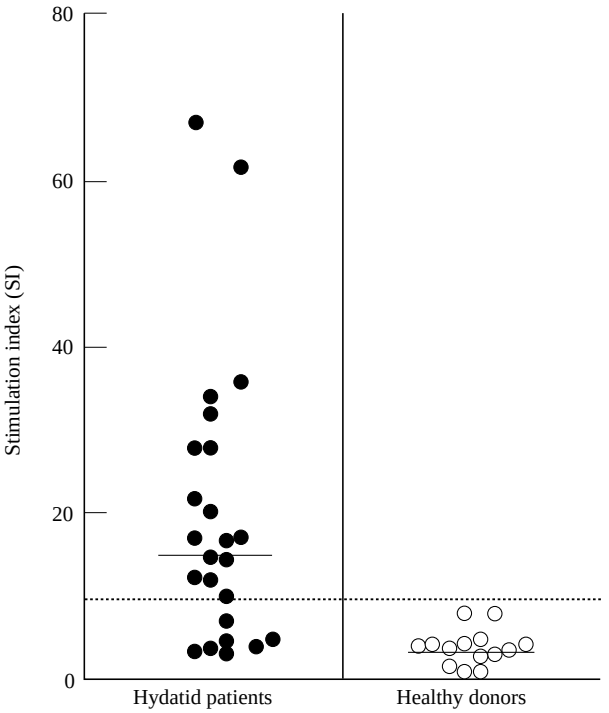

Figure 2 Distribution of HCFAg-driven proliferation in cultures of PBMC from hydatid patients and healthy donors. Horizontal bars show mean values and dotted line represents the positive/negative threshold.

Table 1 HCFAg-driven cytokine production in patients and healthy donors

	Cytokines	IL-5	IL-10	IL-2	IFN-γ
	Time of culture (h)	96	96	72	96
Hydatid patients	Mean (range) in pg/ml	181 (<31–4000)*	89 (<4–1465)	121 (<4–1300)**	22 (<3–815)***
	Number of responder/total patients	17/25	23/25	22/24	15/25
Healthy donors	Mean (range) in pg/ml	48 (<31–163)	141 (39–432)	25 (<4–109)	5 (<3–102)
	Number of responder/total donors	7/23	18/18	7/14	3/22

Statistically significant differences between groups: * $P < 0{\cdot}0028$; ** $P < 0{\cdot}02$ and *** $P < 0{\cdot}01$.

with 12·5 μg/ml of HCFAg for three days in parallel with mitogen stimulated cultures as positive controls, a negligible response was obtained (mean SI = 1·5).

Cytokine production in response to mitogen and parasite antigen

Incubation times for optimal cytokine detection in supernatants were evaluated in a previous kinetic study with antigen- and mitogen-stimulated cultures; concentrations of each cytokine were then measured in supernatants collected at the optimal time (Table 1 and 2).

Levels of IL-2 and IFN-γ in unstimulated cultures were below the limit of detection for each assay in both patients and control groups. In the case of IL-5 and IL-10 similar low concentrations were achieved in non-stimulated cultures (96 h) from both patients and controls (means 37 and 46 pg/ml for IL-5; means 5·8 and 5·3 pg/ml for IL-10 respectively). The mean +2 SD was taken as the threshold for a significant response (57 and 10·5 pg/ml for IL-5 and IL-10 respectively).

HCFAg-driven cytokine production was significantly higher in PBMC cultures from patients than from healthy donors for IL-2 ($P < 0{\cdot}02$), IL-5 ($P < 0{\cdot}0028$) and IFN-γ ($P < 0{\cdot}01$) whereas IL-10 was detected in supernatants from both groups in similar concentrations ($P = 0{\cdot}32$) (Table 1).

PHA stimulated secretion of IL-2, IL-10 and IFN-γ by PBMC from all hydatid patients and healthy donors and concentrations achieved in supernatants for each cytokine did not differ significantly between groups. In contrast levels of IL-5 were significantly higher ($P < 0{\cdot}01$) in supernatants from patients than in those from healthy donors (Table 2).

The analysis of the ratio between IL-5 and IFN-γ levels in cultures stimulated with HCFAg was taken into account for each patient to evaluate predominance of either Th1- or Th2- like response. Results indicate that the ratio IL-5 : IFN-γ was significantly higher ($P < 0{\cdot}02$; $n = 22$) when measured in response to specific stimulation than when measured in mitogen-stimulated cultures with geometric means being 8·2 and 3·0 respectively for patients (Figure 3) and 2·5 for PHA-stimulated cultures from controls ($n = 15$).

Levels of cytokines obtained in supernatants from patients were analysed in relation to clinical features. It is noteworthy that patients with relapses of disease exhibited higher production of IL-5 and lower IFN-γ levels than those with primary disease, resulting in a tendency of higher ratios of IL-5 : IFN-γ in the former group, with

Table 2 PHA-driven cytokine production in hydatid patients and healthy donors

	Cytokines	IL-5	IL-10	IL-2	IFN-γ
	Time of culture (h)	72	96	48	96
Hidatid patients	Mean (range) in pg/ml	723 (164–5808)*	576 (65–1400)	106 (97–3843)	263 (75–2357)
	Number of responder/total patients	23/23	24/24	23/23	23/23
Healthy donors	Mean (range) in pg/ml	323 (91–2987)	783 (109–2238)	787 (56–3500)	180 (66–765)
	Number of responder/total donors	18/18	18/18	16/16	15/15

* Statistically significant difference between groups ($P < 0{\cdot}01$).

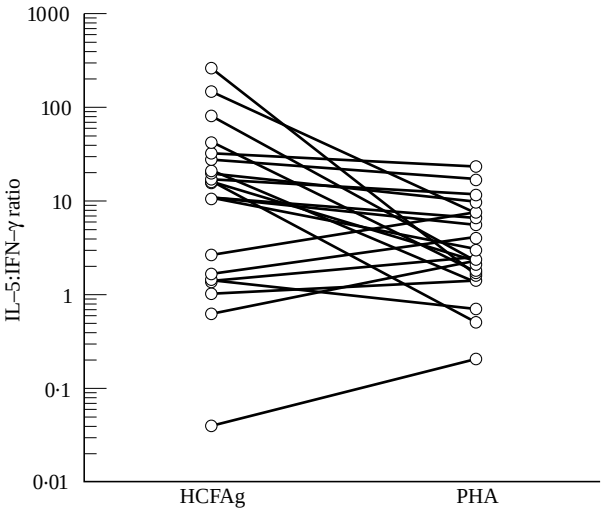

Figure 3 Ratios of IL-5 : IFN-γ levels measured in supernatants of PBMC cultures from hydatid patients. Each point represent the ratio for a single individual when PBMC were stimulated with either HCFAg or PHA.

differences statistically significant in PHA-stimulated cultures ($P = 0{\cdot}01$) (Table 3).

No differences were apparent when patients were divided into two groups according to the stage of cysts.

Specific antibody titres and eosinophil counts

Seventy-one per cent of hydatid patients were positive for specific IgE against HCFAg while 76% of them exhibited specific IgG_4 responses. Significant strong positive correlation were found between IgE and IgG_4 levels ($P = 0{\cdot}0001$, Rh0 $= 0{\cdot}809$) as well as between eosinophil counts and IgE titres ($P = 0{\cdot}05$, Rh0 $= 9{\cdot}453$) (Table 4). No correlation was found between IL-5 levels and serological parameters.

Patients had higher mean levels of eosinophils than uninfected controls ($2{\cdot}6 \pm 1{\cdot}8$ and $1{\cdot}6 \pm 0{\cdot}8$ respectively, $P < 0{\cdot}01$); 33% of patients showed values above mean +2 SD of control group though strong eosinophilia was not assessed in any of them (Table 4).

In order to compare the response profile of patients with primary hydatid disease and those with previous surgery, the group was subdivided for analytical purposes and levels of IgG_4, IgE and eosinophil counts compared. Significantly higher levels of parasite specific IgG_4 ($P = 0{\cdot}004$), IgE ($P = 0{\cdot}003$) and eosinophil counts ($P = 0{\cdot}04$) were obtained in patients with one or more relapses of disease.

DISCUSSION

In this report we compared specific IgG4/IgE response and *in vitro* cytokine production by PBMC from hydatid patients with primary hydatidosis and with relapses of the disease.

A mixed profile including both Th1- and Th2-like cytokines (IL-2, IFN-γ, IL-5 and IL-10) has been found in this study when PBMC from hydatid patients were stimulated with parasite antigen *in vitro*; this finding is consistent with a previous report indicating simultaneous production of IL-10, IL-4 and IFN-γ by PBMC from hydatid patients (Riganò *et al*. 1995). In addition, a broad spectrum of cytokines (IL-2, IL-4, IL-6, interferons and TNF-α) have been shown to be present in serum from patients with hydatidosis (Torcal *et al*, 1996, Touil-Boukoffa *et al*, 1997).

Our data also support the interesting finding of an imbalance in the ratio of Th1 : Th2 towards a predominantly Th2-like response in the group of hydatid patients. When IL-5 and IFN-γ levels were considered, a higher ratio of

Table 3 Analysis of HCFAg- and PHA-driven cytokines production in PBMC cultures with regard to relapses of disease

	IL-5		IFN-γ		IL-5 : IFN-γ ratio	
	HCFAg	PHA	HCFAg	PHA	HCFAg	PHA
Hydatid patients with primary disease	144 (<31–3375)	526 (164–5808)	33 (<3–815)	343 (75–2375)	4·4 (0·04–84)	1·7 (0·2–7·2)
n	13	12	13	11	13	11
Hydatid patients with relapsed disease	231 (<31–4000)	988 (316–2950)	14 (<3–350)	203 (75–1134)	16·4 (1·4–267)	5·9 (0·7–22·7)
n	12	11	12	12	12	11
P (Mann-Whitney)	0·54	0·121	0·35	0·078	0·108	0·011(*)

Data are expressed as geometric mean (range) in pg/ml. *n* indicates the number of samples assayed. (*) statistically significant difference between groups.

Table 4 Clinical and serological characteristics of patients

Patient group (no.)	Stage of cysts*	Specific IgE (kU/ml)	Specific IgG_4 (AU/ml)	Eosinophil counts (%)**
Primary disease	A	43·3	18·6	6·6
(13)	A	5·1	9·6	0·9
	A	24·8	25·7	0·6
	A	6·0	21·2	n.d.
	A	2·6	–	n.d
	I	–	6·8	1·8
	A	–	–	2·1
	A	6·9	10·5	n.d.
	I	–	–	n.d.
	A	–	–	1·9
	I	–	–	0·5
	I	–	–	1·6
	A	–	6·6	2·0
Relapsed disease	A	40·6	23·2	2·6
(12)	I	100·0	27·2	7·1
	A	31·6	21·1	1·9
	A	20·5	15·5	4·0
	I	30·6	19·2	0·7
	A	13·1	13·9	4·7
	I	22·4	14·3	3·4
	A	6·3	15·2	2·7
	A	20·7	27·2	3·6
	A	25·2	7·3	2·4
	A	n.d.	27·2	5·2
	I	2·5	16·2	1·0

* Stage of cysts are referred to as active or inactive according to echographic patterns. ** Eosinophils levels are expressed as percentage of total leukocytes. Horizontal bars indicate levels under cut off. n.d. = not done.

IL-5 : IFN-γ was observed when PBMC from patients were stimulated with parasite antigens in relation to their response to mitogenic stimulation, which is consistent with the possibility of preferential stimulation of Th2-like cells by *E. granulosus* antigen.

In addition, we detected significantly higher levels of IL-5 in supernatants from PHA-stimulated PBMC from hydatid patients than those from healthy donors. Assuming that parasite-specific clones represent a very minor fraction of the peripheral T cell population these results might indicate that non-parasite specific cells producing IL-5 are also preferentially expanded during infection and/or higher levels of this cytokine per cell are being produced.

This elevated production of IL-5 during hydatid infection may be relevant from a clinical point of view and merits further study, as a correlation between IL-5 levels and viability of the larval cestode and progression of disease has been suggested in patients with alveolar hydatidosis (Sturm *et al*. 1995).

The Th2 subset contains the regulatory cells mainly involved in the generation and maintenance of IgE, IgG4 response (Pène 1988, Purkenson & Isakson 1995) and maturation of eosinophils (Coffman 1989, Sher 1990, Finkelman 1991) mediated by IL-4 and IL-5 production. To further assess the nature of regulatory mechanisms in hydatid patients, titres of these specific isotypes as well as eosinophil counts in peripheral blood have also been studied. We confirmed the positive correlation between IgG4 and IgE previously reported (Riganò *et al*. 1995) and in addition we found significant correlation between IgE and eosinophil counts when studying the whole group of patients.

When data from cytokine production and specific antibody levels were evaluated with regard to clinical status, significant differences between patients with primary infection and those with one or several relapses of disease were obtained, with higher levels of specific antibody titres and eosinophil counts in the latter group. In relation to this finding we also observed a tendency to increased production of IL-5 but lower IFN-γ in patients with relapse of disease when PBMC were stimulated with either HCFAg or PHA. These results suggest different patterns of activation of Th2 and Th1 cells (according to values of IL-5 : IFN-γ) depending on clinical status.

Although a longitudinal study is required to assess clinical outcome in patients with primary disease, a putative link between Th2-like response and susceptibility to reinfection could be speculated from current data.

Cytokines secreted by cells of the non-specific immune response during the earliest contact between pathogen and immune cells of the host (Masihi 1994, Garside & Mowat 1995) regulate the differentiation of naive Th precursor cells into Th1 or Th2 phenotypes. High levels of Il-4 and Il-12 are regarded as being the main stimulus evoking Th2 or Th1 differentiation respectively (reviewed by Seder & Paul 1994, Reiner & Seder 1995). Other regulatory cytokines if present in the early response can also contribute to polarization; e.g., IL-10, a cytokine initially defined as an inhibitor of Th1 function (Swain *et al*. 1990, Fiorentino *et al*. 1991), may inhibit IL-12 production by antigen-presenting cells (Hsieh *et al*. 1992, D'Andrea *et al*. 1993). Thus, analysis of cytokines produced by PBMC from non-infected individuals when stimulated with parasite-antigens, might contribute to elucidation of mechanisms of Th differentiation in hydatid disease.

In this report, IL-5, IL-2 and IFN-γ were only produced by PBMC from hydatid patients when cells were incubated with parasite antigens, in contrast significant levels of IL-10 have also been detected in cultures of PBMC from uninfected donors. These data are consistent with the fact that IL-10 is also produced by non-Th cells in PBMC—e.g., monocytes and B-1 lymphocytes (Moore *et al*. 1993).

In this context our results suggest that IL-10 could have been produced by monocytes from healthy donors as no other cytokines only produced by T lymphocytes in PBMC were detected and parasite-driven proliferation after three days of culture was negligible (g. mean SI $= 1{\cdot}5$, $n = 6$) for controls in comparison to patients (g. mean SI $= 7{\cdot}7$, $n = 7$ data not shown). Nevertheless, production of IL-10 by direct stimulation of B-1 population by parasite antigens cannot be ruled out as production of cytokines without proliferation has been previously reported (Evavold & Allen 1991).

In the case of hydatid patients, the putative involvement of B-1 cells as source of Il-10 is consistent with several reports demonstrating auto antibodies in sera (Pini *et al*. 1983, Ben Izhako & Tatarsky 1985, Ameglio *et al*. 1987). Pertinent to this point, it has been reported that carbohydrate moieties from *S. mansoni* antigens contribute to the down-regulation of Th1-response through production of IL-10 by B lymphocytes (Velupillai & Harn 1994). Because of the immunodominance of glucidic epitopes in *E. granulosus* antigens and its involvement in cross-reactivity (Miguez *et al*. 1996), these aspects merit further study as an approach to elucidating the role of defined antigens in the modulation of host-response in hydatid disease.

In conclusion, our results indicate an imbalance through Th2-like response in hydatid patients, interestingly enhanced in those with one or more relapses of disease; in addition, data suggest a putative role of IL-10 produced during first contact with parasite antigens in selecting the response observed in chronic infection.

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