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IL-5 expressed by $CD4^+$ lymphocytes from *Echinococcus multilocularis*-infected patients

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SUMMARY

IL-5 is a major factor inducing differentiation of B lymphocytes into immunoglobulin-producing cells as well as a main regulator of eosinophils. Recently, we have shown that peripheral blood mononuclear cells (PBMC) from patients with alveolar echinococcosis (AE) express IL-5 mRNA after stimulation with crude *Echinococcus multilocularis* (E.m.) antigen. To characterize the observed response in lymphocyte subpopulations, we cultured patients' PBMC in the presence of E.m. crude antigen for 18 h. PBMC were separated from seven patients by fluorescence-activated cell sorting (EPICSorter) into $CD4^+$ and $CD8^+$ subpopulations and from an additional seven patients by magnetic cell sorting (MACS) into $CD4^+$, $CD8^+$ and the $CD4^+/CD8^+$ depleted fractions. mRNA was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) for the cytokines IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, as well as for β-actin as control. IL-4 and IFN-γ expression was positive in all of the patients in the stimulated $CD4^+$ subgroup. IL-5 mRNA expression was detected in eight out of 14 $CD4^+$ samples (58%) and not observed in the other subpopulations, or the unstimulated and healthy controls. Co-expression of other Th2 cytokines in the eight patients expressing IL-5 mRNA was found in five patients for IL-3 and in seven for IL-10. Expression of IL-5 and both Th2 cytokines (IL-3 and IL-10) was only observed in patients judged as critically ill. Out of the six patients who were regarded as cured after radical operation or as stabilized with or without chemotherapy, only two expressed IL-5. Out of those eight patients considered as critically ill, six expressed IL-5 mRNA and five of these co-expressed IL-3 and IL-10. Thus, we conclude that specific antigenic challenge of PBMC from patients with active or previous AE induces an IL-5 response of $CD4^+$ lymphocytes. The expression of Th2-type interleukin mRNA is significantly more frequent in patients clinically judged as progressive. Furthermore, IgE was elevated only in patients regarded as critically ill (six out of eight). In none of the patients were eosinophils elevated. These data support a Th2-type immune response in patients with chronic *E. multilocularis* infection.

Keywords human alveolar echinococcosis IL-5 T cell response Th2 cytokines

INTRODUCTION

The inadvertent ingestion of viable eggs of the cestode *Echinococcus multilocularis* by humans leads to the development of the larval stage. This tumour-like expansion occurs in visceral organs, predominantly in the liver, and is termed alveolar echinococcosis (AE) [1]. The immunological response to *E. multilocularis* is composed of humoral immunity, indicated by the production of specific IgG [2] and IgE [3], and cell-mediated immunity, expressed by the granulomatous infiltration surrounding the parasitic vesicles [4]. Regression or progression of AE is correlated with the cell composition and the ratio of T lymphocyte subpopulations in the local granuloma [5], suggesting a major role for the antigen-specific T cell response in the host–parasite relationship. Cytokines are considered to regulate in an intricate interplay the antigen-specific T cell response [6]. In this context the high IgE levels and the eosinophilia, which are found in a variety of helminthic infections [7], can be attributed to the production of the Th2 cytokines IL-5, IL-4 and IL-13 [8]. The importance of this model for *E. multilocularis* has recently been supported by the finding that a Th1 *versus* Th2 discrimination, with respect to susceptibility and resistance, could be demonstrated in a mouse model [9]. In a previous study, we have shown that the stimulation of unsorted peripheral blood mononuclear cells (PBMC) from *E. multilocularis*-infected patients with crude homologous echinococcus antigen results in an expression of mRNA for cytokines associated with a Th2 response [10]. This correlation was also

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found in a model of PBMC from *E. granulosus*-infected patients stimulated with *E. granulosus* antigen [11] and in the measurement of cytokine production pre- and post-treatment in the same disease [12].

The aim of the present study was to correlate the clinical status of *E. multilocularis*-infected patients with specific Th2 cytokine expression in various subpopulations of PBMC in order to find further hints of a Th2-like response in AE patients and to gain insights into the immune response against *E. multilocularis*.

PATIENTS AND METHODS

Patients

Fourteen patients, eight female and six male, with a median age of 51 years (24–77 years), all chronically infected with *E. multilocularis* and supervised in our out-patient clinic, gave written consent to donate blood for scientific reasons after local ethical committee approval. The clinical classification into three groups was performed as recommended by the French/Swiss/German Working Group on Echinococcosis: group A, patients who have had radical surgical resection of AE lesions without relapse for a period of 2 or more years after surgery ($n = 2$); group B, patients with stationary or regressive course of AE, i.e. a stable course of this chronic disease under continuous chemotherapy with benzimidazoles ($n = 4$); and group C, patients with lesions increasing in size, several complications, relapses or metastasis formation under continuous chemotherapy, defined by a severe or progressive form of the disease ($n = 8$). Seven healthy blood donors (24–58 years old) served as controls. Blood samples were collected under strict precautions in pyrogen-free sterile tubes containing EDTA (Sarstedt, Nümbrecht, Germany).

Mitogenic and antigenic stimulation

Crude *E. multilocularis* protein extract antigen was prepared and used as described [10]. Briefly, metacestodes from *E. multilocularis* (Swiss isolate CH-24) were obtained from *Meriones unguiculatus* experimentally infected by i.p. transplantation of metacestode tissue blocks. Metacestode material was minced three times for 30 s each in sterile PBS pH 7·6 in a polytron PCU-2 blender. Subsequently, the minced material was extracted by one freeze-thaw cycle and two sonications for 30 s each at maximum amplitude. A PBS solution of the crude protein extract was clarified by centrifugation at 30 000 ***g*** at 4°C for 45 min and filtration through a Millipore filter (pore size 5 μm). The extract was dialysed against PBS containing 0·5 M NaCl and thereafter the total protein content was determined by the BioRad protein assay as indicated by the manufacturer (BioRad Labs, München, Germany). For *in vitro* stimulation of sorted PBMC, the *E. multilocularis* antigen preparation was used at a final concentration of 10 μg/ml. This concentration yielded an effective stimulation of lymphocyte reactivity against *E. multilocularis* antigen as confirmed by dose–response studies [10].

Lymphocyte preparation

PBMC were obtained from whole EDTA-blood using lymphocyte-separating solution (Ficoll–Paque; Pharmacia, Uppsala, Sweden) by the method of Böyum [13]. Lymphocytes were washed twice with sterile PBS pH 7·6.

Stimulation of PBMC cultures

The PBMC obtained by centrifugation over Ficoll–Paque were washed twice and 1 ml containing 5×10^6 cells was resuspended in 2-ml wells of flat-bottomed multiwell dishes (Nunc, Roskilde, Denmark) containing either 10 μg of *E. multilocularis* antigen or no stimulation at all, diluted in RPMI 1640 medium supplemented with 2% fetal calf serum (FCS) and antibiotics, 100 U penicillin per ml and 0·1 mg streptomycin per ml (GIBCO BRL, Gaithersburg, MD). The cells were maintained at 37°C in a humidified atmosphere at 5% CO_2. After 18 h, cells were harvested and sorted by either fluorescence-activated cell sorting (EPICSorter; Coulter, Krefeld, Germany) or magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany).

Fluorescence-activated cell sorting

PBMC were labelled with CD4-FITC and CD8-PE antibodies, both purchased from Becton Dickinson (Heidelberg, Germany), incubated for 1 h at 4°C, resuspended in PBS with 5% FCS and sorted in an EPICSorter (Coulter). The median purity was 94·3% in the $CD4^+$ unstimulated cells with a median cell number of $2{\cdot}1 \times 10^6$, 94·7% in the $CD4^+$ stimulated cells with a median cell number of $2{\cdot}3 \times 10^6$, 90% in the $CD8^+$ unstimulated group with a median cell number of $1{\cdot}1 \times 10^6$ and 94·8% in the $CD8^+$ group with a median cell number of $1{\cdot}5 \times 10^6$.

Magnetic cell sorting

Magnetic cell sorting was performed using the magnetic cell separation (MACS) method in a Mini MACS column, according to the recommendations of the manufacturer. PBMC were incubated for 15 min at 4°C with anti-human MoAb for CD4 (Leu-3a) and for CD8 (Leu-2a), both conjugated to superparamagnetic microparticles (diameter $< 0{\cdot}5$ μm) in a concentration of 20 μl antibodies per 10^7 cells. To assess the effectiveness of this sorting, FACS analysis was performed using CD4 and CD8 MoAbs (both from Dako, Hamburg, Germany) and a FACScan from Becton Dickinson. The purity of this sorting was 96·7% for $CD4^+$ cells and 94·7% for $CD8^+$ cells, which is within the normal range according to the manufacturer (Miltenyi Biotec).

RNA isolation

Following sorting, cells were lysed with guanidinium isothiocyanate as described [14] and stored at −70°C prior to preparation of cDNA.

cDNA synthesis

Total RNA was incubated for 30 min at 37°C in a solution containing 50 mM Tris–HCl pH 7·5, 10 mM $MgCl_2$, 40 U/ml RNasin (Promega, Madison, WI), 10 mM dithiothreitol, and 2·5 U of RQ1 DNase (Promega) to remove any contaminating genomic DNA from the preparation. After phenol-chloroform extraction and ethanol precipitation, RNA pellets were resuspended in water. cDNA was synthesized from RNA by priming ≈ 1 μg of total RNA at 37°C for 1 h in a 50 μl total volume containing 1 μg of an oligo(dT) primer, 200 nmol of each deoxynucleoside triphosphate (Pharmacia), and 200 U of Moloney murine leukaemia virus reverse transcriptase (RT; Gibco BRL); 20 mM dithiothreitol and 5 × Moloney murine leukaemia virus RT buffer were added as recommended by the manufacturer (Gibco BRL).

Amplification of cDNA

cDNAs were tested for the presence of cytokine gene sequences by polymerase chain reaction (PCR) performed in a 30-μl total volume with specific primer pairs. Two microlitres of the relevant cDNA were amplified by PCR in the presence of 0·2 μM sense and

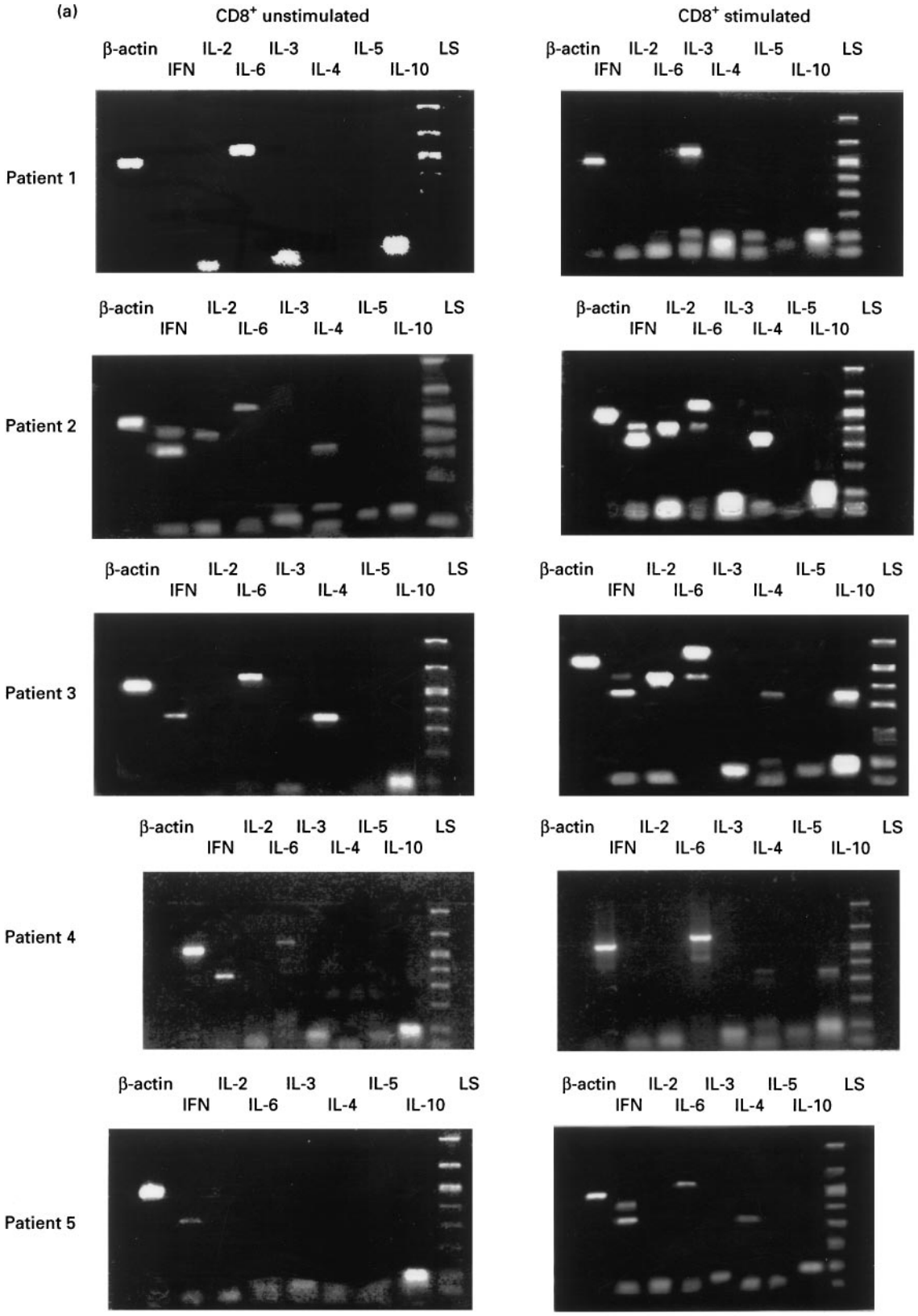

Fig. 1. Polymerase chain reaction (PCR) analysis of cytokine mRNA in sorted peripheral blood mononuclear cells (PBMC) from five *Echinococcus multilocularis*-infected patients. (a) Unstimulated controls after 18 h of cell culture. (b) Cells cultured in the presence of 10 μg/ml crude Echinococcus antigen. Cell sorting was performed by EPICSorter (patient 4) and MACS (patients 1, 2, 3 and 5). PCR products of β-actin, IFN-γ, IL-2, IL-6, IL-3, IL-4, IL-5 and IL-10 were analysed on a 1·5% agarose gel in the presence of appropriate molecular size markers (50, 100, 200, 300, 400, 500, 525, 700 and 1000 bp) (LS).

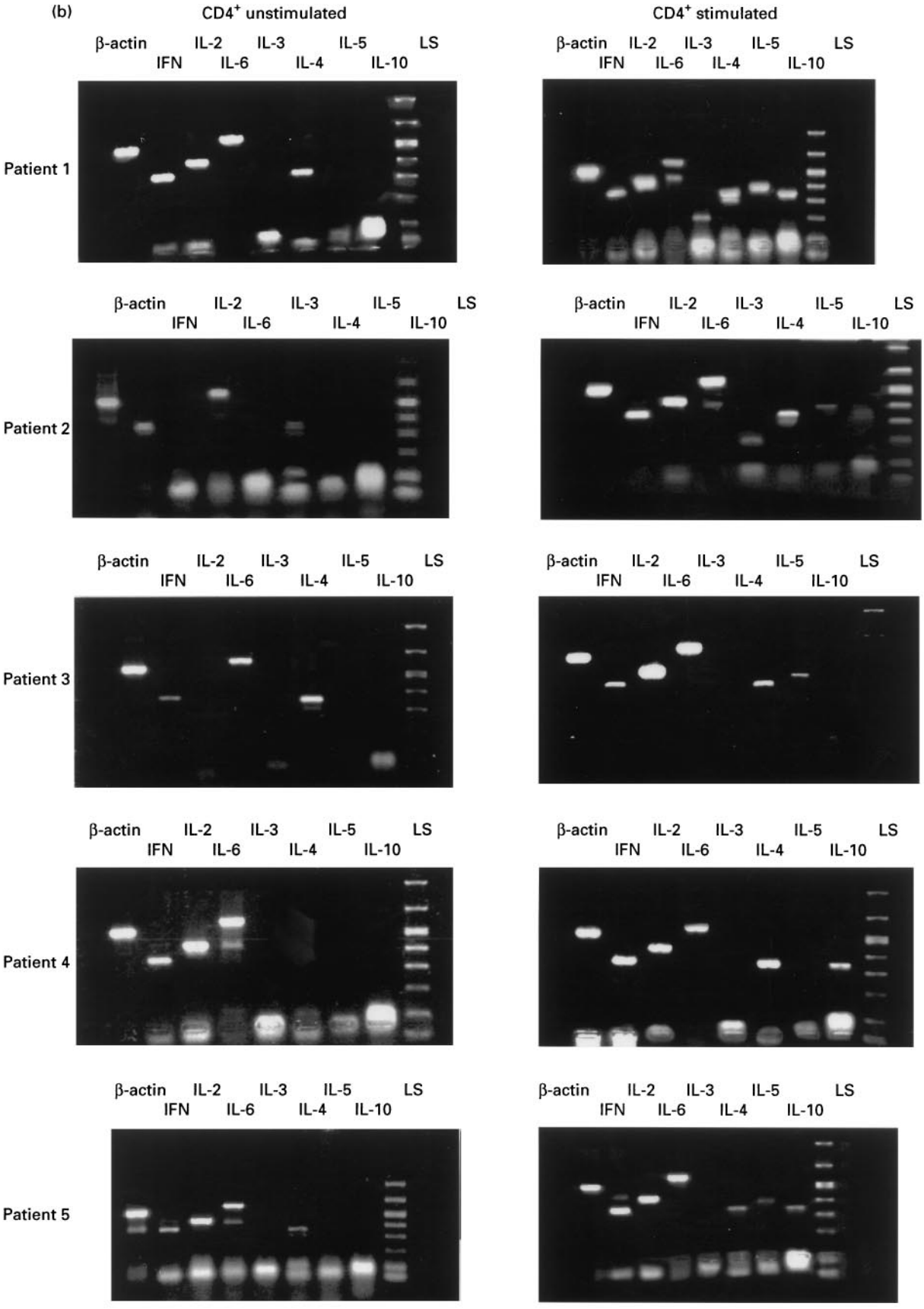

Fig. 1. Continued.

antisense oligonucleotide primers, 2 mM deoxynucleoside triphosphate (Pharmacia, BRL Life Technologies Inc., Gaithersburg, MD), 0·5 U of AmpliTaq polymerase (Perkin Elmer, Branchbureg, NJ) and 10 × PCR buffer. The amplification was performed in a thermal cycler from Perkin Elmer, running a programme of 40 cycles with denaturation at 96°C for 30 s, annealing at 60°C for 1 min, and extension at 73°C for 1·5 min. Oligonucleotide primers specific for β-actin, IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6 and IL-10 were used. Sense and antisense primer sequences were used exactly as described [10], suggesting that the sensitivity should be within the range described by Sorg *et al.* [15]. The PCR products were subjected to electrophoresis and visualized by staining with ethidium bromide.

Quantification of IgE antibody titres

Total IgE titres were determined by Syn^{elisa} test kit (Elias, Freiburg, Germany) as recommended by the manufacturer, with a reference range of 0–120 U/ml.

Statistical analysis

Statistical significance was calculated using the exact Fisher's test with $P \leq 0{\cdot}05$.

RESULTS

Cytokine mRNA expression in cultures of sorted PBMC

The pattern of cytokines produced by the PBMC was analysed by RT-PCR with oligonucleotide primer pairs specific for IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6 and IL-10. The RT-PCR was not performed in a quantitative manner, therefore any band optically identifiable was rated as positive. Representative gels of PCR products from mRNA are demonstrated for the first five patients in Fig. 1a,b. The results of the complete gels are summarized in Fig. 2a,b for both the $CD4^+$ and $CD8^+$ subgroups from the *E. multilocularis*-infected patients. β-actin, IFN-γ, IL-2, IL-6 and IL-4 were detectable in the majority of the unstimulated controls and in the $CD4^+$ and $CD8^+$ subpopulations. IL-3 mRNA was detected in four out of 14 patients in the $CD4^+$ population and in two out of 14 patients in the $CD8^+$ population. IL-10 mRNA was found in eight out of 14 patients in the $CD4^+$ faction and in seven out of 14 patients in the $CD8^+$ population. IL-5 mRNA was not detected in any of the unstimulated sorted cells ($CD4^+$, $CD8^+$ and CD4/CD8-depleted) or the healthy controls. In general, the detection of cytokine mRNA was weaker in the $CD8^+$ subpopulations. In stimulated cultures, cytokine mRNA expression was more frequent in general, but statistical significance was not reached except for IL-5, which was positive for eight patients and detectable only in the $CD4^+$ subpopulation.

In the subgroup of patients expressing IL-5 mRNA in the $CD4^+$ fraction ($n = 8$), co-expression of IL-10 mRNA was observed in seven (87·5%), co-expression of IL-3 in the same group was found in five (62·5%), as was co-expression of both IL-10 and IL-3 mRNA.

A correlation of cytokine expression in $CD4^+$ stimulated cells with the clinical status of the patient is shown in Table 1: patients clinically classified as A ($n = 2$) did not express IL-5 or IL-10 mRNA, one expressed IL-3. Out of the four patients classified as B, two expressed IL-5 mRNA, none IL-3 and three IL-10 mRNA. In the group of patients rated as C ($n = 8$), five patients co-expressed IL-5, IL-3 and IL-10 mRNA, one expressed IL-5 and IL-10, one only IL-10 and one none of the tested cytokines.

IgE above the normal range was found in five patients, all of them clinically classified as C, and all but one expressed IL-5 in the $CD4^+$ stimulated subgroup (data shown in Table 1). Elevated eosinophil levels were absent in all patients.

DISCUSSION

Patients with AE display a clinical spectrum ranging from progression under continuous chemotherapy, to healing after radical surgical removal of the lesion. Primary resistance against the infection is suggested, as patients with antibodies against the *E. multilocularis* antigen Em2 and died-out lesions were reported from regions with high incidence of AE, i.e. Alaska or local endemic areas in France, and regarded as resistant [16]. Sero-epidemiological studies in Switzerland, Alaska and France revealed that only 10–30% of infected and seroconverted patients will develop AE [17]. Several factors might influence this broad spectrum: (i) the parasite exhibits a wide quantitative and qualitative variability of different antigenic profiles [18,19] with unequal virulence [20]; (ii) certain HLA haplotypes may be prone to infection, as HLA-A1 and B35 are dominant in infected patients [21] and others, like HLA-DR11, correlate with a favourable clinical response (Eiermann *et al.*, personal communication); and (iii) the immune response of the host unable to overcome the infection might be inappropriate, either due to specific parasitic products [22] or due to the development of humoral immunity (Th2 type) instead of cellular immunity (Th1 type). Our study refers to the latter point.

The human immune response against infective agents is mainly directed by the activation of T cells from distinct subgroups producing different cytokine panels which mutually inhibit each other. The Th1 panel consists of IFN-γ, IL-2 and tumour necrosis factor-beta (TNF-β), supporting macrophage activation, antibody-dependent cell cytotoxity, production of IgG2a antibodies by B cells and DTH, i.e. cellular immunity, whilst Th2 cells produce mainly IL-4, IL-5, IL-6 and IL-10, supporting humoral immunity [23]. In many chronic infectious diseases the type of T cell reaction, Th1 or Th2, determines the clinical outcome. In leishmaniasis or leprosy the Th1-type cytokine response is associated with healing or protection [24,25]. For helminthic infections this correlation has been demonstrated in a mouse model of *Trichinella spiralis* [26]. In contrast, interferon-gamma (IFN-γ) suppressed the protective immunity of mice against the nematode parasite *Nippostrongylus brasiliensis* [27], which might reflect a protective immunity elicited by a Th2-type response. Multiple factors are responsible for the development of either a Th1- or Th2-type response. Th2 is supported by genetic factors, a high mass of antigen [28], the presence of IL-4 [29] and certain costimulators [30].

Several observations support the assumption that AE patients exhibit a Th2 immune response. First, their capacity to react in a Th1 manner against other infective agents is diminished and is associated with a loss of macrophage function [31], a feature which is generally found in chronic helminthic infections [32]. The mechanisms responsible for the decrease of macrophage function may be the inhibition of cellular immunity by the Th2 cytokines IL-4 and IL-10. It has been shown that IL-4 down-regulates the expression of the IL-2 receptor [33] and inhibits the activation of monocytes and their antimicrobial properties by reducing the capacity to produce IL-1 and TNF-α [34,35]. IL-10 alters the antigen presentation of the macrophages and inhibits the release of

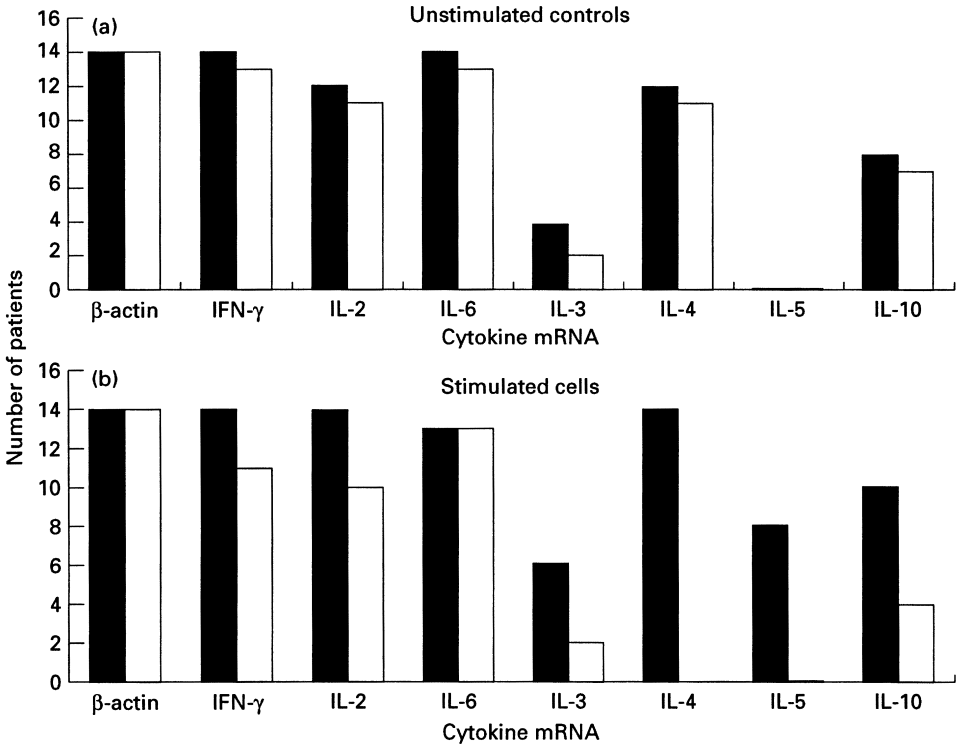

Fig. 2. Cytokine mRNA expression in sorted peripheral blood mononuclear cells (PBMC) from patients with alveolar Echinococcosis ($n = 14$). Freshly isolated PBMC were cultured in the absence (a) or presence (b) of 10 μg/ml of crude *E. multilocularis* antigen. After 18 h of culture, PBMC sorting was performed using EPICSorter or MACS. Total RNA was extracted from sorted cells and reverse transcribed. The cDNA obtained was assayed for the presence of specific cytokine mRNAs as described in Patients and Methods. The polymerase chain reaction (PCR) products were detected by agarose gel electrophoresis in the presence of appropriate molecular size markers. ■, $CD4^+$; □, $CD8^+$.

those cytokines with antimicrobial functions [36]. Second, IgG antibody subclasses supported by Th2 cytokines, i.e. IgG1 and IgG4, comprise the most active subclass of antibodies in response to AE [37]. Third, elevation of IgE which is thought to be mainly induced by IL-5 [38] is found in a large number of AE patients. In our study, six patients, all clinically rated as progressive or severe, had IgE above the normal range. Although IL-5 is associated with eosinophil differentiation, the number of eosinophils in the peripheral blood of AE patients is generally not altered.

In the present study, IL-5, a major Th2-type cytokine, was

Table 1. Characterization of the patients and correlation of their clinical status with cytokine expression and the level of IgE (U/ml) in the stimulated $CD4^+$ subgroup

Patient no.	Gender	Age	First diagnosis	Clinical class	IgE	Sorting	IL-5	IL-3	IL-10	IL-4	IFN-γ
1	F	54	1986	C	228	MACS	+	+	+	+	+
2	M	60	1987	C	42	MACS	+	+	+	+	+
6	M	35	1994	C	>500	MACS	+	+	+	+	+
7	M	55	1972	C	12	MACS	−	−	−	+	+
9	M	66	1994	C	267	EPIC	+	−	+	+	+
11	M	57	1991	C	6	EPIC	+	+	+	+	+
12	F	77	1992	C	253	EPIC	+	+	+	+	+
13	F	55	1993	C	134	EPIC	−	−	+	+	+
3	F	58	1994	B	3	MACS	+	−	−	+	+
4	F	45	1986	B	62	EPIC	−	−	+	+	+
5	F	26	1994	B	81	MACS	+	−	+	+	+
8	M	42	1988	B	13	EPIC	−	−	+	+	+
10	F	62	1993	A	26	MACS	−	−	−	+	+
14	F	24	1992	A	26	EPIC	−	+	−	+	+

M, Male; F, female; +, expression of the interleukin, detected by polymerase chain reaction (PCR) product; −, no detectable PCR product. Patient numbers designate the patients included in the study.

solely detectable in the $CD4^+$ subgroup in eight out of the 14 patients tested (58%). In the stimulation assays performed with unsorted cells from AE patients the rate of IL-5 mRNA expression under otherwise comparable conditions was ≈80% [10]. The slightly reduced frequency in the study presented here may be attributable to the process of sorting, which could reduce the viability of the PBMC. All other cytokine mRNAs were present in each subgroup of stimulated and unstimulated samples, which include the $CD4^+$, $CD8^+$ and the $CD4^+$ and $CD8^+$ depleted cells. This is consistent with the previous finding, that the main source for IL-5 is the activated Th cell population ($CD4^+$ $CD45RO^+$) [38]. Other potential sources are $\gamma\delta$ T cells [39] or $CD8^+$ cells [40]. However, in this study IL-5 mRNA was not detectable in any of the $CD8^+$ population.

In murine systems it was demonstrated that activated $CD4^+$ cells initially do not follow a pure Th1 or Th2 pattern [41,42]. This observation might explain why we find an expression of IL-4 and IFN-γ in all of the stimulated $CD4^+$ subgroups. In contrast, other Th2 cytokines (IL-5, IL-10 and IL-3) are produced significantly more frequently in those patients clinically classified as group C, which represents the critically ill patients ($P \leq 0{\cdot}05$). The over-expression of IL-5, IL-3 and IL-10 may be the result of an expansion or selective activation of the Th2 cell population.

The specific up-regulation of IL-5 mRNA by $CD4^+$ cells might support the assumption that *E. multilocularis* antigen is presented in a T cell-dependent manner by the MHC class II receptor of antigen-presenting cells. Nonetheless, non-peptide antigens presented via CD1 might play an important role in the development of the Th2-type response [43,44].

In conclusion, our data support a correlation between disease severity and expression of Th2 cytokines in AE patients. It is tempting to speculate that the relative susceptibility of chronically infected patients is related to their Th2-like response to *Echinococcus* spp. demonstrated in the restimulation assay. It is not understood whether this pattern of response is induced by factors of the parasite, i.e. surface molecules or soluble products like proteinases which direct the host response towards the unprotective Th2 pattern [27], if immunopathological reactions associated with Th1 are actively avoided, or if a simple malfunction of the immune system occurs. Therefore, to gain further understanding of the various clinical outcomes of this infection, it may be of high interest to find patients with resolved lesions regarded as resistant and to correlate their cytokine expression profile or costimulatory surface molecules of the T cells with those of chronically infected patients.

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