Interleukin-5 Is the Predominant Cytokine Produced by Peripheral Blood Mononuclear Cells in Alveolar Echinococcosis

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An involvement of cellular immunity in alveolar echinococcosis is strongly suggested by the intense granulomatous infiltrations observed around the hepatic parasite lesions. However, the basis of cellular immunoregulation in patients with alveolar echinococcosis is poorly understood. The present report shows a comparative analysis of lymphoid cell function in peripheral blood mononuclear cells (PBMC) of 16 patients with alveolar echinococcosis and of healthy individuals. Our in vitro restimulation studies with crude *Echinococcus multilocularis* antigen demonstrated that PBMC from patients with alveolar echinococcosis were responsive to challenge with parasitic antigen as measured by lymphoid cell proliferation. In this system, we also evaluated cytokine expression at the gene and protein levels after stimulation with *E. multilocularis* antigen. Analysis of cytokine mRNA expression revealed distinct patterns of cytokine expression in patients and normal donors. By using reverse transcriptase PCR, we could demonstrate that the T_H1 cytokine transcripts interleukin-2 (IL-2) and gamma interferon (IFN- γ) are present in PBMC from patients with alveolar echinococcosis. Moreover, it was found that stimulation with *E. multilocularis* antigen induced or enhanced the expression of the T_H2 cytokine IL-3, IL-4, IL-10, and especially IL-5 mRNAs in PBMC from 13 of 16 patients with alveolar echinococcosis. Two patients who were examined after radical surgery, as well as another patient with a stable course of the disease under continuous chemotherapy, were not able to generate the same pattern of cytokine response and had no evidence of IL-5 mRNA synthesis. In contrast to the frequent expression of T_H2 cytokine mRNAs observed in patients with alveolar echinococcosis, PBMC cultures from normal donors showed prominent IL-2 and IFN- γ mRNA expression but weak IL-3, IL-4, and IL-10 mRNA expression. Most interestingly, IL-5 mRNA was substantially absent in PBMC from healthy individuals. In accordance with the mRNA studies, it was found that *E. multilocularis* antigen induced the secretion of large amounts of IL-5 and intermediate amounts of IFN- γ in patients with alveolar echinococcosis, whereas large amounts of IFN- γ and no or threshold amounts of IL-5 were detected in supernatants from healthy individuals. Collectively, the present study provides the first evidence that a T_H2 immune response is gradually activated during the course of *E. multilocularis* infection, indicating a critical role for IL-5 in the manifestation of human alveolar echinococcosis.

Alveolar echinococcosis of the liver is a life-threatening disease caused by the intrahepatic growth of the larval cestode *Echinococcus multilocularis* (12). The clinical signs and symptoms of hepatic alveolar echinococcosis resemble those of hepatic carcinoma or other liver diseases (5, 6, 25, 36). However, despite the public health importance of echinococcosis in areas where the disease is endemic information on the immunological control of *E. multilocularis* infection in humans is sparse. Previous studies of both humans and mice have demonstrated that intense humoral immunity may play a key role in the control of parasite growth (1, 9, 34). Most patients with alveolar echinococcosis develop parasite-specific antibodies in serum, involving immunoglobulin G (IgG), IgA, IgM isotypes (14), as well as IgE-dependent responses which are thought to be involved in granulomatous reactions surrounding the parasitic vesicles (33). Additionally, there is ample evidence that the intense granulomatous infiltration of the periparasitic lesions is a consequence of cell-mediated immunity against *E. multilocularis* (21). It was shown that a regressive or progressive course of alveolar echinococcosis is correlated with the

granuloma cell composition and the relative ratios of T-lymphocyte subpopulations in the lesion, thus suggesting a major role for the antigen-specific T-cell response in the host-parasite relationship (6, 32).

Both antigen-specific T-cell responses and immunomodulatory activities are considered to be regulated by cytokines, which influence the activities of T lymphocytes, B lymphocytes, and other immune effectors in an intricate interplay of positive and negative regulatory signals (3, 30). In particular, two cytokines, interleukin-4 (IL-4) and IL-5, have been found to promote high levels of IgE and induce eosinophilia, which is characteristic of several helminthic infections in mice and humans (10, 18, 31). Therefore, a comprehensive evaluation of the cytokine profile should provide a better insight into the mechanisms involved in regulating the growth of *E. multilocularis* metacestodes. Experimental studies have demonstrated that most patients with alveolar echinococcosis show proliferative responses of peripheral blood mononuclear cells (PBMC) to in vitro stimulation with crude *E. multilocularis* antigen, which is acting as a recall antigen (7, 15, 16). In the experimental mouse model based on secondary alveolar echinococcosis, lymphocyte-dependent mechanisms have been suggested to mediate resistance when metacestode growth is inhibited (6) and to modulate immune system suppression in highly susceptible animals (6, 16, 19). Comparing the IgG subclass profiles

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in resistant and susceptible mice, respective observations indicated a potential T_H1 versus T_H2 discrimination with respect to susceptibility and resistance (16). Thus, the aim of our study was to investigate lymphoid cell function in PBMC from patients with alveolar echinococcosis and from normal individuals. We have assayed the ability of lymphocytes to proliferate upon stimulation with crude *E. multilocularis* antigen and have assessed cytokine production at the levels of both mRNA and protein synthesis.

MATERIALS AND METHODS

Patients. A total of 16 patients with alveolar echinococcosis, including 6 men and 10 women, 9 control patients with cystic echinococcosis, including 6 men and 3 women, and 7 healthy controls, including 4 men and 3 women, were recruited for this study. The ages of the patients with alveolar echinococcosis ranged from 22 to 80 years, with a mean of 50.4 years. Patients with cystic echinococcosis were between 18 and 67 years old (mean age, 42.2 years), and healthy individuals were between 23 and 63 years old (mean age, 43.3 years). Patients with alveolar echinococcosis were resident in an area of southern Germany where alveolar echinococcosis is endemic. Patients with cystic echinococcosis lived in Turkey, Rumania, Russia, and Morocco.

All patients were diagnosed and treated as a part of a multidisciplinary research project centered at the University of Ulm. The patients were seen at regular intervals in the outpatient department and consecutively recruited for the present study. A combination of clinical, serological, and histopathological data was used to define the actual status of the patients. All patients with alveolar echinococcosis had or have had liver involvement. Two of the patients underwent radical surgery and were examined 12 and 18 months later and showed no signs of relapse. The remaining patients were initially classified as inoperable or had relapsed. At the time of investigation, all but one showed a clinically stable course under continuous chemotherapy with benzimidazoles. One patient received an orthotopic liver transplantation 22 years after the first liver surgery for alveolar echinococcosis. A relapse in the peritoneal lymph nodes was histologically verified 2.5 years after transplantation. At the time of investigation, the findings remained unchanged with the patient under continuous anthelmintic treatment. Eosinophilia or a marked increase in IgE levels was not observed in the entire group of patients with AE.

Patients with cystic echinococcosis had various organ manifestations. Seven of the nine patients had undergone one or many operations because of liver, lung, kidney, spleen, or bone involvement and had received postoperative chemotherapy with benzimidazoles. At the time of investigation, the clinical and laboratory check did not reveal any signs of relapse. The remaining two patients developed a stable course while undergoing chemotherapy with benzimidazoles. One patient suffered from multiple abdominal cysts, and another patient had undergone repeated operations for cystic brain echinococcosis in the previous 8 years and remained under chemotherapy with benzimidazoles. Five of these patients with cystic echinococcosis had eosinophilia and elevated levels of IgE.

Blood samples from all patients and healthy individuals were collected and used for determination of in vitro lymphocyte reactivity and cytokine production as described below. Very strict precautions were taken during sample collection and preparation to avoid the presence of impurities in sampling materials. Blood samples were collected in pyrogen-free sterile tubes containing EDTA and containing no endotoxin that would falsely increase cytokine production by blood cells. The protocol used in this study was reviewed by the local ethics committee.

Mitogenic and antigenic stimulants. Crude *E. multilocularis* protein extract antigen was prepared as described previously by Gottstein et al. (13). Briefly, metacystodes from *E. multilocularis* (Swiss isolate CH-24) were obtained from *Meriones unguiculatus* experimentally infected by intraperitoneal transplantation of metacystode tissue blocks. Metacystode material was minced three times for 30 s each in sterile phosphate-buffered saline (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 $30,000 \times g$ and $4^\circ C$ for 45 min and filtration through a Millipore filter (pore size, $5 \mu m$). The extract was dialyzed against PBS containing $0.5 M NaCl$, and thereafter the total protein content was determined by the Bio-Rad protein assay as indicated by the manufacturer. For in vitro stimulation of PBMC, the *E. multilocularis* antigen preparation was used at a final concentration of $25 \mu g/ml$. This concentration yielded an effective stimulation of lymphocyte reactivity against *E. multilocularis* antigen, as confirmed by dose-response studies. To confirm that any observations made with *E. multilocularis* antigen were not an artifact of the preparation described, crude antigen preparation from *Onchocerca volvulus*, kindly provided by P. T. Soboslay (28), was also tested in our experimental system at the same range of concentrations. Phytohemagglutinin (PHA), used at $2 \mu g/ml$ for nonspecific mitogenic stimulation of PBMC, was purchased from Difco Laboratories, Detroit, Mich.

Lymphocyte proliferation assay. PBMC were obtained from whole EDTA-blood by using lymphocyte-separating solution (Ficoll-Paque; Pharmacia, Upp-

sala, Sweden) by the method of Böyum (4). Lymphocytes were washed twice with sterile PBS (pH 7.6), and the number of viable cells was determined by trypan blue exclusion. For the lymphocyte proliferation assay, freshly isolated PBMC were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (100 IU of penicillin per ml and 0.1 mg of streptomycin per ml [Gibco BRL, Gaithersburg, Md.]) at 10^6 cells per ml. A $200\text{-}\mu l$ volume of the lymphocyte suspension was added to triplicate wells of 96-well round-bottom microtiter tissue culture plates (Nunc, Roskilde, Denmark). Cells were stimulated with $5 \mu g$ of *E. multilocularis* protein extract antigen per well or $0.4 \mu g$ of PHA per well. Control, unstimulated PBMC from patients with alveolar echinococcosis and PBMC from normal donors were also tested. On the basis of preliminary kinetics studies, cells were pulse-labeled after 5 days of in vitro stimulation with $1 \mu Ci$ (3.7×10^4 Bq) of [3H]thymidine (Amersham, Brunswick, Germany) and harvested 24 h later. Results are expressed as stimulation indices (SI), which were calculated as the mean counts per minute in stimulated wells divided by the mean counts per minute in control, unstimulated wells.

RNA isolation. Freshly isolated PBMC ($5 \times 10^6/ml$) from whole blood of patients or healthy donors were obtained by centrifugation over Ficoll-Paque and resuspended in 2-ml wells of flat-bottom multiwell dishes, each well containing either $25 \mu g$ of crude *E. multilocularis* antigen per ml or $2 \mu g$ of PHA per ml diluted in RPMI 1640 medium supplemented with 2% fetal calf serum and antibiotics as described above. Control wells contained no antigen or mitogen. The cells were maintained at $37^\circ C$ in a humidified atmosphere of 5% CO_2 . After 24 h, the cells were harvested, lysed with 4 M guanidinium isothiocyanate as described previously (8), and stored at $-70^\circ C$ prior to preparation of cDNA.

cDNA synthesis. Total RNA was incubated for 30 min at $37^\circ C$ in a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 40 U of RNasin (Promega, Madison, Wis.) per ml, 10 mM dithiothreitol, and 2.5 U of RQ1 DNase (Promega) to remove any contaminating genomic DNA from the preparations. After phenol-chloroform extraction and ethanol precipitation, RNA pellets were resuspended in water. cDNA was synthesized from RNA by priming approximately $1 \mu g$ of total RNA at $37^\circ C$ for 1 h in a final volume of $50 \mu l$ containing $1 \mu g$ of an oligo(dT) primer, 200 nmol of each deoxynucleoside triphosphate (Pharmacia), and 200 U of Moloney murine leukemia virus reverse transcriptase (RT); 20 mM dithiothreitol and $5 \times$ Moloney murine leukemia virus reverse transcriptase buffer were added as recommended by the manufacturer (Gibco BRL).

Amplification of cDNA by PCR. cDNAs were tested for the presence of cytokine gene sequences by PCR performed in $30\text{-}\mu l$ volumes with specific primer pairs. Two microliters of the relevant cDNA was amplified by PCR in the presence of 0.2 μM sense and antisense oligonucleotide primers, 2 mM deoxynucleoside triphosphates (Pharmacia, BRL Life Technologies Inc., Gaithersburg, Md.), 0.5 U of AmpliTaq polymerase (Perkin-Elmer, Branchburg, N.J.), and $10 \times$ PCR buffer. The PCR was performed in a Perkin-Elmer thermal cycler, running a program of 40 cycles of denaturation at $96^\circ C$ for 30 s, annealing at $60^\circ C$ for 1 min, and extension at $73^\circ C$ for 1.5 min; oligonucleotide primers specific for β -actin, gamma interferon (IFN- γ), IL-2, IL-3, IL-4, IL-5, IL-6, and IL-10 were used. The following sense and antisense primer sequences were, as described by Sorg et al. (29) and Yamamura et al. (37): β -actin, GTGGGGCGC CCCAGGCACCA and CTCCTTAATGTCACGCACGATTTC; IFN- γ , AGT TATATCTTGGCTTTTCA and ACCGAATAATTAGTCAGCTT; IL-2, AA CTCCTGTCTTGCAATTGCA and GTGTTGAGATGATGCTTTGAC; IL-3, GCCTTTGCTGGACTTCAACA and TTGGATGTCGCGTGGGTGCG; IL-4, CAACTTTGTCCACGGACAC and TCCAACGTACTCTGGTTGG; IL-5, AGGATGCTTCTGCATTGAG and CTATTATCTCCCTGGTGTTC; IL-6, AACTCCTTCTCCACAAGCG and TGGACTGCAGGAATCCTT; IL-10, ATGCCCCAAGCTGAGAACCAAGACCCA and TCTCAAGGGGCTGGGT CAGCTAACCA.

The PCR products were subjected to electrophoresis with $12 \mu l$ of the reaction mixture and $3 \mu l$ of loading buffer and visualized by staining with ethidium bromide. This procedure is not meant to permit a determination of the exact amount of mRNA present in each sample.

Measurement of cytokine production. Cytokine production was measured in culture supernatants from PBMC of patients with alveolar echinococcosis and normal donors. PBMC were isolated from freshly drawn EDTA-treated blood by density gradient centrifugation. The PBMC were cultured at a density of 5×10^6 cells per ml in RPMI 1640 medium supplemented with 20 IU of penicillin-streptomycin and 2% fetal calf serum (Gibco BRL) in the presence of $25 \mu g$ of crude *E. multilocularis* antigen per ml. Supernatants of PBMC cultures were collected after 5, 24, 48, and 72 h of incubation at $37^\circ C$ in a humidified atmosphere containing 5% CO_2 and stored frozen ($-20^\circ C$) until assayed for cytokines. As a negative control, the cells were cultured in the absence of crude *E. multilocularis* antigen. IFN- γ , IL-4, IL-5, and IL-6 cytokine production in culture supernatants was measured by commercially available kits for enzyme-linked immunosorbent assays (ELISAs; Dianova, Hamburg, Germany). The data were presented as mean concentrations from four experiments.

Statistical analysis. Nonparametric statistical tests (the Kruskal-Wallis H test and the Wilcoxon-2 sample test) were used to test statistical differences between patient groups and control subjects with respect to SI of the lymphoproliferative responses. Differences in the levels of cytokine production between patients and normal controls were analyzed by using two-way analysis of variance for each of the cytokines tested. The data used for comparative analysis in this test were adjusted to account for the experimental time of antigenic exposure. The signif-

FIG. 1. Lymphocyte proliferation in freshly isolated PBMC measured in cultures upon stimulation with PHA for 3 days. SI obtained from both groups of patients (group A, patients with alveolar echinococcosis; group B, patients with cystic echinococcosis) and from healthy volunteers (group C) were calculated as counts per minute in stimulated cultures divided by counts per minute in control, unstimulated cultures. The bars indicate the mean SI values of triplicate assays. Individual patient numbers are indicated for all patients (groups A and B) and for healthy controls (group C). Patients 12 and 16 had undergone radical surgery and showed no signs of relapse. The remaining patients were inoperable, had undergone incomplete or palliative surgery, or had received orthotopic liver transplantation (patient 5). All patients were studied while clinically stable under continuous chemotherapy with benzimidazoles.

ificance level for the test of group differences was set to 5%. All analysis was done with SAS software.

RESULTS

PBMC proliferation in response to *E. multilocularis* antigen.

The proliferative responses of PBMC from patients with alveolar echinococcosis and healthy donors used in this study were determined as a control for lymphocyte activation. To examine the effects of *E. multilocularis* antigen preparation on lymphocyte proliferation, PBMC were evaluated for the capacity to incorporate [³H]thymidine upon stimulation with optimal concentrations of crude *E. multilocularis* antigen (25 µg/ml) for 6 days. PBMC cultured in the presence of optimal concentrations of PHA (2 µg/ml) or medium alone served as a control for the level of stimulation.

The results of the lymphocyte proliferation assays are shown in Fig. 1 and 2. A comparison of the results obtained with PHA-stimulated PBMC showed a marked stimulation of lymphocyte proliferation in patients and in normal donors (Fig. 1). As expected, the mean SI calculated for PHA-stimulated PBMC were not significantly different in the two groups of patients and in normal donors (groups A and C, $P = 0.8410$; groups B and C, $P = 0.3143$; groups A and B, $P = 0.1334$). As shown in Fig. 2, increased proliferation was also observed in PBMC from patients with alveolar echinococcosis and cystic echinococcosis when the cells were cultured in the presence of crude *E. multilocularis* antigen. In contrast to PHA stimulation, however, there was an apparent difference between the SI of patients with alveolar echinococcosis (median, 27) and cystic echinococcosis (median, 11) and those of normal individuals (median, 5). The SI of both groups of patients were significantly higher than those of normal donors (groups A and C, P

$= 0.0044$; groups B and C, $P = 0.0290$). However, the SI of patients with alveolar echinococcosis did not differ significantly from those of patients with cystic echinococcosis ($P = 0.0839$), although the SI of patients with alveolar echinococcosis tended to be considerably higher than those of patients with cystic echinococcosis.

Cytokine mRNA expression in PBMC cultures of patients with alveolar echinococcosis. To investigate the pattern of cytokines produced in patients with alveolar echinococcosis, cytokine transcription of PBMC was analyzed 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 primer pairs were selected and synthesized as described by Sorg et al. (29), suggesting that the sensitivity of the RT-PCR used should be of the same range as described on the basis of hybridization analysis. In addition, we assessed the reproducibility and consistency of the experimental conditions used for in vitro stimulation cultures of PBMC and cytokine amplifications (Fig. 3). The determination of RNA preparations derived from PBMC stimulated either with crude *E. multilocularis* antigen (25 µg/ml) or with PHA (2 µg/ml) demonstrated that the conditions under which the PCR was run were suitable to detect any appreciable signal from every cytokine mRNA tested. Cell extracts from PBMC cultures incubated with crude antigen preparation derived from *O. volvulus* (25 µg/ml) were examined in parallel and served as a control to ensure the antigen specificity of the *E. multilocularis* antigen preparation used in our study. As noted above, nonspecific activation of cytokine transcription was not detectable after stimulation with crude *O. volvulus*-derived antigen. PCR amplification with a selected cytokine primer set yielded the same incomplete pattern of cytokine transcripts as detected in specimens from unstimulated PBMC cultures. In

FIG. 2. Lymphocyte proliferation in PBMC measured in cultures of PBMC from patients with alveolar echinococcosis (group A), patients with cystic echinococcosis (group B), and healthy volunteers (group C), upon stimulation with *E. multilocularis* antigen for 6 days. The proliferative responses of PBMC to *E. multilocularis* antigen were expressed as the SI. SI values were calculated as the ratio of counts per minute in experimental, stimulated cultures to counts per minute of control, unstimulated cultures. The bars indicate the mean SI values of triplicate assays. Individual patient numbers are indicated for both groups of patients and normal donors. For descriptions of patients, see the legend to Fig. 1.

particular, IL-5 mRNA amplification was exclusively detectable in PBMC cultures stimulated with crude *E. multilocularis* antigen and might be a highly discriminatory finding for our experimental system.

On the basis of these preliminary assays, we tested cytokine mRNA expression in PBMC of 16 patients with alveolar echinococcosis and 7 normal donors after 24 h of culture in the presence of *E. multilocularis* antigen, acting as a recall antigen. PHA-stimulated PBMC served as a control. To provide meaningful comparisons between patients and healthy donors, we normalized the cDNAs to the β -actin PCR product, which

represents a marker for all cells. Cytokine profiles are shown in Fig. 4 for 7 healthy controls and, representatively, for 9 of 16 patients. The cytokine profiles of the remaining seven patients (patients 10 to 16) are not shown in Fig. 4 but yielded a similar pattern of cytokine transcripts. mRNA phenotyping of PBMC from normal donors by RT-PCR revealed expression of IL-2, IL-6, and IFN- γ mRNAs at elevated levels. Apart from quantitative variations in different PBMC donors, coexpression of IL-2, IL-6, and IFN- γ was detected in PBMC from patients with alveolar echinococcosis, either unstimulated or stimulated with crude *E. multilocularis* antigen.

FIG. 3. PCR analysis of cytokine mRNA in PBMC derived from a patient with alveolar echinococcosis (P) and in PBMC from a normal donor (N). Freshly isolated PBMC were cultured in the presence of crude *E. multilocularis* antigen (Emag) (25 μ g/ml). For a control, PBMC cultures were stimulated by crude antigen derived from *O. volvulus* (Ovag) (25 μ g/ml) or by PHA (2 μ g/ml). After 24 h, RNA was extracted and reverse transcribed (A). The cDNA obtained was assayed for the presence of specific cytokine mRNAs by 40 cycles of PCR as described in Materials and Methods. PCR products (lanes 1 to 6 contain IL-2, IL-3, IL-4, IL-5, IL-6, and IL-10, respectively) were run on a 1.5% agarose gel in the presence of appropriate molecular size markers (rightmost lane: 50, 100, 200, 300, 400, 500, 700, and 1,000 bp). The results are representative of an experiment with PBMC from different donors.

FIG. 4. Cytokine mRNA expression in patients with alveolar echinococcosis (A) and in normal donors (B). Freshly isolated PBMC were cultured in either the presence or absence of PHA (2 μg/ml) or crude *E. multilocularis* antigen (Emag) (25 μg/ml). After 24 h of culture, total RNA was extracted and amplified by RT-PCR with oligonucleotide primer pairs specific for β-actin, IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, and IL-10 cytokines. The PCR products were visualized by agarose gel electrophoresis in the presence of appropriate molecular size markers (rightmost lanes: 50, 100, 200, 300, 400, 500, 525, and 700 bp). Lanes 1 to 9 in panel A indicate the individual patient numbers for nine patients with alveolar echinococcosis (patients 1 to 9). Lanes 1 to 7 in panel B indicate the individual patient numbers for seven healthy controls (controls 1 to 7). For further patient descriptions, see the legend to Fig. 1.

TABLE 1. Parameters selected to characterize patients with alveolar echinococcosis^a

Patient no.	Gender ^b	Age (yr)	Actual clinical status	Expression of IL-5 mRNA
1A	M	58	Stable	++
2A	M	55	Stable	++
3A	F	52	Stable	++
4A	F	64	Stable	++
5A	F	48	Liver transplantation, relapse, stable	++
6A	F	59	Stable	+
7A	M	27	Stable	+
8A	M	40	Stable	—
9A	M	55	Stable	++
10A	M	45	Stable	+
11A	F	75	Stable	+
12A ^c	F	60	12 mo after radical surgery	—
13A	F	24	Recent diagnosis	+
14A	F	53	Stable	+
15A	F	80	Stable	++
16A	F	22	18 mo after radical surgery	—

^a The experiment was performed as described in the legend to Fig. 4. Individual patient numbers are indicated for patients consecutively included in the study.

^b M, male; F, female.

^c All but one patient (12A) were undergoing specific treatment with benzimidazoles.

Figure 4 also shows the profiles of IL-3, IL-4, IL-5, and IL-10 mRNAs. Compared with the intensity of the signals from all other cytokine mRNAs, IL-3, IL-4, and IL-10 mRNAs were weakly detectable in unstimulated and *E. multilocularis* antigen-stimulated PBMC from normal donors. In particular, IL-5 mRNA was substantially absent in PBMC cultures from healthy individuals, either unstimulated or stimulated with crude *E. multilocularis* antigen. In contrast to results obtained with PBMC from healthy controls, antigenic stimulation with crude *E. multilocularis* protein extract induced accumulated levels of mRNAs for IL-3, IL-4, and IL-10 in PBMC from patients. It is of particular interest that IL-5 mRNA levels were significantly elevated in patients with echinococcosis in response to *E. multilocularis* antigen. Some differences in the signal intensity of IL-5 mRNA expression were observed in the entire group of our study patients with alveolar echinococcosis. As indicated in Table 1, high levels of IL-5 mRNA transcripts occurred among 12 patients, all of whom were clinically stable. Patients 13 and 8, belonging to the same clinical category, were weakly positive or even negative for IL-5 mRNA synthesis. As noted above, two patients, patients 12 and 16, who were examined more than 1 year after radical surgery and clinically classified as cured, showed no evidence of IL-5 mRNA synthesis. Altogether, IL-5 mRNA was detected in 81% of samples from all patients analyzed (13 of 16 patients). Specimens from three patients (patients 8, 12, and 16) were negative for T-cell-derived IL-5 mRNA. Thus, these results might reflect a different reactivity of sensitized immune cells in patients with alveolar echinococcosis, possibly related to the status of the disease. In contrast to these findings, mitogenic stimulation of PBMC cultures, induced by PHA (2 µg/ml), revealed a completely different cytokine profile (Fig. 4). mRNAs of all cytokines tested, including IL-5, were expressed at consistent elevated levels in all PBMC cultures, whether obtained from patients or from normal donors. The substantial inability of PBMC from normal donors to produce IL-5 mRNAs in response to *E. multilocularis* antigen could therefore not be ascribed to technical defaults. Thus, there is evidence that ele-

vated expression of IL-5 mRNA, as well as IL-3, IL-4, and IL-10 mRNAs, is associated with the manifestation of chronic infection with *E. multilocularis* larvae.

Kinetics of cytokine mRNA expression. To examine the stability of the cytokine patterns detected in patients with alveolar echinococcosis and in healthy individuals, we next analyzed the temporal pattern of cytokine mRNA expression in PBMC from three patients (patients 10, 14, and 15) and three healthy individuals during stimulation with crude *E. multilocularis* antigen. Total RNA from PBMC was extracted and amplified by RT-PCR at 5, 24, 48, and 72 h after stimulation with crude *E. multilocularis* antigen. The kinetics of mRNA expression (Fig. 5) demonstrated that PBMC cultures from patients chronically infected with *E. multilocularis* produced early and abundant mRNAs for IL-2, IL-6, and IFN-γ, which persisted at high levels for 72 h of culture (Fig. 5A). Similarly, accumulation of IL-2, IL-6, and IFN-γ mRNAs was detected in normal donors as early as 5 to 24 h after stimulation and persisted at elevated levels for the 72 h of culture (Fig. 5B). In contrast, IL-5 mRNA was essentially detectable only in stimulated cultures from patients with alveolar echinococcosis (Fig. 5A). Compared with the kinetics of IL-4 and IL-10 mRNAs, IL-5 mRNA was expressed in patients relatively late (24 h), but its levels also remained elevated even after prolonged incubation (72 h) in the presence of the antigenic stimulant. This temporal pattern of cytokine expression supported the idea that IL-5 mRNA expression might be differently regulated in patients and normal donors.

Cytokine production by PBMC cultures in response to *E. multilocularis* antigen. To determine whether the pattern of selective cytokine mRNA expression in PBMC reflected translation into active cytokines, we measured cytokine concentrations in supernatants of PBMC cultures from four representative patients (patients 10, 11, 14, and 15 [Fig. 6A]) and four normal donors (Fig. 6B), stimulated with crude *E. multilocularis* antigen. IFN-γ, IL-4, IL-5, and IL-6 production was measured by ELISA in supernatants collected at different time intervals after stimulation with *E. multilocularis* antigen and expressed as mean concentrations of four determinations.

As shown in Fig. 6, the measured cytokine production was consistent with cytokine mRNA levels detected in this study. Mean concentrations of IL-6 were markedly elevated in all supernatants tested from patients and normal controls. The levels of IL-6 produced in the presence of *E. multilocularis* antigen were found to be comparable between patients and normal donors ($P = 0.6519$). PBMC from patients secreted approximately 106.6 to 478.2 pg/ml after prolonged culture, and supernatants of PBMC from normal donors were found to contain IL-6 concentrations from 34.6 to 632.3 pg/ml. The mean IFN-γ concentration was markedly elevated in supernatants of PBMC from patients and normal donors in the presence of *E. multilocularis* antigen. Despite similar kinetics of cytokine production, supernatants of PBMC from patients, stimulated with *E. multilocularis* antigen, contained considerably smaller amounts of IFN-γ than did those of control cultures from normal donors. We estimated that the concentrations of IFN-γ in supernatants of PBMC obtained from patients corresponded to approximately 7.2 to 159.2 pg/ml during antigenic exposure, whereas the mean concentration of IFN-γ produced in supernatants of PBMC from normal donors ranged from approximately 5.2 pg/ml at 5 h after stimulation to 268.2 pg/ml at 72 h after stimulation. However, this considerable difference in IFN-γ production did not achieve statistical significance ($P = 0.1756$). Analysis of IL-4 production revealed relatively low levels of IL-4 in supernatants of PBMC from normal donors, ranging from 18 to 19.3 pg/ml after prolonged

FIG. 5. Kinetics of cytokine mRNA expression in *E. multilocularis*-activated PBMC, revealed by RT-PCR. Freshly isolated PBMC from patients with alveolar echinococcosis (A) or normal donors (B) were cultured in the presence of a crude *E. multilocularis* antigen preparation. At the time intervals indicated (5, 24, 48, and 72 h), total RNA was extracted and amplified by RT-PCR with oligonucleotide primer pairs specific for IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, and IFN- γ . β -Actin was the positive transcription control. PCR products were visualized by agarose gel electrophoresis in the presence of appropriate molecular size markers (rightmost lanes: 50, 100, 200, 300, 400, 500, 525, 700, and 1,000 bp). Lanes 1 to 3 in panel A indicate the individual patient numbers of three patients with alveolar echinococcosis (patients 10, 14, and 15). Lanes 1 to 3 in panel B indicate three healthy control subjects who were studied in parallel. For further patient descriptions, see the legend to Figure 1.

culture. Similarly, low titers of IL-4 were detected in supernatants of PBMC from patients. The amount of IL-4 secreted in supernatants of PBMC from patients at different time intervals was estimated to be 20.9 to 24.6 pg/ml, which was significantly higher than the levels of IL-4 in control cultures ($P = 0.0009$). In contrast, IL-5 production was barely detectable in supernatants of PBMC from normal donors, but a strong induction of IL-5 production in response to *E. multilocularis* antigen was found in cultures of PBMC from patients with alveolar echinococcosis. The level of IL-5 production reached 370.5 pg/ml in the cultures from patients, whereas mean concentrations of

IL-5 produced in supernatants of PBMC from normal donors ranged from 2.8 to 4.0 pg/ml. These significant differences in IL-5 production ($P = 0.0128$) are compatible to results of our mRNA studies and support a regulatory role for IL-5 in immune reactions of patients against *E. multilocularis*.

DISCUSSION

In this study, we analyzed lymphocyte function in patients with alveolar echinococcosis, which is necessary for a better understanding of the host-parasite relationship and the mech-

FIG. 6. Production of IFN- γ , IL-4, IL-5, and IL-6 by PBMC from patients with alveolar echinococcosis and from normal donors, stimulated with crude *E. multilocularis* antigen. Freshly isolated PBMC from four patients with alveolar echinococcosis (patients 10, 11, 14, and 15) (A) and four normal donors (B) were cultured in the presence of crude *E. multilocularis* antigen (25 μ g/ml) for extended periods. At 5, 24, 48, and 72 h after stimulation, supernatants of PBMC cultures were collected and cytokine concentrations in supernatants were determined by ELISA. Values are given as the mean cytokine concentrations measured in supernatants derived from four patients or healthy individuals ($n = 4$).

anisms by which host immunity may regulate parasite growth. Useful insights into the immune response against *E. multilocularis* have been gained through evaluation of in vitro lymphocyte proliferation in response to crude *E. multilocularis* antigen (2, 7). Using this model, we first assessed the specificity of the lymphoproliferative response in the PBMC from a representative number of patients with alveolar or cystic echinococcosis and compared it with that observed in PBMC from healthy individuals. The results of our study demonstrate significant quantitative differences in lymphocyte reactivity in the two diseases. The reactivity of PBMC to crude antigen was highest in patients with alveolar echinococcosis, irrespective of their clinical status and the localization of their parasite lesions. However, intermediate and very low levels of reactivity were more frequently observed in patients with cystic echinococcosis. However, in most of our control subjects, there was no significant reactivity of the PBMC to antigenic stimulation compared with PHA stimulation. These findings exclude a nonspecific mitogenic effect of the *E. multilocularis* antigen preparation used in our model. Despite relatively strong antigenic cross-reactions with PBMC from *E. granulosus*-infected patients, a high degree of sensitivity of the blood lymphocyte responsiveness to *E. multilocularis* antigen exists in alveolar echinococcosis. This finding is in agreement with previous results obtained by a very similar method of proliferation assay (2, 7, 15). Thus, our in vitro stimulation cultures of PBMC provided an attractive system for further investigation of immunoregulation in human echinococcosis.

One of the key functional parameters involved in modulation of immune responses in mammalian is the nature of cytokines produced by different subsets of immune cells (17, 23, 24, 26, 27). To define the role of cytokine regulation in alveolar echinococcosis, we analyzed cytokine mRNA expression and cytokine production in *E. multilocularis* antigen-stimulated

PBMC cultures. Our data, presented in this paper, revealed two distinct patterns of cytokine mRNA expression in patients with alveolar echinococcosis and in healthy individuals. To our knowledge, this is the first report documenting simultaneous examination of multiple cytokine mRNAs in humans infected with cestodes. The cytokine patterns in PBMC were evaluated by RT-PCR. mRNA phenotyping demonstrated that mRNAs encoding IL-2, IFN- γ , and IL-6 were present in patients infected with larval *E. multilocularis* and in healthy volunteers. However, mRNAs encoding cytokines resembling those of T_H2 cells, i.e., IL-4, IL-5, and IL-10, were more strongly expressed in antigen-stimulated PBMC of patients than in those of normal donors. In particular, IL-5 mRNA was markedly induced in PBMC of patients upon reactivation with *E. multilocularis* antigen but was virtually absent in PBMC of normal donors. The lack of detection of IL-5 mRNA in PBMC from these normal control donors is not likely to be due to the insensitivity of our methods, because we have shown that expression of IL-5 mRNA can be readily detected in PBMC of a small number of normal donors upon PHA stimulation. Therefore, it is likely that PBMC of normal individuals failed to express IL-5 mRNA in response to *E. multilocularis* antigen. Elevated expression of some T_H2 cytokines, including IL-5, might thus contribute to specific changes in the immune response of patients with alveolar echinococcosis, suggesting some form of selection for distinct cytokine-producing cells. Although we cannot differentiate whether these cytokine patterns observed in patients with alveolar echinococcosis are the cause or the result of disease, our current studies at least indicate quantitative variations of cytokine expression between individual patients, which might be associated with their different courses of the disease. Within the group of 16 patients studied, 2 patients had undergone radical surgery 12 or 18 months before participating in our study and were considered cured after clinical examinations

and computed tomography. The remaining 14 patients were clinically stable under continuous chemotherapy but still had active lesions. All but one of these patients with active lesions exhibited high or intermediate levels of IL-5 mRNA expression. The status of cured patients, however, was characterized by a lack of IL-5 mRNA expression. Thus, it seems possible that detection of IL-5 mRNA in patients with alveolar echinococcosis is associated with the viability status of *E. multilocularis* metacystodes and the control of larval proliferation. However, the variability of the clinical course of alveolar echinococcosis is rather extensive and makes it difficult to draw definite conclusions from these observations. In our small group of study patients, the reported association between IL-5 mRNA expression and the clinical status of the disease is a novel observation and thus might contribute to our further understanding of the immunology of alveolar echinococcosis in humans.

Kinetics of mRNA expression demonstrated that the distinct cytokine patterns detected in patients with echinococcosis and in normal individuals were highly stable and remained conserved even after prolonged culture in the presence of *E. multilocularis* antigen. Consistent with cytokine mRNAs was the actual cytokine production, because on contact with *E. multilocularis* antigen, PBMC of patients produced large quantities of IL-5 but lower levels of IFN- γ than did PBMC of normal individuals, whereas PBMC of normal donors produced elevated levels of IFN- γ but practically no IL-5. All blood leukocytes, including lymphocytes, macrophages, and neutrophils, have the potential to produce a broad spectrum of cytokines (3). However, the inverse correlation between the levels of IL-5 and IFN- γ detected in patients during antigen-induced reactivation of PBMC indicates that, analogous to the mouse model (16, 24), functionally different lymphocyte populations might be involved in the immune reactions of patients against *E. multilocularis* and that among the responding cells a T_H2 response is preferentially activated. Reports on other human helminthic infections, such as schistosomiasis, onchocerciasis, and lymphatic filariasis, demonstrated that in each infection the proportion of CD4⁺ lymphocytes induced by parasitic antigens to secrete T_H2 cytokines was significantly higher than in uninfected individuals (11, 28, 35, 38). This raises the possibility that individuals infected with helminths have expanded populations of antigen-specific lymphocytes, which have the capacity to specifically produce T_H2 cytokines (22). However, it is not clear if the increased production of IL-5 in alveolar echinococcosis is due to a clonal expansion of T_H2 lymphocytes or to an increased level of cellular activation. Moreover, other cell types certainly also contribute to the observed cytokine patterns, as suggested by the prompt and abundant IL-6 production in both patients and uninfected individuals. Previous reports on other infectious diseases indicate that the proinflammatory cytokines IL-6 and IL-10 could be the products of mononuclear phagocytes but may also be produced by B and T lymphocytes (3). Moreover, in patients with alveolar echinococcosis, T-cell activation may not only be restricted to CD4⁺ lymphocytes but may also include CD8⁺ lymphocytes (32). CD8⁺ lymphocyte levels were reported to be markedly elevated in the periparasitic tissue of patients and were also identified as antigen-specific suppressors of the immune response in murine alveolar echinococcosis (16, 19, 20). Thus, the functional importance of different lymphocyte subsets, including CD8⁺ cells, to immunosurveillance in echinococcosis remains the subject of further studies.

Taken together, our results indicate that production of T_H2 cytokines, including IL-5, specifically increases in *E. multilocularis*-infected individuals upon reactivation with parasitic anti-

gen. Although we cannot clearly differentiate T_H1 and T_H2 immune responses in patients with alveolar echinococcosis, the imbalance between IL-5 and IFN- γ production at least indicates that the immune response to *E. multilocularis* is regulated by a balance between T_H1- and T_H2-type cytokines and that a gradual shift in the balance toward T_H2 responses may contribute to the long-standing manifestation of *E. multilocularis* infection in humans.

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