

Variation of Cytokine Patterns Related to Therapeutic Response in Diffuse Cutaneous Leishmaniasis

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BOMFIM, G., NASCIMENTO, C., COSTA, J., CARVALHO, E. M., BARRAL-NETTO, M., AND BARRAL, A. 1996. Variation of cytokine patterns related to therapeutic response in diffuse cutaneous leishmaniasis. *Experimental Parasitology* 84, 188–194. Diffuse cutaneous leishmaniasis is a rare entity characterized by disseminated cutaneous nodules associated with specific anergy to leishmanial antigens. A low but not absent IFN- γ expression by cells present in cutaneous lesions has been documented during the active phase of diffuse cutaneous leishmaniasis. In this study we confirm this observation, and extend it by showing a similar pattern in peripheral blood mononuclear cells and the variation of mRNA cytokine expression pattern during different stages of the disease. During active disease, patients did not express mRNA for IFN- γ , while expressing mRNA for IL-2, IL-4, and IL-10. In contrast, an expression of IFN- γ and low IL-10 was observed after treatment-induced transient healing of cutaneous lesions. In three patients we have been able to analyze a third PBMC sample obtained after clinical relapse, documenting in all of them decreased IFN- γ expression with no expression of IL-10. Although there was an association between the appearance of IFN- γ expression and clinical improvement, with marked expression of IFN- γ mRNA and decreased expression of mRNA for IL-10 after treatment, this was not sufficient to prevent relapse in these patients. Therefore, it is possible that factors other than the cytokines characteristic of the Th1 and Th2 balance are implicated in the inability of diffuse cutaneous leishmaniasis patients to mount an anti-*Leishmania* immune response causing clinical improvement. © 1996 Academic Press, Inc.

INDEX DESCRIPTIONS AND ABBREVIATIONS: leishmaniasis; cytokines; therapy; cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; IFN- γ , interferon-gamma; IL, interleukin; PBMC, peripheral blood mononuclear cells; DEPC, diethylpyrocarbonate; PCR, polymerase chain reaction.

INTRODUCTION

Human cutaneous leishmaniasis displays a spectral distribution, ranging from responsive mucosal leishmaniasis to the anergic form of diffuse cutaneous leishmaniasis (DCL), with localized cutaneous leishmaniasis (LCL) representing an intermediate state.

DCL is a rare manifestation, characterized by a selective lack of cell-mediated immunity against the parasite, resulting in extensive cutaneous involvement by nonulcerated nodules or plaques containing abundant parasites (Costa *et al.* 1992). DCL is a challenge to anti-parasite

therapy. Following several anti-*Leishmania* therapeutic schemes, DCL patients exhibited clinical responses characterized by significant reduction of cutaneous lesions, but virtually all of them relapsed a few months after therapy (Costa *et al.* 1992).

Experimental studies showed that immune response against intracellular parasites seems to be mediated by two functional subpopulations of CD4⁺ cells, distinguished by their pattern of cytokine production (Th1 and Th2) (Yamamura *et al.* 1991; Romagnani 1992). In murine leishmaniasis, resistance has been associated with Th1-type reactions (production of IL-2 and IFN- γ) and susceptibility with Th2-type patterns (production of IL-4, IL-5, and IL-10) (Scott 1991; Cáceres-Dittmar *et al.* 1993).

Studies in human active DCL showed a con-

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sistent low expression of IFN- γ and IL-2 mRNA associated with high IL-10, IL-5, and IL-4 mRNA in cells from cutaneous lesions (Pirmez *et al.* 1993).

In this study we took advantage of the possibility of investigating a relatively large group of DCL patients during different stages of the disease. Evaluation of the mRNA for IFN- γ , IL-2, IL-4, and IL-10 from PBMC was performed in a total of six DCL patients during active disease, and compared to the pattern obtained during transient healing; in three patients it was also possible to evaluate cytokine patterns after clinical relapse.

PATIENTS AND METHODS

Patients. Six patients with DCL were evaluated in this study. They were treated with several anti-leishmanial regimens, including antimony gluconate, pentamidine, and amphotericin B. Clinical characteristics of the patients are presented in Table I. During their initial evaluation for this study, all patients exhibited active DCL, characterized by the presence of multiple nonulcerated nodules distributed in large areas of the body surface, and had been given anti-leishmanial drugs for at least 3 months. Further immunological evaluations were performed in six patients during clinical remission of their skin lesions and in three patients during relapse.

Lymphocyte separation and cell cultures. PBMC were obtained from heparinized venous blood layered over a Hypaque-Ficoll gradient (LSM; Organon Teknica Corporation, Durham, NC) as previously described (Horwitz *et al.* 1977). After washing three times in 0.9% NaCl, PBMC were resuspended in RPMI 1640 culture medium (GIBCO BRL, Grand Island, NY) supplemented with 200 IU/ml penicillin, 100 μ g/ml streptomycin, and 10% type AB human sera. A total of 3×10^6 cells were cultured in microtiter plates (Falcon; Becton-Dickinson Co., Oxnard, CA) with or

without 20 μ g of *Leishmania* antigen or phytohemagglutinin (PHA) (GIBCO; in a final dilution of 1:10). Cultures were maintained at 37°C in 95% air and 5% CO₂ for 3 days.

Measurement of cytokine secretion. Culture supernatants of antigen or PHA-stimulated PBMC were harvested after 24, 48, and 72 hr of culture, and were analyzed by ELISA using a quantitative sandwich technique "Quantikine" (RD Systems, Minneapolis, MN 55413) for IL-10 with the minimum detectable dose of 4.4 pg/ml. Levels of IFN- γ were measured by Cytoscreen immunoassay kit (BioSource International, Camarillo, CA 93012) with a minimum detectable dose of <4 pg/ml.

Preparation of cells for reverse transcriptase-polymerase chain reaction (RT-PCR). PBMC at a concentration of 2×10^6 cells/ml were kept nonstimulated or stimulated with 10 μ g/ml *Leishmania* antigen. At 24, 48, and 72 hr of incubation cells were resuspended in 0.75 M guanidium thiocyanate, sodium citrate, pH 7.0, and 20% Sarcosyl and stored at -70°C.

Procedures to avoid nucleic acid contamination. Procedures were performed in three different rooms, separating preamplified samples from postamplified products. Gloves, tubes, and tips for pipettes used for RNA extraction and PCR were disposable. A special pipette set was used for RNA extraction and another for cDNA synthesis, and PCR reagents, including water, used in the PCR mixture were previously aliquoted to avoid repeated freezing and thawing. For each PCR analysis, one positive and one negative control were used. Between PCR tests all pipettes and racks were decontaminated by UV light and washed with 2% sodium hypochlorite.

RNA extraction. Total RNA was isolated from *Leishmania* antigen-stimulated PBMC at 24, 48, and 72 hr of culture. Cells from 48 hr of culture showed higher cytokine expression and are shown in this report. A phenol-chloroform extraction of RNA was carried out following a standard protocol (Chomczynski and Sacchi 1987; Saiki *et al.* 1990) by adding 0.04 ml of 2 M sodium acetate, pH 4.0, 0.4 ml of saturated phenol, and 0.2 ml of chloroform:isoamyl alcohol at a 24:1 dilution. RNA was precipitated in 0.05 ml of 3 M NaAc, pH 7.0, and 1 ml of 95% ethanol, kept

TABLE I
Clinical Characteristics of Diffuse Cutaneous Leishmaniasis Patients

Patient	Age year/sex	Duration of disease in years	Number of cutaneous lesions	Transient clinical improvement	Relapse
1	28/M	4	>500	Yes	No
2	31/M	9	168	Yes	Yes
3	16/M	12	20	Yes	Yes
4	16/M	14	51	Yes	Yes
5	21/M	15	86	Yes	Yes
6	12/F	5	40	Yes	Yes

Note. M, male; F, female.

overnight at -70°C , later thawed and centrifuged for 20 min at 4°C . The pellet was dried at 37°C after two washings with 70% ethanol, and resuspended in DEPC-treated water. RNA concentration was determined spectrophotometrically and its integrity evaluated through electrophoresis in 2% agarose gel.

Single-stranded cDNA synthesis. One microgram of total RNA was reverse transcribed by addition of 2.5 units of RNAsin (Promega, Madison, WI), 25 ng of oligo(dT)12-18 and 250 μM dNTPs (both from Pharmacia LKB Biotechnology, Piscataway, NJ), and 200 units of Moloney murine leukemia virus RNAse H-reverse transcriptase, 10 mM dithiothreitol, and 5 \times reverse transcriptase buffer (GIBCO BRL/Life Technologies Gaithersburg, MD) in a final reaction volume of 20 μl . The reaction was then incubated at 37°C for 1 hr and boiled for 10 min after the addition of 180 μl of DEPC-treated water.

Polymerase chain reaction. Five microliters of 1:20 cDNA of each sample (after normalization to β -actin) were amplified by PCR for IL-2, IFN- γ , IL-4, and IL-10 in a 20- μl mixture containing 200 μM dNTPs, 0.2 μM 3' and 5' external primers, 2.25 mM MgCl_2 , 1 \times Gene Amp PCR buffer, and 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT). All reaction tubes included a 40- μl mineral oil overlay. A cDNA sample from a mixed lymphocyte culture from normal individuals was included as a positive control of cytokine expression for each PCR reaction. Cycling parameters for β -actin, IL-2, IFN- γ , and IL-4 amplifications were 30 cycles at the following temperatures: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 30 sec, with a last cycle of 72°C for 2 min. The amplification conditions for IL-10 consisted of 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and 30 sec in a thermal cycler reactor (National Labnet Company, Woodbridge, NJ). Cytokine-specific PCR was performed using oligonucleotide primers (kindly provided by Steve Reed) as previously described (Ehlers and Smith 1991). Sequences of external primers were as follows: β -actin, 5' TGACGGGGTACCCACACTGTGCCATCTA and 5' CTAGAAGCATTCGCGTGGACGATGGAGGG (product size 661 bp); IFN- γ , 5' ATGAAATATACAAGT-TATATCTTGGCTTT and 5' GATGCTCTTCGACCTC-GAAACAGCAT (product size of 501 bp); IL-2, 5' ATGTACAGGATGCAACTCCTGTCTT and 5' GTCAGTGT-TGAGATGATGCTTTGAC (product size of 458 bp); IL-4, 5' ATGGGTCTCACCTCCCAACTGCT and 5' CGAACACTTTGAATATTTCTCTCAT (product size of 456 bp); and IL-10, 5' TCTCAAGGGGTGGGTGAGTATCCCA and 5' ATGCCCAAGCTGAGAACCAAGACCCA (product size of 351 bp). PCR products were separated on 1.5% agarose gels and transferred to Nytran (Schleicher and Schuell, Keene, NH) by Southern blotting.

Cytokine probes. Inserts from plasmids containing the human sequences IL-2, IL-4, IL-10, IFN- γ , and β -actin were isolated as described (Ghalib *et al.* 1993; De Waal *et al.* 1991). DNA fragments were excised from agarose gels, electroeluted, and purified as described (Sambrook *et al.*

1984). Probes were labeled with fluorescein using a random primer DNA labeling kit (ECL Amersham Life Science, Arlington Heights, IL).

Hybridization of amplified products by Southern blot. Hybridization was detected by ECL (ECL Amersham Life Science). Briefly, nylon membranes containing PCR products were incubated with fluoresceinated probes and washed three times at 56°C with antibody wash solution provided by the manufacturer. Antifluorescein peroxidase conjugate, diluted 1:1000, and the manufacturer's detection system were applied before film exposure for 10 min.

RESULTS

Results are presented for each phase of the disease, i.e., the active, transient remission, and relapse phases. During the active phase of disease no patient exhibited detectable IFN- γ mRNA. All patients exhibited signals of IL-2 mRNA although with varying different intensity. Four of the six patients had high expression of IL-4 mRNA, whereas all of them exhibited mRNA for IL-10 (Fig. 1, column A). In three patients (1, 3, and 6) the signal for IL-10 mRNA was very intense and in the other patients much weaker. Strong IL-10 mRNA signals did not always coincide with intense signal for IL-4, since patients 2 and 5 had little IL-10 mRNA and a very strong signal for IL-4 mRNA. Patient 3 had intermediate signal for IL-4 and a very intense signal for IL-10.

High IFN- γ mRNA expression was observed during the transient healing phase in all but patient 1. In contrast, significant reductions during the transient healing period were observed in IL-10 mRNA. On the other hand, there were no changes for IL-4 mRNA expression in the majority of patients evaluated. It is noteworthy that patient 1 had clinical remission with marked reduction of IL-10 without alteration in other cytokine signals (Fig. 1, column B).

During active disease the majority of patients showed very intense mRNA expression for IL-4, except for patients 3 and 4. A high expression of IL-4 mRNA was observed during healing phase. Only patient 6 had very weak expression of IL-4 mRNA during healing phase of the disease.

Relapse of cutaneous lesions was documented in three of six patients during the period

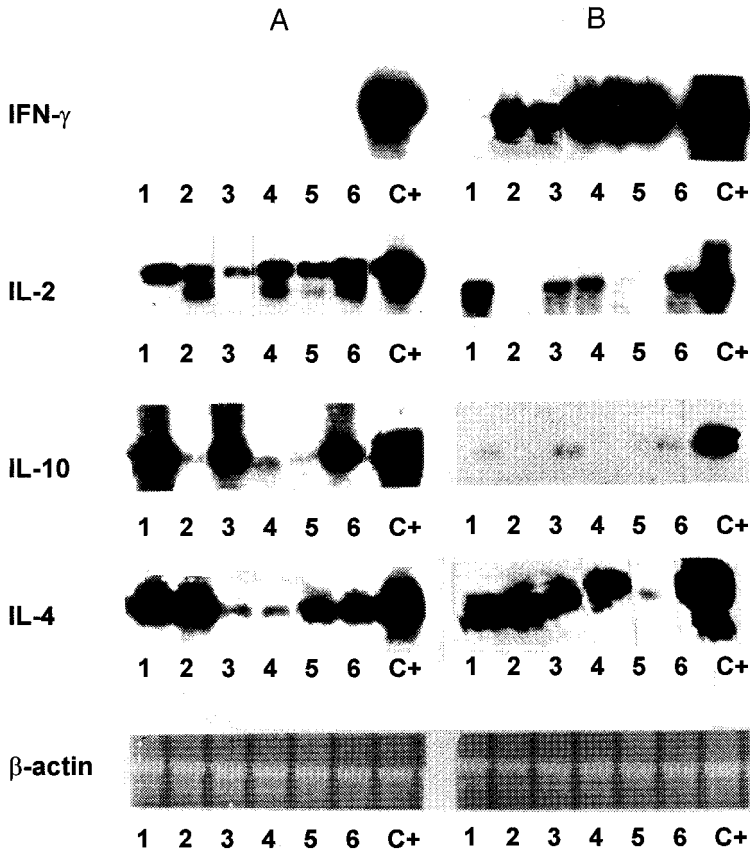


FIG. 1. Cytokine patterns in diffuse cutaneous leishmaniasis. Peripheral blood mononuclear cells from six patients with active DCL were evaluated by RT-PCR for cytokines. (A) Cytokine expression during active disease, before treatment; (B) cytokine expression during clinical remission of cutaneous lesions, after treatment. Numbers 1 to 6 are patients; C+ is a positive control of cytokine expression by PCR.

of observation (patients 4, 5, and 6). Patient 6 had a significant decrease in IFN- γ mRNA expression, whereas patients 4 and 5 continued to show intermediate signals for this cytokine during relapse (Fig. 2). All patients showed a marked reduction of IL-10 mRNA expression at the beginning of the relapse phase. Strong IL-4 mRNA expression was observed in patient 6, while very little message was seen in patients 4 and 5. There was an inverted cytokine pattern for IFN- γ and IL-10 mRNA expression during active and relapse phases in DCL patients.

Cytokine levels were determined by ELISA in supernatants from patients' lymphocytes stimulated with *Leishmania* antigen or PHA. High levels of IL-10, but not IFN- γ , were de-

tected in active DCL patients following antigen-stimulation (Tables II and III). Immediately after clinical remission IFN- γ levels increased, and IL-10 was not detectable. Except for patients 5 and 6, this pattern of cytokine production paralleled the findings of cytokine mRNA detection by PCR.

DISCUSSION

The results presented in this report provide the first evidence for a change in cytokine pattern, in the same patients, in correlation with disease status in DCL. Recently, transient alterations in immune reactivity, as assessed by lymphocyte proliferation and antibody response, have been documented after ivermectin treat-

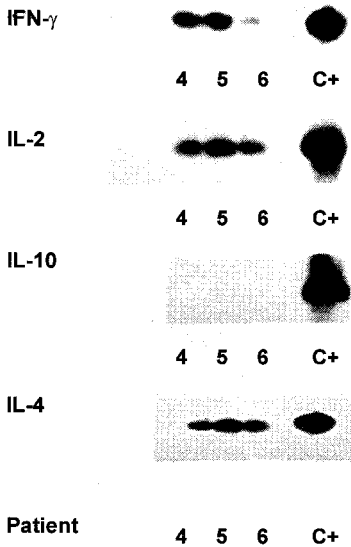


FIG. 2. Cytokine pattern during relapse of diffuse cutaneous leishmaniasis. Peripheral blood mononuclear cells from three patients (4, 5, and 6) with relapse of cutaneous lesions were evaluated.

ment of onchocerciasis (Steel *et al.* 1994) and these changes may be due to modification of antigen load. Our findings show that some cytokines, mainly IFN- γ mRNA and IL-10, have a pattern coinciding with clinical changes in human leishmaniasis. The absence of IFN- γ mRNA during the active phase of disease was followed months later, after a favorable therapeutic response, by an increase of mRNA expression and production of IFN- γ on the supernatant of antigen-stimulated cells. Interestingly, in only one of six patients in this study, a direct

TABLE II
Levels of IFN- γ (pg/ml) in PBMC Supernatant

Patient	Clinical condition	Leishmanial antigen	PHA
2	Before therapy	<limit	992
	After therapy	49	1200
3	Before therapy	<limit	1200
	After therapy	20	NP
4	Before therapy	<limit	1181
	After therapy	46	NP

Note. PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cells; NP, not performed; <limit, lower than minimum detectable dose.

TABLE III
Levels of IL-10 (pg/ml) in PBMC Supernatant

Patient	Clinical condition	Leishmanial antigen
1	Before therapy	855
	After therapy	<limit
4	Before therapy	535
	After therapy	<limit
5	Before therapy	550
	After therapy	<limit
6	Before therapy	100
	After therapy	<limit

Note. PBMC, peripheral blood mononuclear cells; <limit, lower than minimum detectable dose.

correlation between cutaneous lesion healing and IFN- γ mRNA signal was not observed (patient 1). The role of IFN- γ is questionable, however, considering that its presence did not prevent relapse. Cytokines do not act as isolated molecules, and the relative amounts of other products that potentiate or neutralize their action are of utmost importance in understanding the final result. It is possible that the marked decrease in IL-10 potentiates the effect of locally produced IFN- γ .

Experimentally IL-10 has been implicated in inhibiting cytokine production by Th1 cells (Fiorentino *et al.* 1991). IL-4 (Nabors and Farrel 1994) and TGF- β have also been shown to counterbalance the IFN- γ effect, whereas IL-12 stimulates IFN- γ production. It is possible then that cytokines not evaluated in the present study may also have influenced the course of disease. Production of IFN- γ by cells obtained from cutaneous lesions in DCL has been shown (Cáceres-Dittmar *et al.* 1993) concomitant with a high expression of IL-4, IL-5, and IL-10. A predominance of IL-4 expression was also reported in lesions from DCL patients from Brazil (Pirmez *et al.* 1993). The clinical and therapeutic status of these patients at the time of biopsy were not given clearly. In our study we found a predominance of IL-10 and IL-4, with no expression of IFN- γ and variable expression of IL-2 mRNA.

Despite small differences, the present observation made with PBMC reflected the general

pattern observed in studies using cells obtained from lesions (Cáceres-Dittmar *et al.* 1993; Pirmez *et al.* 1993). The single cytokine that best reflected disease status was IFN- γ which was absent during active disease or relapse, and positive during remission of clinical manifestations. We cannot, from the present findings, attribute a major protective role to this cytokine, since even in the presence of IFN- γ , patients experienced relapses. IL-10 was highly expressed during active DCL, with a marked reduction of expression during clinical improvement in most but not all patients. IL-10 may play a crucial role in the disease status, suppressing IFN- γ action, considering its powerful suppressive action in the skin (Berg *et al.* 1995).

Dissociation between the production of IL-2 and IFN- γ during active disease was an important finding in all patients in the present study. At this stage, all patients exhibited mRNA for IL-2, but not for IFN- γ . A similar finding had been reported in murine leishmaniasis (Scott 1991). The contrary situation, IFN- γ in the absence of message for IL-2, has also been reported in human leishmaniasis in lesions from DCL (Cáceres-Dittmar *et al.* 1993) and in LCL (Melby *et al.* 1994). Differences in antigen signaling and cell responses may be involved in this dissociation. Another possibility is that different cell sources are involved in the production of these cytokines. NK cells are able to produce IFN- γ (Kos and Engleman 1996) and B cells may be a source for IL-2.

In the present study, in only two cases, an elevated expression of IL-10 was accompanied by high IL-4 expression, showing that different patients may use diverse immunomodulatory mechanisms even in a highly selected group of patients with similar anergic disease. Such findings indicate that strategies of immune intervention have to be individually tailored.

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