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Determination of the cytokine profile in American cutaneous leishmaniasis using the polymerase chain reaction

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SUMMARY

The lymphokine profiles were determined in the skin lesions of the three distinct clinical forms of American cutaneous leishmaniasis (ACL), using a reverse transcriptase polymerase chain reaction (RT-PCR) and primers for various lymphokines. The message for interferon-gamma (IFN-γ), tumour necrosis factor-beta (TNF-β), and IL-8 was expressed in the three clinical forms of ACL. IL-1β mRNA was expressed in most localized (LCL) and mucocutaneous (MCL) leishmaniasis, but in only few of the diffuse cutaneous leishmaniasis (DCL). IL-2 mRNA was detected in about half of the lesions, with more prominent values for MCL. IL-4 mRNA was present in most lesions from the three clinical forms, but markedly increased in DCL. IL-5 and IL-10 mRNAs were expressed in all MCL and in half of the DCL lesions and weakly expressed in LCL lesions. IL-10 mRNA was more abundant in MCL lesions. In contrast, IL-6 and TNF-α mRNAs were expressed in a large number of LCL. In MCL, IL-6 mRNA was expressed in most cases and TNF-α mRNA in all the cases. In DCL, IL-6 mRNA was absent and TNF-α mRNA was weakly expressed. These results suggest that most T cells present in the MCL and DCL lesions secrete a mixture of type 1 and type 2 cytokine patterns, but in DCL granulomas type 2 cytokines predominate. In LCL the cytokine patterns show a mixture of type 1 and type 0 with a preponderance of IFN-γ over IL-4, and low levels of IL-5 and IL-10. The lack of IL-6 and TNF-α mRNAs, and the low expression of IL-1β in DCL lesions suggest a defect in the antigen-processing cells that may account for the state of unresponsiveness in these patients.

Keywords cytokine leishmaniasis polymerase chain reaction T lymphocytes

INTRODUCTION

Leishmaniasis constitutes a range of acute or chronic cutaneous or visceral diseases caused by protozoan parasites of the genus *Leishmania*. The New World form, American cutaneous leishmaniasis (ACL) is a chronic granulomatous disease with a spectrum of clinical manifestations. In localized cutaneous leishmaniasis (LCL) an adequate cell-mediated immune response is mounted against the parasite, and the presentation of the disease is restricted to well defined skin lesions. In contrast, diffuse cutaneous leishmaniasis (DCL) is characterized by a selective anergy of the specific cell-mediated immunity, resulting in extensive involvement of the skin, nasopharyngeal mucous tissue and some lymph nodes, with abundant parasites [1–4]. A proportion of ACL patients develop mucocutaneous leishmaniasis (MCL), which is characterized by exacerbated cell-mediated immunity, and destructive lesions of the oral and nasopharyngeal cavities [2–4]. These variations in the immune response to the parasite in the human disease, and the existence of experimental models in different inbred strains of mice, have made leishmaniasis an excellent prototype for studying the immunoregulatory processes involved in infectious diseases.

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Analysis of the immune response in genetically susceptible BALB/c and resistant C57BL/6 mice infected with *Leishmania*, has shown that both resistance and susceptibility are mediated by functionally distinct CD4$^+$ T lymphocytes [5]. The production of IL-2 and interferon-gamma (IFN-γ) is diminished with progression of the disease [6–8], whereas IL-4 is increased during infection [8]. These differences in lymphokine secretion fit the definition of Mosmann *et al.* [9] for the division of chronically stimulated T helper cells into T helper cells type 1 (Th1) and type 2 (Th2). Th1 lymphocytes produce IL-2 and IFN-γ, whereas Th2 cells preferentially secrete IL-4, IL-5, IL-6 and IL-10 [9,10]. In murine leishmaniasis, resistance has been associated with Th1 and susceptibility with Th2 [11]. Recently, other lymphokine patterns have been found in T cell clones and

normal T cells [10,12]. A phenotype designated Th0 that produces IL-2, IL-4, IL-5, IL-3, IL-10, GM-CSF and IFN-γ is common among short-term stimulated clones. The phenotypes, Thp and Thm, are 'virgin' and 'memory' IL-2 producing cells respectively, distinguished by the expression of CD45R isoforms [10].

We have previously demonstrated a decrease in the number of IL-2-positive (IL-2 producing) cells in DCL lesions [13]. Recently, we have shown differences in the numbers of 'memory' ($CD4^+CD45RO^+$) and 'virgin' ($CD4^+CD45RA^+$) T cells in the granulomas of LCL, MCL and DCL [14]. These differences in the cellular component of the immune response in ACL are almost certainly associated with changes in the lymphokine secretion pattern, and information concerning this will provide insight into the immunocompetent cells required for overcoming unresponsiveness and activating antigen-presenting cells to eliminate the *Leishmania* parasites. In the present study we used the reverse transcriptase polymerase chain reaction (RT-PCR) to determine the lymphokine profile in lesions of ACL.

PATIENTS AND METHODS

Patients

Groups of patients with either LCL ($n=10$), MCL ($n=9$) or DCL ($n=10$) were studied in the Instituto de Biomedicina. The patients were diagnosed by established clinical, epidemiological and histopathological criteria [15]. Parasitological confirmation of the clinical diagnosis was based on Giemsa staining of smears from biopsies, culture of minced biopsy material on blood agar base slants containing 15% defibrinated rabbit blood and 200 U of penicillin/ml, inoculation of hamsters with biopsy macerate, and histopathological examination of Giemsa and haematoxylin-eosin stained sections. LCL patients had less than 5 months of evolution. The patients were not under treatment at the time of study.

Skin biopsy specimens were embedded in OCT compound (Miles Scientific, Elkhart, IN), snap-frozen in liquid nitrogen and stored at $-70°C$ until examination.

Preparation of samples for PCR

The PCR procedure was carried out according to Yamamura *et al.* [16]. Thirty-to-forty frozen sections (5 μm) were cut with a cryostat and placed in 1·5-ml microcentrifuge tubes containing 0·5 ml digestion buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7·0, 0·5% sarcosyl, 0·1 M 2-mercaptoethanol). The microtome and knife were carefully cleaned with ethanol between each block to prevent sample-to-sample contamination.

RNA extraction

A phenol-chloroform extraction of RNA was carried out by adding 0·05 ml 2 M sodium acetate pH 4·0, 0·5 ml phenol (buffer-saturated), 0·1 ml chloroform:isoamyl alcohol (24:1). The RNA was precipitated in isopropanol, kept for 30 min at $-20°C$, and centrifuged for 15 min at 4°C. The pellet was washed twice with 75% ethanol and resuspended with 250 μl diethylpyrocarbonate (DEPC)-treated water containing 25 U RNAse inhibitor (Boehringer Mannheim, Penzberg, Germany), 30 μl 10 × buffer (10 × buffer: 400 mM Tris-HCl, pH 7·9, 100 mM NaCl, 60 mM $MgCl_2$) and 20 U RNase-free DNase (Promega Corp., Madison, WI), incubated for 20 min at 37°C. The RNA was extracted with 0·3 ml phenol, 0·5 ml chloroform:isoamyl alcohol (24:1). The aqueous phase was taken and 0·03 ml 3 M sodium acetate pH 6·0, 20 μg glycogen, and 0·8 ml ice-cold 100% ethanol were added, mixed, stored for 30 min at $-70°C$ and centrifuged for 15 min. The pellet was air-dried after washing twice with 75% ethanol.

Reverse transcription

The nucleic acid pellet was dissolved in DEPC-treated H_2O containing 1 μl RNase inhibitor (Boehringer Mannheim), 4 μl 5 × buffer (250 mM Tris-HCl, pH 8·3, 375 mM KCl, 50 mM dithiothreitol, 15 mM $MgCl_2$), 2 μl dNTP (10 mM), 2 μl oligo-dT (0·5 μg/μl) (Pharmacia, Uppsala, Sweden), and 2 μl AMT reverse transcriptase (2·5 U/μl) (GIBCO-BRL, Bethesda, MD), volume mix 20 μl. Each sample was then incubated at 42°C for 1 h.

Primer selection

Primer pairs for CD3δ, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, TNF-α, TNF-β, IL-6, IL-8 and β-actin (described in Table 1) provided amplification products of 309, 356, 248, 266, 372, 405, 352, 124, 162, 190, 300 and 540 bp respectively. Primers for each cytokine were selected from published cDNA sequences and were constructed following previously described guidelines [17].

Polymerase chain reaction

In order to compare the results between different individuals and clinical forms of leishmaniasis, the cDNAs are normalized based on the expression of β-actin or CD3δ PCR products, depending on whether monokines or interleukins were to be detected. Two microlitres of the cDNA were amplified with each primer pair, separately. Each reaction contained (in a total of 25 μl) 2 μl cDNA, 2·5 μl 10 × buffer (100 mM Tris-HCl, pH 9·0, 500 mM KCl, 15 mM $MgCl_2$, 0·1% gelatin), 2·5 μl dNTP (2 mM) (Pharmacia), 0·5 μl $MgCl_2$ (50 mM), 0·5 μl of each primer (50 pM/μl), 0·5 μl Taq polymerase (5 U/μl) (Promega). All reaction tubes included a 25 μl mineral oil overlay. Cycling parameters for the mRNA amplification were 1 min at 94°C, 2 min at 65°C for 35 cycles, except for the amplification of IL-4 that was for 40 cycles, and IL-10 that was 1 min at 94°C, 2 min at 55°C for 40 cycles in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT).

Analysis of amplified DNA

After PCR amplification, 8 μl of the samples + 2 μl of a tracking dye were fractionated over a 1·7% agarose gel containing 1 μg/ml ethidium bromide. PCR products were verified by probing samples transferred to Hybond-N nylon membrane (Amersham, Aylesbury, UK), with a ^{32}P-labelled internal oligonucleotide. This kinase end-labelled probe was synthesized consisting of internal sequences complementary to the amplified product. Blots were hybridized for 4 h at 55°C, washed for 15 min at room temperature with 2 × SSPE (3 M NaCl, 0·2 M NaH_2PO_4, 0·02 M EDTA-Na_2) and 0·1% SDS, followed by 1 × SSPE and 0·1% SDS at 55°C for 15 min, 0·1 × SSPE and 0·1% SDS at 55°C for 10 min, and exposed to x-ray film.

Relative quantification of PCR product

Nylon membranes were scanned and the amount of radioactivity bound to PCR products present in each lane was deter-

Table 1. Sequences of cytokine amplification primers

Primers	5′ specific	3′ specific
IL-1β	GACACATGGGATAACGAGGC	ACGCAGGACAGGTACAGATT
IFN-γ	AGTTATATCTTGGCTTTTCA	ACCGAATAATTAGTCAGCTT
IL-2	ACTCACCAGGATGCTCACAT	AGGTAATCCATCTGTTCAGA
IL-4	CCTCTGTTCTTCCTGCTAGCA TGTGCC	CAACGTACTCTGGTTGGCTTCC TTCAC
IL-5	ATGAGGATGCTTCTGCATTTG	TCAACTTTCTATTATCCACTCG GTGTTCATTAC
IL-6	ATGTAGCCGCCCCACACAGA	CATCCATCTTTTTCAGCCAT
IL-10	ATGCCCCAAGCTGAGAACCAA GACCCA	TCTCAAGGGGCTGGGTCAGCTA TCCCA
TNF-α	TCTCGAACCCCGAGTGACAA	TATCTCTCAGCTCCACGCCA
TNF-β	CCTCACACCTTCAGCTGCCC	GAGAAACCATCCTGGAGGAA
IL-8	ATGACTTCCAAGCTGGCCGTG	TTATGAATTCTCAGCCCTCTTC AAAAACTTCTC
β-actin	GTGGGGCGCCCCAGGCACCA	CTCCTTAATGTCACGCACGATT TC
CD3δ	CTGGACCTGGGAAAACGCATC	GTACTGAGCATCATCTCGATC

mined using an AMBIS radioanalytic imaging system (Automated Microbiology Systems Inc, San Diego, CA). The relative intensity of individual bands was expressed as a percentage of the most intense band, among cytokine blots, assigned the value of 100. The statistical analysis among the three forms of ACL for each lymphokine was performed using the Mann–Whitney test.

Quantification of cytokine plasmid DNA by PCR

PCR analysis of 10-fold serially diluted plasmids containing IL-2, IL-4, IFN-γ, IL-6, IL-10 and TNF-α cDNAs with visualization by ethidium bromide staining indicated that our PCR procedure was sensitive to the order of 10^2–10^3 copies for each cytokine [16]. Furthermore, the intensity of the PCR product increased according to the number of copies of starting plasmid to at least 10^9 copies. These PCR products were transferred to nylon membrane, probed and quantified by radioanalytic imaging. There was a log-linear correlation between the number of starting copies and the quantity of PCR products throughout the range investigated. These results show that our PCR conditions provide meaningful comparison of the small amounts of cytokine mRNAs present in lesions.

Several controls were employed to ensure accurate comparisons of the different samples studied. Upon PCR amplification of serial 10-fold dilutions of sample cDNAs a concomitant decrease in the PCR product was observed. Similarly, varying the number of PCR cycles did not change the relative differences between samples. These studies show that our PCR conditions lie on the exponential phase of amplification. PCR of RNA from biopsies and buffer alone did not yield product confirming the absence of extraneous cDNA or PCR product contaminating the samples.

RESULTS

The PCR allowed the detection and semiquantitative assessment of changes in mRNA levels of various lymphokines in the three distinct clinical forms of ACL (Fig. 1). For meaningful comparisons of the small amounts of cytokine mRNAs present in the lesions, cDNA samples were normalized by PCR using primers for the CD3δ molecule, which is universally present in T cells, and primers for the β-actin molecule present in all cells. The normalization allowed the use of equivalent amounts of cDNA for each assay.

The PCR analysis showed different lymphokine profiles for the three clinical forms of ACL. IFN-γ mRNA was expressed in most of the patients (9/10 LCL, 9/9 MCL and 10/10 DCL). The quantitative analysis for this lymphokine showed considerable amounts for the three clinical forms of leishmaniasis (Figs 2 and 3). IL-2 mRNA was detected in about the same number of patients (4/10 LCL, 4/10 DCL and 5/9 MCL), but the quantitative values were significantly higher in MCL than in LCL ($P \leqslant 0{\cdot}05$). Even though the values for IL-2 mRNA in LCL patients are low, prominent bands were observed in some of the patients. IL-4 mRNA was expressed in most ACL lesions (4/4 LCL, 4/4 DCL and 3/4 MCL) with significant differences between LCL/DCL and MCL/DCL ($P \leqslant 0{\cdot}05$) (Fig. 2). IL-10 mRNA was present in the three clinical forms of ACL, with more prominent bands in MCL and DCL specimens. The quantitative analyses showed statistical differences between LCL/MCL and MCL/DCL ($P \leqslant 0{\cdot}05$), with the highest relative ct/min values for the MCL lesions. IL-5 mRNA expression in LCL lesions was visually undetectable and the quantitative values were very low. This lymphokine was, however, observed in the other two forms (4/4 MCL and 2/4 DCL). The quantification showed statistically significant differences between LCL/MCL and LCL/DCL ($P \leqslant 0{\cdot}05$). TNF-β mRNA was expressed in most ACL lesions (3/4 LCL, 4/4 MCL and 4/4 DCL), with stastistically significant differences between MCL and DCL ($P \leqslant 0{\cdot}05$). IL-1β was expressed in most of the LCL and MCL lesions (8/10 LCL and 8/9 MCL) and only a few DCL specimens (4/10). The number of patients expressing TNF-α mRNA was about the same for the three clinical forms (2/3 LCL, 3/3 MCL and 2/3 DCL), but the electrophoretic bands of the DCL specimens were weak. IL-6 mRNA was expressed in most LCL and MCL lesions (2/3 LCL and 2/3 MCL), but absent in DCL. IL-8 mRNA was expressed in most

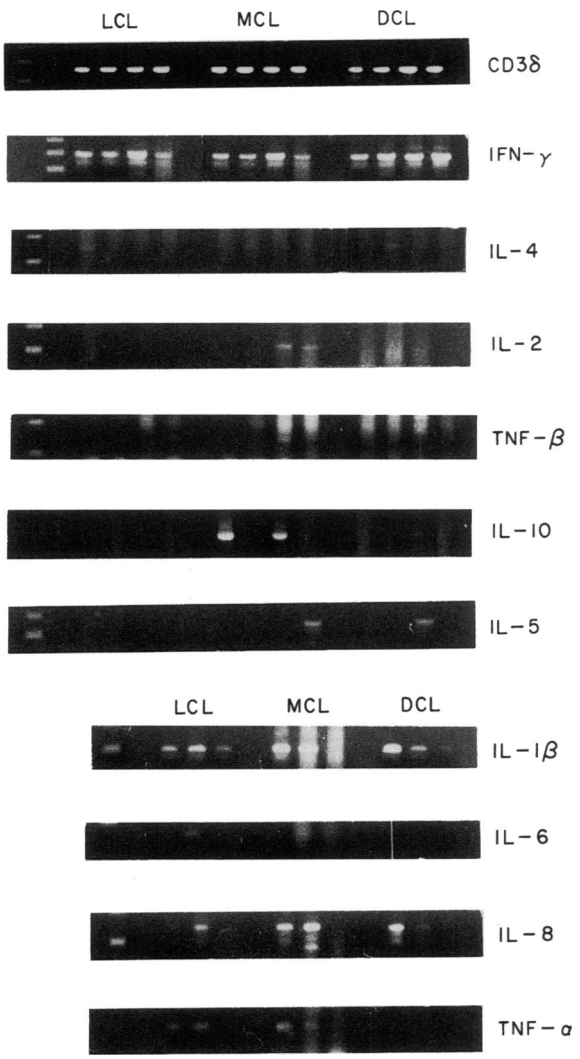

Fig. 1. Polymerase chain reaction (PCR) amplification of cytokine and CD3δ mRNA fragments of representative lesions from patients with localized (LCL), mucocutaneous (MCL) and diffuse cutaneous leishmaniasis (DCL). Lane 1 from left is a 1 kb DNA ladder. Ethidium bromide-stained 1·7% agarose gel. Positive samples: CD3δ, all; IFN-γ, all; IL-4, lanes 2, 5, 7, 9–12; IL-2, lanes 2, 8–10, 12; TNF-β, lanes 5, 8–10; IL-10, lanes 6, 8, 12; IL-5, lanes 9, 12; IL-1β, all; IL-6, lanes 3, 6, 7; IL-8, lanes 2, 3, 5, 6, 8, 9; TNF-α, lanes 2, 3, 5, 6.

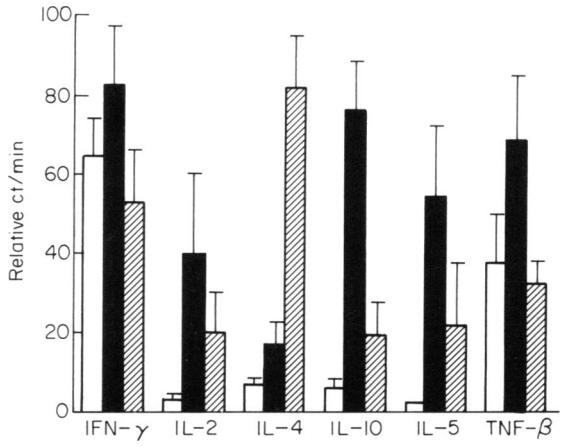

Fig. 2. Quantification of cytokine polymerase chain reaction (PCR) products in American cutaneous leishmaniasis lesions. The cDNA derived from lesions were normalized to CD3δ. The products were electrophoresed, transferred to nylon membranes and hybridized with a radiolabelled oligonucleotide probe. The transfers were then scanned with an automated β scanner. □, Localized cutaneous leishmaniasis (LCL); ■, mucocutaneous leishmaniasis (MCL); ■, diffuse cutaneous leishmaniasis (DCL).

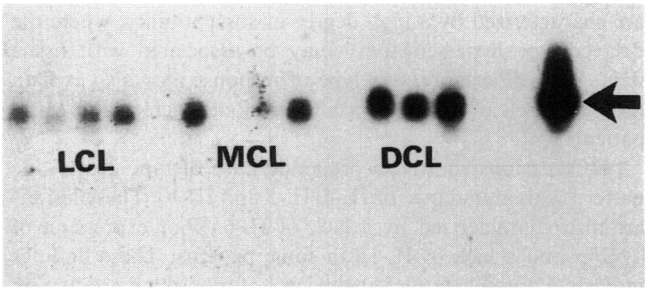

Fig. 3. Hybridization of IL-4 polymerase chain reaction (PCR) products with a radiolabelled internal oligonucleotide probe from human leishmaniasis lesions. LCL, Localized cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis. Note from left 4/4 positive LCL patients, 3/4 positive MCL patients and 3/3 positive DCL patients. Weal signals are observed in lane 2 and lane 3 of LCL and MCL groups respectively. Control is IL-4 plasmid DNA (arrow).

of the ACL lesions analysed (2/3 LCL, 2/3 MCL and 2/3 DCL) with more prominent bands in the MCL samples.

DISCUSSION

PCR allows the identification of cytokine mRNA present in relatively small numbers and in low copy numbers, and characterized by a short half-life [16,18,19]. Therefore, as is the case for other proteins, variations in the rate of cytokine synthesis are related to differences in the number of available mRNA templates [16,19]. Different strategies have been proposed for the quantification of cytokine mRNA expression using PCR amplification of reverse-transcribed RNA [18]. Most approaches take advantage of the fact that the radioactive signal of the PCR product can be quantified by densitometric measurement of the autoradiographs, or by image scanning of the nylon membrane. In the present study, we used an imaging system in which equivalent sensitivity of PCR detection for each cytokine was obtained based on previous titration of cytokine plasmid cDNAs [16]. In addition, equivalent amounts of cDNA were used for each assay based on a prior amplification of CD3δ and β-actin molecules. Our quantitative PCR relies on the efficiency of reverse-transcription, specific PCR amplification for each cytokine, and the assurance that the primers and probes do not detect genomic DNA. Once these conditions are established, the procedure offers some advantages over more complex approaches such as competitive PCR, which requires an internal standard to be co-amplified with the sample in the same tube.

Cutaneous leishmaniasis is an excellent model to understand the various regulatory mechanisms involved in protozoal immunity. In leishmaniasis, T helper-inducer CD4$^+$ lymphocytes have been associated with effector mechanisms promoting

resistance or susceptibility [11,20]. These apparently contradictory findings may be explained by the existence, in the mouse, of two functionally distinct T helper cells, the Th1 and Th2 subsets [9]. Heinzel *et al.* [8] demonstrated a correlation between the presence of mRNA for lymphokines associated with Th1 cells and resistance, and those associated with Th2 cells and susceptibility. In addition, they demonstrated that *in vivo* IL-4 depletion reverses the susceptibility of BALB/c mice, and eventually resolves most skin lesions [21].

In the present study, we determined the presence of mRNA for various lymphokines in the three clinical forms of human ACL. In LCL, we observed a predominant expression of mRNAs for IFN-γ, IL-1β, TNF-α, IL-6, IL-8, TNF-β and IL-2 in some cases, and a low expression of IL-4, IL-5 and IL-10. This lymphokine profile corresponds to patterns observed in most human CD4$^+$ T cell clones established from healthy individuals after polyclonal activation [22,23]. Basically, two clones have been identified in humans, a predominant clone that produces IL-2, IL-4, IL-5, IFN-γ and GM-CSF, and a second clone that produces IL-3, IL-6, TNF-α, TNF-β and GM-CSF. The former resembles the Th0 pattern described in mice.

MCL lesions showed a mixture of type 1 and type 2 cytokine patterns, characterized by a high abundance of IL-2, IL-4, IL-5 and IL-10. These profiles may coincide with the high proportion of CD4$^+$ T cells observed in these granulomas [14]. These lesions are characterized by a high degree of susceptibility, where the delayed-type hypersensitivity may be associated with tissue damage. Furthermore, this type of response may also explain the high levels of parasite-specific antibodies detected in MCL patients [24].

DCL lesions showed a preponderance of type 2 cytokine pattern with abundance of IL-4, IL-5 and IL-10. These lesions are also characterized by a lack of IL-6, poor expression of TNF-α and a lack of IL-1β in some patients. These findings indicate a possible failure at the level of monokine production by the antigen-presenting cells in the DCL lesions. The presence of IFN-γ in the DCL lesions may contribute to the lack of message for IL-1 in these patients, since IFN-γ is known to down-regulate IL-1 production [25]. The lack of IL-6 in DCL may be the result of the poor expression of IL-1β and TNF-α, since these cytokines are necessary to induce IL-6 production [26]. In addition to Th2-like cells, the DCL granuloma also contains 'virgin' T cells in larger numbers than in LCL lesions [14].

The type 2 cytokine pattern observed in MCL and DCL lesions may be the result of a chronic stimulation by the *Leishmania* parasite, whereas the type 1 and type 0 cytokine pattern demonstrated in LCL lesions may be due to a 'short-term' stimulation, since all the patients studied had less than 5 months of evolution.

The cytokine pattern of ACL granulomas is very similar to that found in lesions of leprosy, where the tuberculoid form is characterized by a type 1 response and the lepromatous form by a type 2 response [16]. In addition, our results are similar to those found in Brazilian leishmaniasis by Pirmez *et al.*, who demonstrated a type 1 pattern in LCL and a mixture of type 1 and type 2 in MCL (personal communication).

The present study suggests that the outcome of cutaneous leishmaniasis is usually determined by a predominant T cell group and the pattern of cytokines produced. Future studies in our laboratory will be directed to determining the role of antigen-presenting cells and their accessory signals in the pathogenesis of leishmaniasis. In addition, the detection by PCR of cytokine receptors, adhesion molecules and other activation molecules will provide further insight into the immunoregulatory mechanisms required for overcoming unresponsiveness in cutaneous leishmaniasis.

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