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T-Cell Subpopulations, Expression of Interleukin-2 Receptor, and Production of Interleukin-2 and Gamma Interferon in Human American Cutaneous Leishmaniasis

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Leukocyte subpopulations, the expression of the interleukin-2 (IL-2) receptor, and the production of IL-2 and gamma interferon (IFN-γ) were studied in the peripheral blood mononuclear cells of American cutaneous leishmaniasis patients that had been stimulated in vitro with either leishmanial antigen or mitogen (phytohemagglutinin M). The 75 patients examined were classified as having either the localized (LCL; 66 patients), mucocutaneous (MCL; 5 patients), or the rare diffuse (DCL; 4 patients) form of the disease. Patients with DCL, who are characterized by their defective cell-mediated immune response to leishmanial antigen, failed to express the IL-2 receptor and did not produce IFN-γ when exposed to the antigen but did so when stimulated by phytohemagglutinin M. Both LCL and MCL patients showed strong proliferative responses to leishmanial antigen; these were by far the greatest in MCL patients. Both groups had significantly increased IL-2 receptor expression and IFN-γ production after exposure to either antigen or mitogen, and these were highest in the MCL patients. Concerning the leukocyte subpopulations evaluated (CD2, CD4, CD8, CD20, MO_2), the most significant findings were a decrease of both $CD4^+$ cells and the CD4/CD8 ratio in MCL patients compared with the other groups. Considering IL-2 production, in response to phytohemagglutinin M both MCL and LCL patients showed amounts of IL-2 comparable to those of the controls. Our results help explain the anergy of T cells from DCL patients to leishmanial antigen, which could lead to a defective production of IFN-γ and possibly contribute to their incapacity to kill the *Leishmania* parasite. Concerning MCL patients, the significantly increased expression of IL-2 receptor, decreased expression of the CD4 (helper-inducer of suppression) phenotype, and elevated IFN-γ production might partially explain the state of hypersensitivity and mucosal damage exhibited by these patients.

American cutaneous leishmaniasis (ACL) is a disease caused by infection with a flagellated protozoan that invades and grows within macrophages. Leishmanias of the complexes *Leismania mexicana* and *L. braziliensis* are the principal agents of the disease in Central and South America (14, 18). The infection manifests itself in three general clinical forms. Localized cutaneous leishmaniasis (LCL) is characterized by limited and ulcerated skin lesions that either heal spontaneously or after treatment with pentavalent antimonial salts. Mucocutaneous leishmaniasis (MCL), the intermediate form, is characterized by lesions that contain few parasites but that are progressive and can cause severe histological, anatomical, and functional disruption of the oral, nasal, pharingeal, or laryngeal mucous membranes. These mucosal lesions are often refractory to therapy (27). Diffuse cutaneous leismaniasis (DCL), the malignant form, occurs very rarely and is characterized by the presence of progressive nonulcerated nodules, rich in parasites, which are resistant to treatment (7).

In previous studies (2, 3) we demonstrated that patients with DCL lack in vivo and in vitro responsiveness to leishmanial antigens and have a marked leishmanial antigen-induced suppression of in vitro mitogenic responses. This anergy is not due to an involvement of prostaglandins in the suppression of the specific immune response (4). In contrast, patients with MCL have a hyper-responsiveness to the parasite antigen and do not demonstrate either suppressor activity or prostaglandin-dependent inhibitory mechanisms (2, 3). Patients with LCL have moderate responses to leishmanial antigens, and we demonstrated (4) that an indomethacin-sensitive, prostaglandin-dependent suppressor mechanism is operating in these patients.

It has been well established in a variety of systems that the proliferative response of T lymphocytes upon their exposure to antigens or mitogens is the result of two major events: the generation of the T-cell growth factor, or interleukin-2 (IL-2), and the induction of T-cell responsiveness to this mediator (30), controlled by the expression of an inducible cell-surface receptor (15, 26). In turn, the IL-2 generated also acts as a regulator and mediator of gamma interferon (IFN-γ) induction (10). Recently, a critical role has been reported for IFN-γ as the major lymphokine mediating macrophage activation to enable the killing of leishmanias in vitro (25, 29).

To better understand the cellular events involved in the activation of T lymphocytes and the generation of IFN-γ in leishmaniasis patients, we have examined, with monoclonal antibodies, the occurrence of different T-cell phenotypes (CD2, CD4, CD8) as well as IL-2 receptors (Tac) in phytohemagglutinin M (PHA)- or leishmanial antigen-stimulated peripheral blood leukocytes. In addition, we have undertaken preliminary evaluation of IL-2 and IFN-γ production in PHA- or antigen-stimulated supernatants from patients with either of the three clinical forms of ACL.

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MATERIALS AND METHODS

Study subjects. (i) Patients. A total of 75 patients (34 females and 41 males) with ACL were evaluated in the Instituto de Biomedicina, Caracas, Venezuela. They were classified on the basis of clinical and histopathological criteria (6) as suffering LCL with one (56 patients) or more (10 patients) lesions, MCL (5 patients), and DCL (4 patients). None had received treatment during the period immediately before evaluation, with the exception of one patient with DCL, who, despite the treatment, had shown no effective clinical improvement at the time of the study. The extremely rare occurrence of the DCL form of the disease prevented the evaluation of larger numbers in this group.

(ii) Normal controls. A total of 44 individuals were studied (5 females, 39 males); samples were obtained from the Municipal Blood Bank of the Vargas Hospital, Caracas.

Antigen preparation. *Leishmania* extracts were kindly provided by J. L. Avila (Instituto de Biomedicina, Caracas). These were obtained from a previously described (2) strain of *L. mexicana* subsp. *mexicana*.

The protozoa were cultured in minimal essential medium containing 2.5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), although the last passage was in a medium free of extraneous proteins (32).

The promastigotes were collected by centrifugation (1,800 $\times$ *g*, 20 min) at 4°C, washed twice with phosphate-buffered saline (PBS), suspended to a concentration of 25 $\times$ 10^6 cells per ml, and autoclaved (125°C, 15 min). Protein concentrations were determined by the method of Lowry et al. (17).

Separation and culture of mononuclear cells. Human peripheral blood leukocytes were separated from heparinized blood by centrifugation over Ficoll-Hypaque, washed three times, and suspended at concentrations of 2 $\times$ 10^6 or 3 $\times$ 10^6 cells per ml. The tissue culture medium used was RPMI 1640 (GIBCO) containing 100 U of penicillin per ml and 100 μg of streptomycin per ml, supplemented with 5% heat-inactivated, pooled human AB serum.

The cells suspended at 3 $\times$ 10^6 cells per ml were used for the evaluation of the surface markers with no stimulation. The cells suspended at 2 $\times$ 10^6 cells per ml were cultured in flat-bottomed microdilution plates (Linbro Chemical Co., New Haven, Conn.) for lymphocyte proliferation assays.

Lymphocyte proliferation assay. A standard microtest procedure was employed. PHA (B grade; Calbiochem, San Diego, Calif.) was used at a concentration of 10 μg/ml. The *Leishmania* preparation was used at a final concentration of 2.5 $\times$ 10^5 parasites per well.

After 3 days of exposure to the mitogen or 6 days of exposure to the antigen, 1 μCi of [3H]thymidine was added to some wells to measure lymphocyte proliferation, 6 h before harvesting onto glass fiber strips and counting by liquid scintillation. The results were expressed as the stimulation index, defined as the counts per minute of antigen or mitogen test divided by that of control wells.

In addition, samples of the PHA- or antigen-stimulated cells were recovered at day 3 for mitogen stimulation and day 6 for antigen stimulation to evaluate surface markers. These samples were centrifuged and suspended at 3 $\times$ 10^6 cells per ml.

Monoclonal antibodies. Mouse monoclonal antibodies were used at optimal concentrations predetermined by checkerboard titrations. Each sample was stained with a panel of monoclonal antibodies that included the pan-T-cell marker CD2, the inducer-helper T-cell marker CD4, the suppressor-cytotoxic T-cell marker CD8, the pan-B-cell marker CD20, and the monocyte-macrophage marker MO_2; all of these were from Coulter Immunology and were used at a dilution of 1/100. We also used an antibody to IL-2 surface receptor (Tac) at 1/5,000, kindly provided by T. A. Waldman, National Cancer Institute.

Negative controls consisted of the omission of the primary antibody or the use of an antibody of irrelevant specificity at the same protein concentration.

Slide preparation. Samples (1 ml) containing 3 $\times$ 10^6 cells, either nonstimulated or stimulated with antigen or mitogen, were centrifuged at 300 $\times$ *g* for 10 min in Eppendorf tubes and suspended in 250 μl of PBS with 1% bovine serum albumin. Samples (10 μl) of this supension were smeared onto glass slides (Clay, Adams), dried at room temperature, and then stored at −20°C until staining.

Before immunostaining, the slides were fixed in fresh reagent-grade acetone at room temperature for 10 min and allowed to air dry. Slides were then washed for 5 min in PBS with 1% bovine serum albumin.

Immunoperoxidase staining. The immunoperoxidase method used was a biotin-avidin system (13) as modified by Hofman et al. (12). The slides were sequentially incubated with normal horse serum diluted at 1/20 in PBS buffer for 30 min at 25°C, then primary mouse monoclonal antibody, biotinylated horse antimouse antibody (50 μg/ml) (Vector, Burlingame, Calif.), and avidin-biotin-peroxidase complex (Vectastain kit; Vector). Five-minute washes with PBS were performed between each incubation.

The specimens were then incubated with aminoethyl carbazole in the presence of hydrogen peroxide for 10 min. After a 5-min wash they were counterstained with methyl green for 6 min, washed again, and mounted in glycerol-gelatin.

The positive cells in a total of 200 cells were counted under standard light microscopy, and the percent positive for each surface marker was calculated.

The results were also expressed as absolute numbers, referring to the total number of cells per milliliter of blood of the subject.

Preparation of supernatants for IL-2 and IFN-γ determinations. Cells (5 $\times$ 10^6 in 3 ml of medium) were stimulated either with PHA or leishmanial antigen, at the same concentrations used for the proliferation assay, for 24, 48, 72, and 120 h in 6-well tissue-culture plates (Limbro Co., New Haven, Conn.) at 37°C in a 5% CO_2–95% air atmosphere. Control wells contained cells with only culture medium. Culture supernatants were collected by centrifuging the cell suspension at 400 $\times$ *g* for 15 min. The supernatants were filtered through a Millipore filter (0.45-μm pore size) and stored at −20°C until assay.

IL-2 assay. IL-2 activity was quantitated by using a murine cytotoxic T-lymphocyte line (CTLL) kindly provided by U. Kees (Clinical Immunology Research Unit, Princess Margaret Hospital for Children, Western Australia). Samples of 4,000 cells were suspended in 10% fetal calf serum–RPMI 1640 and plated in 96-well flat-bottom tissue-culture plates for 24 h. Supernatant samples or standard IL-2 preparations were added to triplicate wells at various dilutions (from 1/2 to 1/256).

Quantitation of IL-2-induced CTLL proliferation was achieved by the colorimetric assay of Mosmann (22). 3-(4, 5-Dimethyl thiazol-2,yl)-2,5-diphenyl tetrazolium bromide from Sigma Chemical Co. (St. Louis, Mo.) was dissolved in PBS at 5 mg/ml; at the time indicated, 10 μl per 100 μl of medium was added to the well and incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was

TABLE 1. Clinical profile and lymphocyte reactivity to PHA and *L. mexicana* antigen of leishmaniasis patients and controls

Patients or controls (*n*)	No. of patients		Age (yr)	Duration of illness (mo)	Leukocyte count (10^6/ml)	Stimulation index[a]	
	Male	Female				PHA	*L. mexicana*
LCL (66)	38	28	33.3 ± 2.4	3.36 ± 0.61[b]	6.19 ± 2.14	38.89 ± 3.76	9.04 ± 1.45[c,d]
MCL (9)	4	5	24.0 ± 6.5	96.60 ± 25.44	6.37 ± 3.14	27.92 ± 3.55	15.89 ± 3.92[d]
DCL (4)	2	2	29.5 ± 8.8	96.00 ± 84.24	4.19 ± 1.75[c]	26.57 ± 13.74	2.58 ± 0.83
Controls (44)	39	5	29.9 ± 1.4		6.00 ± 3.28	39.08 ± 5.91	2.86 ± 0.48

[a] The counts per minute for unstimulated cells at days 3 and 6 were 402 ± 187 and 472 ± 239 cpm, respectively.
[b] $P < 0.001$ to 0.05 for LCL patients versus MCL and DCL patients.
[c] $P < 0.05$ for LCL patients versus DCL patients.
[d] $P < 0.001$ to 0.01 for LCL and MCL patients versus DCL patients and controls.

added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature the plates were read on a Dynatech MR580 Microelisa reader at a wavelength of 570 nm. The IL-2 activity was expressed as described by Gillis et al. (11).

IFN-γ assay. IFN-γ levels were determined by two methods. One was the conventional cytopathic effect inhibition assay with a human amnion cell line (WISH) as the indicator cells and vesicular stomatitis virus as the challenge virus (33). Antiviral activity was estimated by using a laboratory interferon standard that had been calibrated against the international reference standard from the National Institutes of Health, Bethesda, Md. (Hu IFN-γ; 4,000 reference units per ampoule). The antiviral activity, expressed in IFN units, was calculated as the reciprocal of the highest dilution of the sample that reduced the number of viral plaques by 50%. The other method used was a solid-phase sandwich radioimmunoassay (5), with two murine monoclonal antibodies specific for human IFN-γ (16), developed by Centocor, Malvern, Pa. (I MRX-IFN-γ radioimmunoassay). This assay is specific for IFN-γ, and the lower limit of sensitivity is 0.1 to 0.3 U/ml. Because the standard deviations of triplicate samples were less than 10% of the mean (5), differences of >20% between groups within the same experiment can be considered significant.

Statistical analysis. Paired or conventional Student *t* tests were used to compare groups. The Person *r* coefficient was used to determine the significance of the correlation between the different parameters studied.

RESULTS

Patient groups and lymphoproliferative studies. The clinical profiles of the different groups of patients and controls studied and their lymphocyte proliferative responses to PHA or *Leishmania* antigen are shown in Table 1.

Patients in all three clinical groups had active disease at the time of study. In the LCL group, 56 patients had one ulcer and 10 had two or more lesions. There were no statistically significant differences in the mean ages of the patient groups or the controls. There was, however, a significant difference in the duration of illness in the LCL group compared with that of the MCL and DCL groups (P, <0.001 to 0.05). Also, a significantly ($P < 0.05$) decreased leukocyte count was found in DCL patients compared with LCL patients.

Concerning the response to PHA, no statistically significant differences were found between the various groups. The responses of MCL and DCL patients tended, however, to be decreased. In contrast, the response to leishmanial antigen was significantly increased (P, <0.001 to 0.05) in LCL and MCL patients compared with DCL patients and controls, being greatest in the MCL patients.

Leukocyte subpopulations in nonstimulated mononuclear cells. The mean percentages of expression of surface markers (CD4, CD8, Tac, CD20) and the CD4/CD8 ratio of peripheral blood mononuclear cells from the three groups of patients with ACL and controls are shown in Table 2. We found a significant decrease of $CD4^+$ cells ($P < 0.05$) and of the CD4/CD8 ratio (P, <0.02 to 0.05) in patients with MCL compared with those of the other groups of patients and the controls. The percentage of cells expressing receptors for IL-2 (Tac) was significantly increased (P, <0.001 to 0.05) in DCL patients compared with those in patients with LCL or MCL and the controls. This group also showed a significant increase ($P < 0.001$) of the CD20 pan-B phenotype when compared with results in LCL patients and controls. No significant differences were found between the patient or control groups for the suppressor-cytotoxic cells (CD8). Also, there were no significant differences for the pan-T-lymphocyte marker (CD2) or for the monocyte-macrophage (MO_2) cells (results not shown).

It is of interest that there were no significant differences between the controls and LCL patients for any of the surface markers tested.

TABLE 2. Leukocyte surface markers in nonstimulated mononuclear cells from leishmaniasis patients and controls

Cell source (*n*)	% (mean ± SEM) of cells		CD4/CD8 ratio (mean ± SEM)	% (mean ± SEM) of cells	
	$CD4^+$	$CD8^+$		Tac^+	$CD20^+$
Controls (25)	44.4 ± 1.9	32.6 ± 1.8	1.47 ± 0.09	1.8 ± 0.3	7.7 ± 1.1
LCL (41)	43.5 ± 1.4	31.1 ± 2.3	1.59 ± 0.10	2.2 ± 0.3	10.9 ± 1.5
MCL (5)	36.9 ± 2.8[a]	23.8 ± 4.7	1.16 ± 0.14[b]	2.6 ± 1.2	13.7 ± 2.7
DCL (4)	46.9 ± 2.0	28.7 ± 3.7	1.45 ± 0.05	7.7 ± 1.6[c]	19.2 ± 0.9[d]

[a] $P < 0.05$ for differences between MCL patients versus all other groups.
[b] $P < 0.02$ to 0.05 for differences between MCL patients versus all other groups.
[c] $P < 0.001$ to 0.05 for differences between DCL patients versus all other groups.
[d] $P < 0.001$ for differences between DCL patients versus LCL patients and controls.

TABLE 3. Leukocyte surface markers in *Leishmania* antigen-stimulated mononuclear cells from leishmaniasis patients and controls

Cell source (*n*)	% (mean ± SEM) of cells		CD4/CD8 ratio (mean ± SEM)	% (mean ± SEM) of cells	
	$CD4^+$	$CD8^+$		Tac^+	$CD20^+$
Controls (25)	46.9 ± 3.6	35.2 ± 3.1	1.42 ± 0.13	18.6 ± 4.5	7.8 ± 1.7
LCL (41)	46.8 ± 2.6	37.6 ± 2.8	1.36 ± 0.09	28.1 ± 3.5[a]	19.9 ± 3.4[b]
MCL (5)	40.1 ± 4.2	31.3 ± 2.7	1.24 ± 0.16	43.1 ± 6.7[c]	ND[d]
DCL (4)	49.4 ± 4.1	34.1 ± 4.1	1.46 ± 0.06	9.7 ± 2.2	16.1 ± 6.9

[a] $P < 0.001$ to 0.05 for differences between LCL patients versus DCL patients and controls.
[b] $P < 0.005$ for differences between LCL patients and controls.
[c] $P < 0.02$ to 0.001 for differences between MCL patients versus all other groups.
[d] ND, Not determined.

When the results were expressed as the absolute number of cells per milliliter of peripheral blood, we found results essentially similar to those presented as percentages (results not shown). The only difference compared with when percentages were considered was that the number of MO_2 cells was significantly ($P < 0.05$) lower in the DCL patients ($0.24 \times 10^6 \pm 0.07 \times 10^6$) compared with the other patient groups (LCL patients; $0.36 \times 10^6 \pm 0.08 \times 10^6$; MCL patients, $0.44 \times 10^6 \pm 0.15 \times 10^6$).

Leukocyte subpopulations in leishmanial antigen- and PHA-stimulated mononuclear cells. We previously reported that there are profound differences in the proliferative responses of the three groups of patients to leishmanial antigen (2). Therefore, we characterized the leukocyte subpopulations under conditions of antigenic stimulation (Table 3). The most important finding was that the percentage of cells with receptors for IL-2 from DCL patients was significantly decreased (P, <0.001 to 0.05) when compared with those of LCL and MCL patients. Even though not statistically significant, the value in DCL patients was also half that of the controls. In contrast, a significant increase ($P < 0.05$) of these cells was found in LCL and MCL patients compared with the controls, being by far the greatest in the MCL group (P, <0.02 to 0.001).

We also observed an increase of B cells ($CD20^+$) in LCL and DCL patients that was only statistically significant ($P < 0.005$) for the LCL group when compared with controls.

When the subpopulations were evaluated in PHA-stimulated cells, no significant differences were found for any of the T-cell subsets evaluated between the three groups of patients and controls. The only significant difference observed was an increase ($P < 0.05$) of B cells (CD20) in all patient groups, when compared with controls (results not shown).

We also considered it interesting to compare by the paired Student t test the CD4/CD8 ratios and percentages of Tac^+ cells between nonstimulated cells and mitogen- or antigen-stimulated mononuclear cells for each group of leishmaniasis patients and controls.

The percentages of Tac^+ cells after PHA stimulation were 40.7 ± 2.5% in LCL patients, 29.1 ± 8.4% in MCL patients, 35.3 ± 12.4% in DCL patients, and 43.1 ± 4.0% in the controls. A significant increase occurred in the percentage of Tac^+ cells in all the groups after mitogenic stimulation (P, < 0.001 to 0.005) compared with nonstimulated cells (Table 2).

When the cells were stimulated with leishmanial antigen (Table 3), a significant increase in the percentage of Tac^+ cells occurred in LCL and MCL patients and controls (P, <0.001 to 0.05) but not in DCL patients when compared with results with nonstimulated cells (Table 2).

It should be noted that the increase in Tac^+ cells under PHA stimulation was significantly higher (P, <0.001 to 0.05) than that under antigenic stimulation for controls and DCL and LCL patients but not for MCL patients. In the last group the increase in Tac^+ cells was higher under antigenic stimulation than that under PHA stimulation.

Considering the CD4/CD8 ratio, both controls (ratio, 1:16 ± 0.08) and LCL patients (ratio, 1.21 ± 0.11) showed a significant decrease (P, <0.001 to 0.05) under mitogenic stimulation when compared with results with the other two experimental conditions (Tables 2 and 3).

Production of IFN-γ and IL-2. Table 4 shows the results of the evaluation of IFN-γ and IL-2 production in 10 patients with LCL, 5 patients with MCL, 2 patients with DCL, and 4 controls. We measured the IFN-γ in supernatants collected 48, 72, and 120 h after PHA or antigenic stimulation by using the biological assay. Similarly, in some samples collected 120 h after antigenic stimulation we evaluated the production of IFN-γ by using a radioimmunoassay that is specific for IFN-γ. The correlation between the results obtained by the two methods was highly statistically significant ($r = 0.94$; P

TABLE 4. IFN-γ and IL-2 in PHA- and leishmanial antigen-stimulated supernatants from leishmaniasis patients and controls[a]

Supernatant source (*n*)	IFN-γ (U/ml) in supernatants						IL-2 (U/ml) in PHA-stimulated supernatants[b]	
	Non-stimulated	PHA stimulated			Antigen stimulated			
		48 h	72 h	120 h	72 h	120 h	RIA[c] (120 h)	48 h	72 h
Controls (4)	3.75 ± 2.4	240 ± 56[d]	160 ± 56	480 ± 113[d]	7.5 ± 2.5	10.0 ± 4.0		44.7 ± 29.2	24.7 ± 17.3
LCL (10)	2.86 ± 1.5	150 ± 38[d]	220 ± 78[d]	142 ± 29[d]	26.3 ± 9.9[d]	45.7 ± 1.6[d]	35.2 ± 11.7	31.9 ± 7.5[e]	31.7 ± 8.3[e]
MCL (5)	2.5 ± 2.2	560 ± 71[d]	1,060 ± 503	2,000 ± 500[d]	80.0 ± 30.9	176.0 ± 62.6[d]	181.5 ± 91.2	52.3 ± 34.3	6.9 ± 5.1
DCL (2)	0.0				1.25 ± 1.23	2.5 ± 2.5	3.0		

[a] Results are means ± standard errors of the means.
[b] Mean value of unstimulated supernatants for IL-2 was 6.57 ± 1.51 U/ml.
[c] RIA, Radioimmunoassay.
[d] $P < 0.001$ to 0.05 for differences between PHA- or antigen-stimulated supernatants and nonstimulated supernatants.
[e] $P < 0.001$ for differences between PHA-stimulated supernatants and nonstimulated supernatants.

< 0.001). In the same supernatants we evaluated the level of IL-2 48 and 72 h after PHA and antigenic stimulation.

In the supernatants of cells stimulated with PHA we found that controls and LCL or MCL patients produced significantly ($P < 0.001$ to 0.05) more IFN-γ than unstimulated cultures at virtually all of the times evaluated. Significantly higher levels of IFN-γ were also found in the supernatants of MCL patients ($P < 0.001$ to 0.02) when compared with those in supernatants of LCL patients and controls at 48 and 120 h.

When the cells were stimulated with leishmanial antigen we found about 10 times less IFN-γ in the supernatants of the patients and controls compared with when these had been stimulated with PHA; these differences were statistically significant ($P < 0.01$ to 0.05). Despite this, the levels of IFN-γ in the supernatants from LCL and MCL patients were significantly greater ($P < 0.001$ to 0.05) than those of unstimulated cell supernatants, particularly at 120 h. This was not the case for the controls.

With the radioimmunoassay at 120 h we demonstrated significantly higher ($P < 0.05$) IFN-γ levels in MCL patients compared with those in LCL patients. With the radioimmunoassay, when standard deviations of triplicate samples are less than 10% of the mean, differences of >20% between groups within the same experiment can be considered significant.

It is of interest to note that a significant positive correlation was found between the IFN-γ production under PHA stimulation and the PHA-induced lymphoproliferative response ($r = 0.49$; $P < 0.05$). This was also the case for antigenic stimulation of IFN-γ production and the lymphoproliferation ($r = 0.69$; $P < 0.01$).

We were only able to study two patients with DCL for IFN-γ production, and the levels measured in antigen-stimulated supernatants were very low in both (range, 0 to 5.0 U/ml) at the two times tested. However, when one of the patients was stimulated with PHA, 20.0, 80.0, and 20.0 U/ml were found at 48, 72, and 120 h, respectively. These values were comparable to those in LCL patients or controls under the same conditions.

When IL-2 was quantitated in PHA-stimulated cell supernatants, we found comparable levels of IL-2 in LCL and MCL patients and the controls at 48 h. These levels were only statistically significantly ($P < 0.01$) elevated over those in unstimulated cultures in LCL patients, probably because of the low numbers in the other two groups. At 72 h there was a decrease in the IL-2 levels in the supernatants from MCL patients. In fact, when results from all of the patients were taken together and the production of IFN-γ was correlated with the levels of IL-2 in the same supernatants, we found at 48 h a significant positive correlation ($r = 0.51$; $P < 0.05$) between these. In contrast, at 72 h we observed a significant inverse correlation between these same parameters ($r = -0.59$; $P < 0.01$).

DISCUSSION

In this study we analyzed various aspects of immune function in patients with ACL in an attempt to better define the basis of the types of in vitro and in vivo response to leishmanial antigen that characterize the different clinical forms of the disease. In particular, we have commenced to elucidate the reasons for the anergy of DCL patients and the hyperreactivity observed in MCL patients.

Based upon the view that IL-2 provides the necessary signal for T cells activated by antigen to enter the S phase through an interaction with specific membrane receptors (9, 26, 31), we examined peripheral blood leukocytes from ACL patients for the expression of IL-2 receptors (Tac antigen) after challenge with leishmanial antigen and PHA. This was paralleled by an evaluation of a number of other T-cell subsets, including CD2, CD4, and CD8.

The results indicated that in spite of a normal percentage of CD4 and CD8 cells, T cells from DCL patients, but not T cells from MCL or LCL patients, failed to express IL-2 receptors upon stimulation with leishmanial antigen. This is not due to a nonspecific defect of T cells from DCL patients, since exposure of the same patients to PHA resulted in Tac expression that was equivalent to that seen in T cells from MCL or LCL patients and controls stimulated with the same mitogen. This complements the finding of Modlin et al. (19), who demonstrated that although LCL and DCL granulomas contain a comparable mixture of helper and suppressor phenotypes, the DCL granulomas have a level of IL-2-positive cells that is lower by 1 order of magnitude. In a recent study, Sacks et al. (28) demonstrated that depletion of leu-2^+ ($CD8^+$) T cells does not reverse the specific unresponsiveness that also occurs in Kala-Azar patients.

An interpretation of these results is that the failure of T cells from DCL patients to proliferate upon challenge with leishmanial antigen is partly due to their failure to express IL-2 receptors, which is an early requisite in the sequence of events leading to the clonal expansion of antigen-reactive cells and the elaboration of lymphokines (24).

It is important to note that a small proportion of the control group demonstrated a low level of lymphocyte proliferation after exposure to the *Leishmania* antigen as well as a significant increase in the number of Tac^+ cells after antigenic stimulation. This could be due to prior or subclinical infections with either *Leishmania* species or other parasites with cross-reactive antigens.

It has been demonstrated that mouse and human macrophages are capable of killing *Leishmania* cells in the presence of supernatants of activated lymphocytes (25, 29). IFN-γ is considered to be the lymphokine that mediates this macrophage-mediated killing of the parasite (29, 34). In the present study IFN-γ production by antigen-stimulated mononuclear cells was determined with both a conventional bioassay and a commercially available IFN-γ radioimmunoassay. Since the results of these assays were well correlated, we used the biological assay to test most of the supernatants.

We were unable to demonstrate significant production of IFN-γ by antigen-stimulated PBL from the two DCL patients that were studied. However, when one of these patients was tested by PHA stimulation, significant amounts of IFN-γ in the supernatants were measured. Since IL-2 binding to its receptor is a requirement for IFN-γ production (8), and DCL patients have a diminished expression of Tac in response to the leishmanial antigen but not to PHA, this finding is not unexpected.

The failure to secrete IFN-γ in response to leishmanial antigen could be an important factor in the inability of the DCL patients to control their infection, as has been suggested by Murray et al. (23) in the case of a patient with extensive leishmaniasis.

The pathogenesis of the mucosal lesions in ACL is little understood, and no experimental model for the study of this form of the disease has been developed. These patients are characterized by a particularly high response in vitro and in vivo to leishmanial antigen (5) and a depressed suppressor-cell function (3, 4). In the present study we demonstrated that these patients had a significantly decreased level of the CD4 inducer-helper T cells and normal levels of CD8 sup-

pressor-cytotoxic T cells. We also observed that these patients had a significant increase in Tac^+ cells upon stimulation with leishmanial antigen compared with LCL patients. This was even significantly higher than after mitogenic stimulation. In fact, we found a significant positive correlation between Tac^+ cells and the antigen-induced proliferative response when all of the patient results were taken together.

Carvalho et al. (1) have also reported a decreased number of CD4 cells in patients with MCL. We postulate that because the monoclonal antibody that recognizes the CD4 phenotype is directed both to the inducer of suppression subpopulation and to the helper cells (20), it is possible that the population which is deficient in MCL patients is the former. Now that monoclonal antibodies specific for this function are available (21), this possibility may be clarified in future studies.

We also demonstrated that MCL patients produced significant amounts of IFN-γ, both upon mitogenic and antigenic stimulation, when compared with that in unstimulated cultures, and that the levels of IFN-γ in both types of supernatant were significantly higher than those in LCL patients.

Our present results support our previous suggestion that MCL patients have a hypersensitivity to the leishmanial infection (3). These patients possibly have an exacerbated immune response due to the long time that they have been exposed to the parasite (1), but they are unable to completely eliminate the infection and lack adequate immune regulation (3, 4). This could allow an exaggerated clonal expansion of reactive T cells, which in turn can stimulate the production of IFN-γ. It is possible that IFN not only plays a role in the activation of the macrophages to kill the parasite but also participates in the pathogenesis of the disease. For example, it is well known that lesions of MCL patients are typical immune granulomas, with abundant lymphocytes and activated macrophages and with epithelial differentiation and the formation of giant cells. However, the lesions contain very few parasites. It is difficult to believe that the few parasites present could be the direct cause of the extensive damage of the mucous membranes that occurs in this form of the disease.

Patients with LCL, who control their infection without suffering progressive tissue damage and who show moderate in vitro and in vivo responses to leishmanial antigen (2), had CD4/CD8 ratios similar to those of the controls. They showed significant increases of Tac^+ cells upon mitogenic or antigenic stimulation compared with unstimulated cells, but, in contrast to MCL patients, this was higher with mitogenic stimulation than with antigenic stimulation. This is perhaps to be expected considering the polyclonal nature of mitogenic stimulation. They also had significant but moderate production of IFN-γ in response to mitogenic or antigenic stimuli when compared with unstimulated cultures. All of these results suggest that these patients have an adequate immune response, with stimulatory and suppressive activities well balanced. This may allow an effective and protective immune response against the parasite, in which the participation of IFN-γ probably plays a central role.

Considering IL-2 production, it has been demonstrated that IL-2 generated endogenously in the process of T-lymphocyte activation by mitogens or antigens acts as a regulator and mediator of IFN-γ induction (35). We observed that supernatants obtained 48 h after mitogenic stimulation of MCL and LCL patients as well as the controls had similar amounts of IL-2 that were significantly greater than those in unstimulated cultures. However, at 72 h we observed a decrease in the IL-2 level, particularly in MCL patients but also in the controls. It is interesting to note that at 48 h a significant positive correlation was found between IL-2 and IFN-γ production, but, in contrast, at 72 h a significant inverse correlation between these two parameters occurred. This might indicate a consumption of IL-2 to induce IFN-γ production, which becomes evident at 72 h.

The significant increase of B cells ($CD20^+$) in the three groups of patients, but particularly in those DCL, upon mitogenic or antigenic stimulation is consistent with the fact that these patients produce specific antibodies against the parasite, with the highest levels in DCL (36). No protective role for these antibodies has been found in leishmaniasis.

In conclusion, our results suggest reasons why T cells from DCL patients do not respond to leishmanial antigen. This is coupled to a defective production of IFN-γ, which might be one of the reasons for their incapacity to kill the *Leishmania* parasite. However, they do not explain the failure of leishmanias to induce the formation of IL-2 receptors in these patients. Conceivably, circulating leishmania-specific suppressor cells in DCL patients could prevent T cells from expressing Tac antigen upon exposure to leishmania antigen. We have, in fact, demonstrated elevated suppressor cell activity in DCL patients (3). However, other causes should also be taken into consideration; these include genetic nonresponsiveness, clonal deletion, defective antigen presentation by the macrophages, and defective IL-1 or IL-2 secretion. Further studies are required to clarify the underlying cause of the anergy in DCL patients.

Concerning patients with MCL, we have found a significantly increased expression of IL-2 receptors, decreased CD4 cells, and elevated IFN-γ production, all of which could account for the state of hypersensitivity exhibited by these patients. It is possible that IFN plays a role in the pathogenesis of the disease, but further study of why the parasite is not definitively eliminated is required.

We are presently monitoring a group of patients longitudinally to determine whether changes in the parameters measured occur after treatment and cure of the lesions and then to determine whether they provide an indication of the mechanisms involved in the defense against the parasite.

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