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Intermediate or chronic cutaneous leishmaniasis: leukocyte immunophenotypes and cytokine characterisation of the lesion

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Abstract: The American cutaneous forms of leishmaniasis include immune-responder individuals with localised cutaneous leishmaniasis (LCL) and non-responder individuals with diffuse cutaneous leishmaniasis (DCL). Patients with intermediate or chronic cutaneous leishmaniasis (ICL) have increased morbidity due to the length of their illness, atypical forms and areas of compromise. In the present study, we evaluated the expression of the leukocyte antigens (CD4, CD8, CLA: cutaneous lymphocyte antigen, CD69, CD83 and CD1a) and cytokines (IFN-γ, IL-4, IL-10 and TGF-β1) in the lesions of patients with ICL ($n = 18$) using an immunocytochemical procedure. ICL results were compared with the information for LCL ($n = 19$) and DCL ($n = 4$). The numbers of CD4+ and CD8+ T cells in ICL were similar to those of LCL lesions, but significantly different ($P \leq 0.05$) from DCL lesions. LCL lesions have about half the numbers of early activated CD69+ cells as ICL, but most are CLA+ skin homing memory T cells, whereas ICL lesions have the highest number of CD69+ T cells, but about one-third of these cells expressed CLA. This suggests that the granuloma of ICL patients contains many activated T cells that are unprimed to cutaneous-launched antigens, thus contributing to an aberrant immune response. In contrast, DCL granulomas presented the lowest numbers of activated CD69+ and CLA+ cells, associated with the characteristic tolerogenic state of these patients. The immunolocalisation of cytokines showed a mixed cytokine pattern in ICL lesions with many positive cells for IL-10, TGF-β1, IL-4 and IFN-γ, with a preponderance of the first two, and different from the prevalent Th1 and Th2 responses associated with LCL and DCL lesions, respectively. CD1a+ Langerhans cells were decreased ($P \leq 0.05$) in both ICL (271 ± 15 cells/mm^2) and DCL (245 ± 19 cells/mm^2) as compared to LCL (527 ± 54 cells/mm^2) epidermis. The percentage of IL-10+ epidermal Langerhans cells in ICL (33.69), from the total CD1a+ population, was higher than in LCL (17.45). In addition, fewer CD83+ primed Langerhans cells were present in ICL epidermis. The diminished participation of epidermal Langerhans cells, causing a defective signalling by the epidermis, in ICL lesions may account for the tissue-damaging state observed in these patients.

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Introduction

Leishmaniasis is a disease caused by flagellated protozoa of the genus *Leishmania*, and is characterised by different clinical, histopathological and immunological features. Of these, cell-mediated immunity is generally believed to be of dominant influence in determining the outcome of the disease. Infection may be restricted to the skin in cutaneous leishmaniasis, limited to the mucous membranes in mucocutaneous leishmaniasis, or spread throughout the reticuloendothelial system in visceral leishmaniasis. In the Americas, the cutaneous form includes immune-responder individuals with

acute or localised cutaneous leishmaniasis (LCL) and non-responder individuals with disseminated or diffuse cutaneous leishmaniasis (DCL) (1, 2). The intermediate area of the clinical spectrum, intermediate or chronic cutaneous leishmaniasis (ICL) is characterised by single or multiple lesions with atypical development and by plaques with many ulcers. Mucocutaneous leishmaniasis (MCL) is also included in this group. Patients with both ICL and MCL present strong cell-mediated immunity and few parasites in lesions. They usually accomplish clinical cure by the simultaneous application of chemotherapy with N-methylglucamine antimoniate and immunotherapy with a mixture of heat-killed *Leishmania* promastigotes and BCG (*Mycobacterium bovis*, Bacille Calmette-Guerin) (3–5). ICL patients have an increased morbidity due to the prolonged natural history of their illness and to a plethora of atypical clinical manifestations. Confusing names such as verrucous, sarcoidal, chromomycoid, psoriasiform, keloidal, eczematous, varicelliform and carcinoma-like leishmaniasis have been given to these atypical forms.

Leishmaniasis is an excellent model to study the extremes of a host/parasite relationship, particularly the diversity of the immune response associated to the genetic background of the host. We have shown the importance of the epidermis as an immunoregulatory site in the three classical forms of American cutaneous leishmaniasis, and the way epidermal cell signalling is crucial in determining the type of cytokine-related immune response to be generated against *Leishmania* parasites (6–8). This study characterised the leukocyte immunophenotypes, activation-associated molecules including the cutaneous lymphocyte antigen (CLA), and cytokine patterns in lesions of patients with ICL, and compared the results with those obtained for LCL and DCL patients.

Materials and methods

Patients

Patients with ICL ($n = 18$), DCL ($n = 4$) and LCL ($n = 19$) forms were studied in the Instituto de Biomedicina. The patients were diagnosed using established clinical, epidemiological and histopathological criteria (2). ICL patients were identified by the prolonged natural history of their disease and the presence of single or multiple lesions with verrucous, sarcoidal or vegetative development and by plaques with numerous ulcers. Parasitological confirmation of the clinical diagnosis was based on Giemsa or haematoxylin-eosin staining of smears from biopsies, culture of minced biopsy material on blood agar slants containing 15% defibrillated rabbit blood and 200 units of penicillin/ml, and the inoculation of hamsters with the biopsy macerate. Parasite characterisation to a subgenus level was achieved using a polymerase chain reaction (9). LCL patients had less than 5 months of evolution. The patients were not under treatment at the time of study, except DCL patients, who manifest their characteristic features even after therapy.

Skin biopsy specimens were embedded in Cryomatrix™ resin (Shandon, Pittsburgh, PA, USA), snap-frozen in liquid nitrogen and stored at −70°C until examination. Frozen sections (5 µm) were cut with a cryostat and air-dried overnight before the immunostaining procedure.

Antibodies

All antibodies used were diluted in a modified phosphate-buffered saline (PBS), pH 7.2 (10). For the leukocyte immunophenotype characterisation, murine monoclonal antibodies directed to the following human cell surface antigens were used: CD4 (B66.6, T helper at 1:1000) and CD8 (B116.1, T suppress-or/cyto-toxic at 1:1000) donated by Dr G. Trinchieri (The Wistar Institute, Philadelphia, NJ, USA); CD1a (Langerhans cells at 1:100) and CD69 (X63Ag8.653 at 1:250) purchased from Biodesign International, Kennebunkport, ME, USA.; CD83 (HB15 at 1:1500) donated by Dr T. F. Tedder (Dana-Farber Cancer Institute, Boston, MA, USA); CLA (HECA 452 at 1:100) donated by Dr L. Picker (University of Texas, South-western Medical Center, Dallas, TX, USA). Ovine polyclonal antibodies were used to detect human IFN-γ (AF-204-NA at 1:100) and Interleukin-4 (AF-285-NA at 1:100); and murine monoclonal antibodies to identify Interleukin-10 (MAB217 at 1:100) and TGF-β1 (MAB240 at 1:100, recognising the active form of the molecule); all purchased from R&D Systems Inc., Minneapolis, MN, USA.

Immunohistology

A variation of our previously described (11, 12) immunostaining procedure was used. Briefly, the samples were hydrated in PBS and sequentially incubated for 30 min at room temperature with primary mouse monoclonal antibody or for 120 min at 37°C with goat polyclonal antibody; biotinylated goat antimouse IgG at 1:150 (BRL, Gaithersburg, MD, USA) or biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min; and Vectastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) at 1:100, 30 min incubations were carried out in a Shandon Coverplate™ system (Pittsburgh, PA, USA), and 5-minute washes with PBS were done between steps. The reactions were developed for 10 min with 90 µM H_2O_2 and 3-amino- 9-ethyl-carbazole (AEC) (final concentration 0.88 mM), which was dissolved in 50 mM N,N-di-methylformamide in 0.1 M acetate buffer, pH 5.2 or 3 min in Vector® NovaRed™ substrate. The sections were then washed and counterstained with Harris' haematoxylin. Omission of the primary antibody or incubation with an antibody of irrelevant specificity at the same protein concentration were used as controls.

Leukocyte quantification

Cells were counted using a light microscope (Leica, Wetzlar, Germany) connected to a colour video monitor, calibrated to determine the number of cells/mm^2 in epidermis and dermal infiltrates. Only cells with a visible nucleus and showing red immunostaining were counted as positive. For obtaining a representative sample of the lesions, four non-serial alternate sections were immunostained and counted for each cell marker. All the fields of interest were counted in each section at a magnification of 1000X, giving $2–4 \times 10^4$ cells per section. Percentages of each phenotype were calculated. A previous count of the nucleated cells in a haematoxylin and eosin-stained section showed about 4000 cells/mm^2 of infiltrate. The values of CD4, CD8, CD1a and CD83 for LCL patients were taken from two previous studies of the group, using the same primary and secondary antibodies as in the present study (7, 13). In addition,

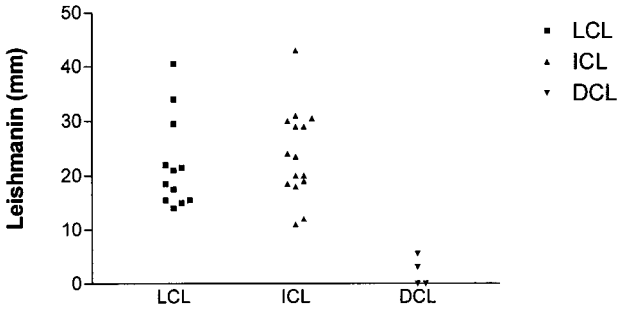

Figure 1. Leishmanian skin test in patients with localised (LCL), intermediate (ICL) and diffuse (DCL) cutateous leishmaniasis.

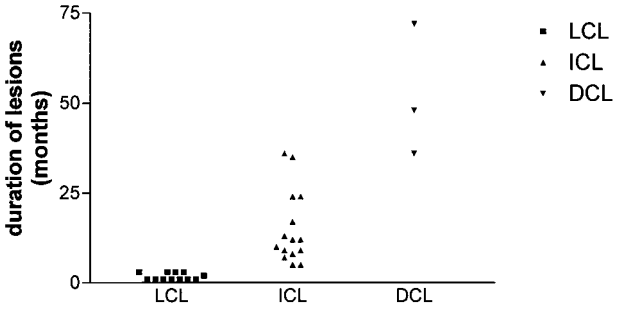

Figure 2. Duration of the lesions in patients with localised (LCL), intermediate (ICL) and diffuse (DCL) cutaneous leishmaniasis.

present CLA and CD69 detection was performed on the same patients from the two previous studies.

Statistical analysis

All the information was expressed as mean ± standard error of the mean (SEM). The means were calculated based on individual values for each patient. Comparisons between groups were made with the non-parametric Mann–Whitney test and Student's *t*-test for unpaired samples. The *P*-values less than 0.05 were considered significant. The Kruskal–Wallis non-parametric test was used for the analysis of the variance to compare variability within groups. Correlations between variables were analysed using Spearman rank coefficient. All tests were performed using GraphPad InStat 3.02 (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

Results

The histological analysis of skin biopsies of the patients confirmed the diagnosis of LCL, ICL and DCL. The clinical examination of the ICL patients showed that 23 had verrucous plaques, 31 multiple ulcers, 31 sarcoidal plaques and 15 sarcoidal plaques and ulcers. The leishmanin skin test was strongly positive (23.90 ± 2.15 mm, mean ± standard error) in these patients, but it was not significantly different from that of LCL patients (22.04 ± 2.42 mm) (Fig. 1). In contrast, the duration of lesions was significantly longer in ICL (15.07 ± 2.62 months, mean ± standard error) than LCL (1.75 ± 0.28 months), but less than DCL (52.00 ± 10.58 months) (Fig. 2). The PCR characterisation of the parasites showed that 80.0% of ICL patients were infected with *Leishmania (Viannia) braziliensis* and 20.0% with *Leishmania (Leishmania) mexicana* (14).

Epidermal CD1a+ Langerhans cells were reduced ($P \leq 0.05$) in both ICL (271 ± 15 cells/mm^2) and DCL (245 ± 19 cells/mm^2) as compared to

Table 1 Density of leukocyte immunophenotypes phenotypes in localised, atypical chronic and diffuse cutaneous leishmaniasis

	Localised cutaneous leishmaniasis n = 19	Intermeiate cutaneous leishmaniasis n = 18	Diffuse cutaneous leishmaniasis n = 4	$P \leq 0.05$
Epidermis				
CD1a	527 ± 54 *	271 ± 15	245 ± 19	LCL-ICL LCL-DCL
CD83	349 ± 64	55 ± 10	25 ± 7	LCL-ICL LCL-DCL
Dermis				
CD4	1536 ± 232 * (38.40)	1468 ± 38 (36.70)	990 ± 45 (24.75)	LCL-DCL ICL-DCL
CD8	1575 ± 196 * (39.38)	1394 ± 37 (34.85)	725 ± 41 (18.13)	LCL-DCL ICL-DCL
CLA	458 ± 34 (11.45)	363 ± 22 (9.07)	91 ± 21 (2.27)	LCL-ICL-DCL
CD69	537 ± 80 (13.42)	1018 ± 37 (25.45)	523 ± 28 (13.07)	LCL-ICL ICL-DCL
CD83	1082 ± 98 ** (27.05)	213 ± 18 (5.33)	170 ± 24 (4.25)	LCL-ICL LCL-DCL

Values are expressed as cells/mm^2 (mean) ± SEM (respectively,% of the designated cells ± SEM).
SEM = standard error of the mean
CLA = cutaneous lymphocyte antigen
* From Martínez-Arends *et al.* 1991
** From Tapia et al. 1994

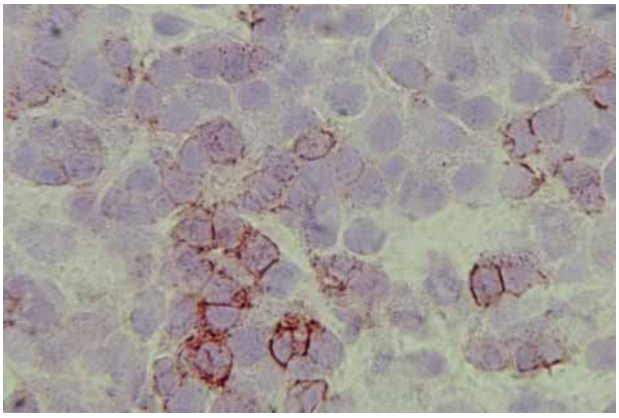

Figure 3. Activated CD69 + cells in a granuloma of intermediate cutaneous leishmaniasis. Avidin-biotin immunoperoxidase. Original magnification 800×.

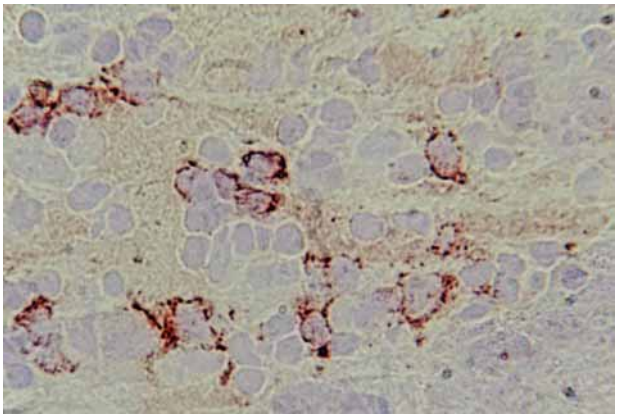

Figure 4. Cutaneous lymphocyte antigen (CLA) in a granuloma of intermediate cutaneous leishmaniasis. Avidin-biotin immunoperoxidase. Original magnification 800×.

LCL (527 ± 54 cells/mm^2). A similar tendency was observed for the CD83+ cells, a subgroup of activated also termed mature Langerhans cells (Table 1). The percentages of CD83+ cells in the CD1a + population were 20.30 for ICL, 66.22 for LCL and 10.20 for DCL.

The density of CD4+ T helper-inducer cells and CD8+ T suppressor-cytotoxic T cells in ICL lesions were similar to those found in LCL lesions, but significantly different ($P \leq 0.05$) from DCL lesions (Table 1). The CD4/CD8 ratio was 1.05 for ICL, 0.98 for LCL and 1.39 for DCL. In addition, ICL lesions presented significantly higher numbers of CD69+ activated T cells (1018 ± 37 cells/mm^2) than LCL (537 ± 80 cells/mm^2) and DCL (523 ± 28 cells/mm^2) lesions (Table 1, Fig. 3). The numbers of CLA+ skin-specific infiltrating cells were 363 ± 22 cells/mm^2 for ICL, 458 ± 34 cells/mm^2 for LCL and 91 ± 21 cells/mm^2 for DCL (Table 1, Fig. 4). In consequence, the percentages of CLA+ cells from the CD69+ population were 35.66, 85.29 and 17.40 for ICL, LCL and DCL, respectively. Similarly, the percentages of CLA+ cells from the CD83+ infiltrating population, were 42.33 for LCL and 53.53 for DCL, whereas for ICL CLA+ cells outnumbered CD83+ cells (Table 1).

The cytokine immunolocalisation of IFN-γ, IL-4, IL-10 and TGF-β1 in the lesions of LCL and ICL, showed a preponderance of IFN-γ+ cells over IL-4, and low density of TGF-β1 and IL-10+ cells, in LCL. In contrast, ICL granulomas showed fewer IFN-γ+ cells and more IL-10+ and TGF-β1+ positive cells (Table 2, Figs 5, 6 and 7). The density of IL-4+ cells was very similar in LCL and ICL (Table 2). In addition, the numbers of IL-10+ epidermal Langerhans cells were similar for ICL and LCL (Table 2), but when calculated from the total CD1a count gave the percentages of 33.69 for ICL and 17.45 for LCL. IFN-γ and IL-4 positive cells were mainly localised in the periphery, and TGF-β1 in the mantle of the granuloma. Many cytokine-producing cells were observed in the

Table 2 Density of cytokine-producing cells in the granulomas of localised and intermediate cutaneous leishmaniasis

	Localised cutaneous leishmaniasis $n = 6$	Intermediate cutaneous leishmaniasis $n = 6$	$P \leq 0.005$
IFN-γ	2063 ± 195 (51.57)	1073 ± 82 (26.82)	LCL-ICL
IL-4	1556 ± 199 (38.90)	1713 ± 107 (42.82)	ns
TGF-β1	794 ± 96 (19.85)	1560 ± 117 (39.00)	LCL-ICL
IL-10	969 ± 63 (24.22)	1750 ± 100 (43.75)	LCL-ICL
IL-10-Langerhans cells	92 ± 18 (17.45)	125 ± 17 (33.69)	ns

Values are expressed as cells/mm^2 (mean) ± SEM (respectively,% of the designated cells ± SEM).
Percentage of IL-10 + epidermal Langerhans cells were calculated taking the numbers of CD1a + Langerhans, for each clinical form, as 100%.
ns = not statistically different
SEM = standard error of the mean

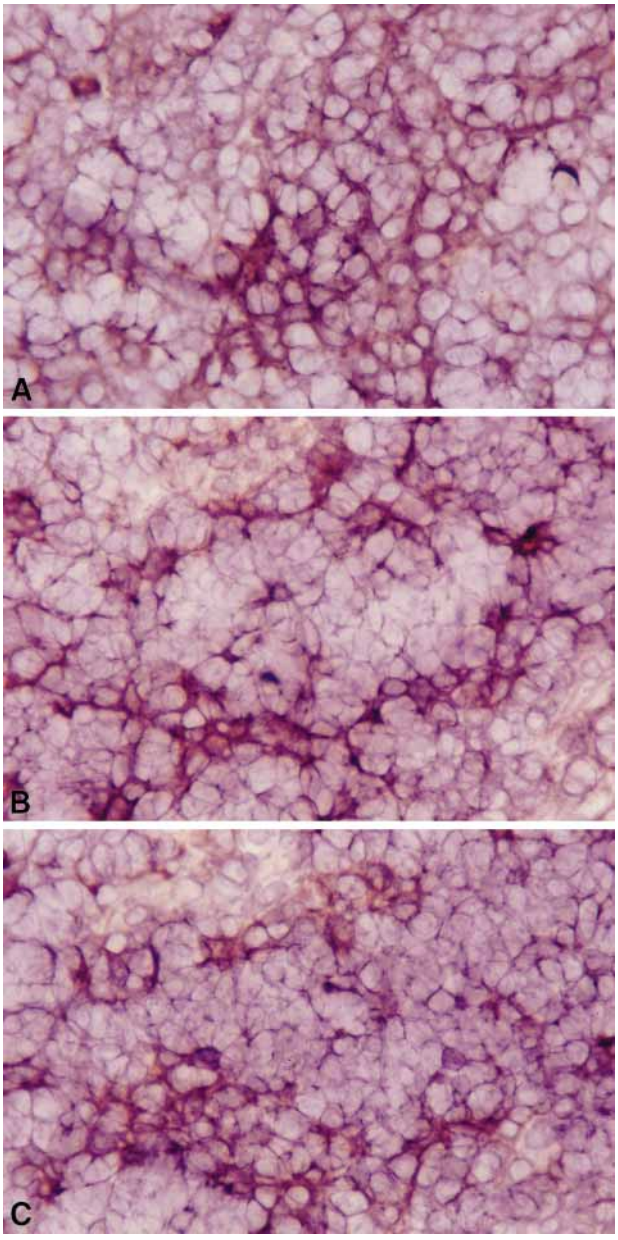

Figure 5. Cytokine expression, demonstrating numerous specifically stained cells, in lesions of intermediate cutaneous leishmaniasis. A. IFN-γ, B. IL-4, C. TGF-β1. Avidin-biotin immunoperoxidase. Original magnification 500×.

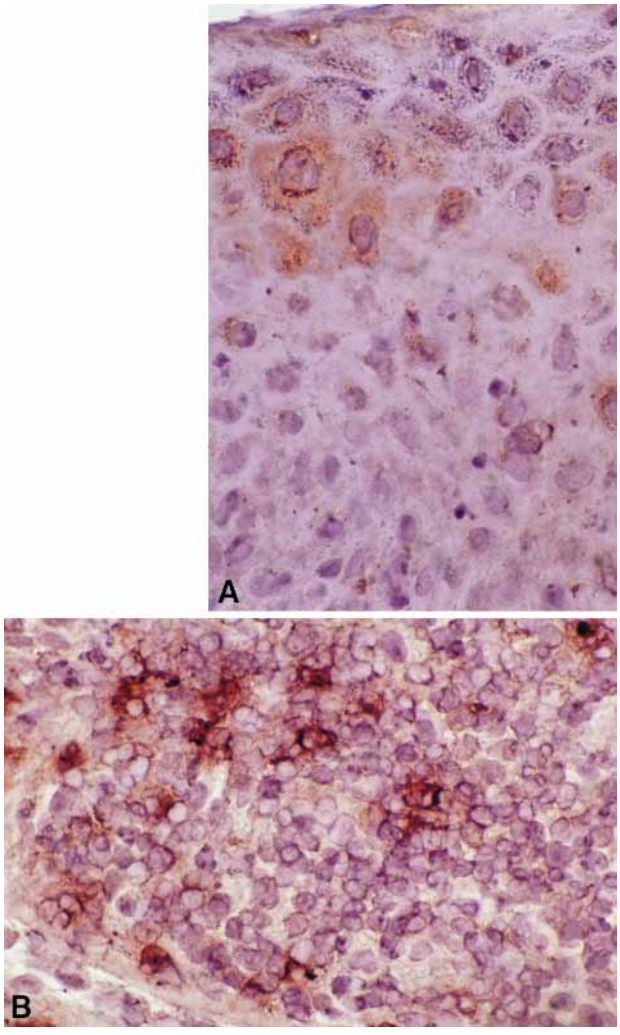

Figure 6. IL-10 + cells in a lesion of intermediate cutaneous leishmaniasis. A. Epidermis, B. Granuloma. Avidin-biotin immunoperoxidase. Original magnification 500×.

stroma surrounding the granuloma (Fig. 7). The analysis of the cellular percentages suggests that multiple Th1 and Th2 cytokines can be produced simultaneously in an individual T cell.

Discussion

The middle portion of the American tegumentary leishmaniasis spectrum, which comprises MCL and ICL, is characterised by vigorous cellular immune responses against *Leishmania* antigens, tendency to relapse and resistance to antileishmanial treatment that usually required combination of pentavalent antimonials and immunotherapy (5, 14, 15). In previous studies, we identified inadequate epidermal homing that leads to a proinflammatory state and subsequent tissue damage in patients with MCL (6–8). In the present study, we analysed a group of patients with ICL characterised by chronic and aberrant lesions, strong leishmanin skin test reactions, no mucosal involvement and long course of infection. These patients presented large and diverse clinical manifestations and a prolonged time span of their illness, as previously described (5, 6). No differences from LCL patients were observed on the size of the leishmanin skin test reaction.

In ICL epidermis, the reduced number of

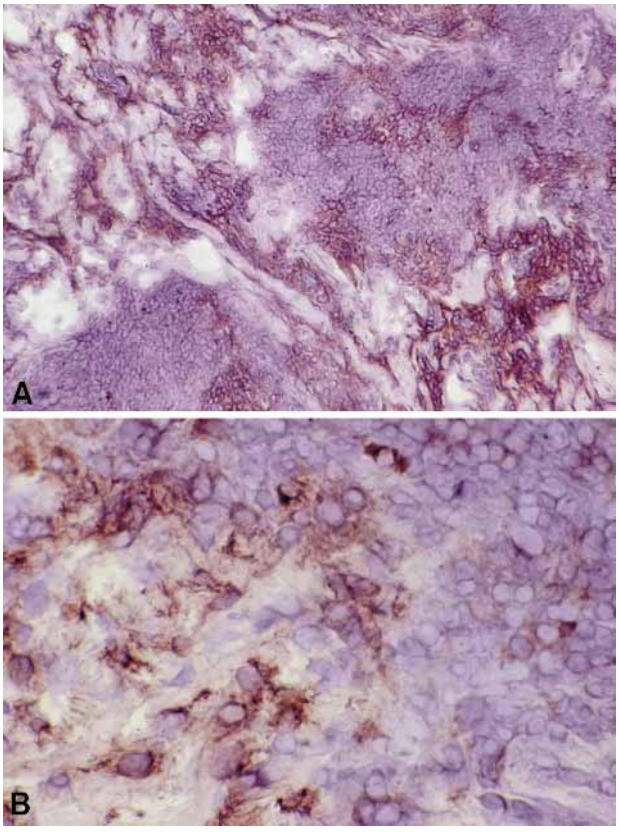

Figure 7. IFN-γ + cells in a lesion of intermediate cutaneous leishmaniasis. Notice positive cells in the connective tissue surrounding the granuloma and in the mantle of the granuloma. Avidin-biotin immunoperoxidase. A. Original magnification 100×, B. Original magnification 500×.

CD1a+ Langerhans cells, with the high proportion of IL-10+ and low numbers of CD83+ Langerhans cells, may provide inadequate epidermal accessory signals, which generate an ineffective immune response against the *Leishmania* parasite. The low density of CD1a+ Langerhans cells in the epidermis of ICL lesions may reflect the selective migration of antigen-primed Langerhans cells from the epidermis to regional lymph nodes, as previously suggested for MCL patients (13). Moreover, we have previously shown that the numbers of primed CD83+ Langerhans cells are important in the development of an appropriate Th1 response in LCL patients (6, 7). Recently, it has been shown that CD83+ Langerhans cells, which are usually in close contact with CD4+ T cells (16), are essential for the recruitment and survival of antigen-specific T lymphocytes (17). Together, the results emphasize the important role of dendritic cells in *Leishmania* infection.

CLA identified skin homing memory T cells (18, 19). The higher proportion of these cells in LCL and ICL over DCL, may be associated to effector capabilities of each clinical form. Thus, when compared with the number of early activated CD69+ T cells, we found that LCL lesions have about half the numbers of CD69+ cells as ICL, but most are CLA+ cells, whereas ICL lesions have the highest number of early activated CD69+ T cells, but about one-third of these cells expressed CLA. This suggests that the granuloma of ICL patients contain many activated T cells that are unprimed to cutaneous-launched antigens, thus contributing to an aberrant immune response. In contrast, DCL granulomas presented the lowest numbers of activated CD69+ and CLA+ cells, associated with the characteristic tolerogenic state of these patients (5, 6).

The present study confirmed our previous results regarding the lymphocyte expression of CD83 in LCL and DCL granulomas (7), and showed that as in MCL, these cells are also decreased in ICL. However, LCL lesions, contrasting with ICL and DCL, presented more CD83+ than CD69+ infiltrating cells. This may be due to the recruitment of antigen-specific Langerhans cells that migrate in association with T lymphocytes (16, 20, 21). Although the expression of CD83 by activated B and T lymphocytes is not yet understood, our observations, and the fact that CD83 values are similar to those showed for CD18 (LFA-1β) (7), suggest an association of this molecule with antigen-primed cells.

In this study, we standardized an immunocytochemical procedure for the *in situ* detection of IFN-γ, IL-4, IL-10 and TGF-β1. The results confirmed a Th1 type response in LCL lesions (22–24), with a predominance of IFN-γ+ cells, considerable numbers of IL-4+ cells and fewer TGF-β+ cells. In ICL, as in MCL lesions (22, 23), we found a mixed cytokine pattern with predominance of IL-10 and TGF-β1, a negligible increase of IL-4 and a significant decrease of IFN-γ+ cells as compared to LCL. Our results confirm recent evidence that part of the infiltrating cells may produce more than one cytokine simultaneously (25). An important observation was the demonstration of several cytokine-producing cells in the stroma surrounding the granuloma, suggesting those arriving T cells are rich in cytokine content. These observations show the advantage of using an immunocytochemical technique over a polymerase chain reaction on denatured tissue.

The present investigation further emphasizes the concept of the epidermis as an important homing site for primed T cells. In this system, Langerhans cells and keratinocytes provide accessory signals that may govern the effector phase of the immune response. Failures at this site will cause a defective immune response that is unable to eliminate the

pathogen, or produce tissue damage. In MCL, mucosal lesions frequently appear years after primary cutaneous manifestations, possibly emerging after trauma (Koëbner phenomenon) or recurrent bites of sandflies (infected or not), characterised by scarcity of parasites and a proinflammatory (Th1 + Th2) response, which causes the characteristic tissue damage (6, 7). Here, we showed a diminished participation of epidermal Langerhans cells in ICL lesions that may account for the tissue-damaging state observed in these patients. The hyper-reactive granuloma containing many activated but unprimed T cells that produce a mixed cytokine pattern may be the consequence of defective signalling from the epidermis.

Acknowledgements

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