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Immunocytochemical characterization of immune cells in lesions of American cutaneous leishmaniasis using novel T cell markers

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Some recently defined lymphocyte immunophenotypes were determined in lesions of patients with American cutaneous leishmaniasis (ACL). New monoclonal antibodies have allowed the demonstration of cell surface antigens of T lymphocytes, such as CD45RA and CD45RO, which recognize different maturational stages of the same T CD4+ cell subgroup: 'virgin' (CD4+CD45RA+) and 'memory' (CD4+CD45RO+) T cells respectively. The CD4/CD8 cell ratios were higher in mucocutaneous leishmaniasis (MCL) than in localized cutaneous leishmaniasis (LCL) lesions. Diffuse cutaneous leishmaniasis (DCL) has the highest values of 'virgin' T cells; LCL and MCL patients have lower values, similar to each other. 'Memory' T cells were higher in MCL than in LCL or DCL. The ratio of 'memory'/'virgin' T cells was 7.9 for LCL, 9.6 for MCL and 2.5 for DCL. The highest value for IL-2 receptor positive cells (CD25) was observed in LCL, whereas single CD45RO-immunoreactive cells showed a peak value in DCL patients. HLA-DR+ cells were present in all three clinical forms of ACL. MCL patients showed a lack of epithelial Langerhans cell (CD1a+) in the nasal mucosa.

Key words: Avidin-biotin-immunoperoxidase; Granulomas; Leishmaniasis; T cell phenotypes

Introduction

American cutaneous leishmaniasis (ACL) is a chronic granulomatous disease with a spectrum of clinical manifestations, produced by intracellular parasites of the *Leishmania* genus. Localized cutaneous leishmaniasis (LCL), the most common form of the disease, has few organisms within well defined lesions, which generally heal after treatment, or spontaneously. In contrast, diffuse cutaneous leishmaniasis (DCL) occurs infrequently and the lesions, which are characterized by extensive involvement of the skin, naso-bucopharyngeal mucous tissue and some lymph nodes contain abundant parasites. DCL patients, tend to have relapses after treatment (Convit et al., 1972; Convit, 1974). A small proportion of ACL patients develop mucocutaneous leishmaniasis (MCL) characterized by destructive lesions of the oral and nasopharyngeal cavities. This form does not respond well to chemotherapy and there is a high rate of relapse (Convit and Pinardi, 1974).

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The leishmanial antigen-specific cell-mediated immune response is depressed in DCL (Convit et al., 1972; Convit and Pinardi, 1974; Castés et al., 1983, 1984), and usually enhanced in MCL (Castés et al., 1983, 1988). Castés et al. (1988) have demonstrated that T lymphocytes from patients with DCL fail to express the IL-2 receptor and do not produce INF-τ when exposed to *Leishmania* antigen. In contrast, LCL and MCL patients have a significant increase in IL-2 receptor expression and IFN-τ production after exposure to specific antigens.

Within ACL lesions, Modlin et al. (1985) have shown that cells expressing the IL-2 receptor (Tac positive) are present in approximately equal numbers in LCL and DCL. Differences were only observed in the number of IL-2 positive (IL-2 producing) cells, which were significantly decreased in DCL. These results further confirm the unresponsiveness of T cells from DCL patients to leishmanial antigens.

Morimoto et al. (1985a,b) have associated suppressor-inducer and helper-inducer activities with the expression on T cells of the CD45RA and CD29 molecules, respectively. However, this concept of two functional subpopulations within the CD4+ subset has recently been reinterpreted, since apparently the markers recognize different maturational stages of the same cell subset (Sanders et al., 1988; Serra et al., 1988). The CD4+CD45RA+ cells previously known as T suppressor-inducer cells, are now referred to as 'virgin' or 'naive' T cells, whereas the CD4+CD29+ cells previously described as T helper-inducer cells are designated primed or 'memory' T cells.

A related antigen CD45RO (UCHL1) (Smith et al., 1986; Akbar et al., 1988) apparently recognizes the same cell subset as CD29, thus identifying 'memory' T cells.

This study explores the relationships between cell types in the different forms of ACL, using a series of recently characterized monoclonal antibodies, and immunoperoxidase techniques.

Materials and Methods

Patients

Patients with LCL ($n=11$), MCL ($n=6$) and the rare DCL form ($n=2$) were studied in the Instituto de Biomedicina. The patients were diagnosed by established clinical, epidemiological and histopathological criteria (Convit, 1974). 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 units of penicillin/ml, inoculation of hamsters with biopsy macerate, and histopathological examination of Giemsa and hematoxylin-eosin stained sections. LCL patients had less than 1 year of evolution. The patients were not under treatment at the time of study.

Skin biopsy specimens were embedded in OCT compound (Miles Scientific, U.S.A.), 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.

Monoclonal antibodies

All monoclonal antibodies used were diluted in a modified phosphate-buffered saline (PBS), pH 7.2. (Hofman et al., 1982). These antibodies recognized the following mononuclear cell markers: CD4 (HP 2.6, T helper/inducer at 1:2500) kindly donated by Dr. A. Moretta (Ludwig Institute, Lausanne, Switzerland); CD8 (B116.1, T suppressor/cytotoxic at 1:2500) and Ia (B 33.1.1, HLA-DR-MHC class II antigens at 1:500) kindly donated by Dr. G. Trichieri (The Wistar Institute, Philadelphia, U.S.A.); CD1a (Langerhans cells at 1:100) Becton Dickinson, Inc, Mountain View, U.S.A.; Tac (CD25) (anti-IL-2 receptor at 1:1500) kindly provided by Dr. T.A. Waldmann (N.C.I., NIH, Bethesda, U.S.A.); CD45RA (3AC5, T cell sub-groups at 1:100) provided by Dr. J. Ledbetter (Oncogene Corp., Seattle, U.S.A.); and CD45RO (UCHL1, T cell subgroups as undiluted supernatant) provided by Dr. P.C.L. Beverly (I.C.R.F. Human Tumour Immunology Group, London, U.K.).

Immunoperoxidase staining

Single and double immunoperoxidase staining was performed. Single immunostaining was carried out as previously described (Gross et al., 1988; Tapia et al., 1988). Briefly, the samples were hydrated in PBS and sequentially incubated for 30 min with primary mouse monoclonal antibody, biotinylated goat anti-mouse IgG at 1:150 (BRL, Gaithersburg, U.S.A.) for 15 min, and streptavidin-horseradish peroxidase conjugate (BRL, U.S.A.) at 1:300, 30 min. 5-min washes with PBS were performed between incubations. 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*-dimethylformamide in 0.1 M acetate buffer, pH 5.2. The sections were then washed and counterstained with Mayer's hematoxylin. Controls consisted of omission of the primary antibody or the use of an antibody of irrelevant specificity at the same protein concentration.

The double-staining procedure was carried out using two consecutive immunoperoxidase techniques. In the first staining sequence, the primary monoclonal antibody was an anti-CD4 and the development was carried out using 3,3-diaminobenzidine (DAB) intensified with $CuSO_4$ (Hsu and Soban, 1982), which produced a dark brown colour. In the second staining sequence, the primary monoclonal antibody was an anti-CD45RA, developed with AEC, which produced a red product. Double-stained cells were identified when a combination of brown and red colours could be seen on the same cell. Controls were incorporated to demonstrate that the second labelling system did not cross-react with antibodies already added as part of the first sequence. The antibodies used in the double staining procedures were diluted as for single staining procedures.

'Memory' T cells were determined by substracting the number of CD4+CD45RA+ cells from the total of CD4+ cells, based on a previous study which suggests that CD45RA and CD45RO (CD29) cells are mutually exclusive populations (Bos et al., 1989).

Leukocyte quantification

Cell counting was carried out using a light microscope with a millimetered scale (Carl Zeiss, F.R.G.), calibrated to determine the number of cells/mm^2 in epidermis

and dermal infiltrates. Only cells with a visible nucleus and showing red immunostaining for single staining and dark brown-reddish colour for double staining were counted as positive. In order to obtain a representative sample of the lesions, four non-serial alternate sections are immunostained and counted for each cell marker. All the fields of interest were counted in each section at a magnification of 1000×, giving $2–4 \times 10^4$ cells per section. Percentages of each phenotype were calculated. There are approximately 4000 cells/mm^2 of infiltrate, according to a previous count of the nucleated cells in a hematoxylin and eosin-stained section.

Statistical analy

All the information was expressed as mean ± standard error of the mean (SEM). The means were calculated on the basis of individual values for each patient. Comparison between groups was made with Student's t test for unpaired samples. Any P value less than 0.05 was considered significant. The degree of correlation between groups was calculated using Pearson's correlation method for LCL patients and Kendall's rank correlation method for MCL patients.

Results

The histological analysis of skin biopsies of the patient groups confirmed the clinical diagnosis of ACL.

The immunocytochemical study showed that different LCL patients have similar values for T helper-inducer CD4+ cells and T suppressor − cytotoxic CD8+ cells, with a CD4/CD8 ratio of 0.99 ± 0.08 (Table 1, Fig. 1). No particular topographical distribution in the lesions was observed for these two phenotypes. CD4+ CD45RA+ cells were, however, predominantly restricted to the mantle surrounding the granuloma. In the epidermis a few spherical lymphocyte-like cells expressing either CD4, CD45RA, Tac or CD45RO antigens were also observed. Statistical analysis showed a positive correlation between the numbers of T helper-inducer (CD4+) cells and T suppressor cytotoxic (CD8+) cells, as well as between T suppressor-cytotoxic (CD8+) and Tac+ cells ($p \leq 0.05$).

The CD4/CD8 ratio in MCL lesions was 1.24 ± 0.09, indicating a higher proportion of T helper-inducer (CD4+) cells than T suppressor-cytotoxic cells (Table 1, Fig. 1). A few CD45RO+ cells were observed in the mucosal epithelium. An interesting finding was the lack of epithelial Langerhans cells (CD1a+) in the nasal mucosa of these patients. A positive correlation was found between T suppressor-cytotoxic cells (CD8+) and Tac+ cells ($p \leq 0.05$).

DCL patients showed a CD4/CD8 ratio of 0.77 ± 0.04, indicating a higher proportion of T suppressor-cytotoxic cells (Table 1, Fig. 1). In some sections CD45RA+ cells were observed towards the basal membrane of epidermal papillae.

The comparison between LCL and MCL lesions showed higher values for the latter in relation to the number of CD45RA+ cells and the CD4/CD8 ratio (Table 1) ($p \leq 0.05$).

The highest value for CD25+ cells (668 ± 220 cells/mm^2) was observed in LCL patients, whereas CD45RO+ cells showed a maximal value (2156 ± 802 cells/mm^2)

TABLE 1

Mononuclear cell densities in the lesions of American cutaneous leishmaniasis

Phenotype	Localized cutaneous leishmaniasis	Muco-cutaneous leishmaniasis	Diffuse cutaneous leishmaniasis	Differences between LCL and MCL[a]
CD4	1536±232 (38.89±5.81)	1882±182 (47.05±4.56)	1472±507 (36.79±12.69)	NS
CD8	1575±196 (39.37±4.92)	1339±218 (33.48±5.46)	1875±556 (46.88±13.90)	NS
Tac (CD25)	668±220 (16.69±5.51)	244±51 (6.11±1.29)	391 (9.78)	NS
CD45RA (3AC5)	764±110 (19.11±2.77)	1367±205 (34.18±5.13)	1651±1078 (41.28±26.95)	$p \leq 0.05$
CD45RO (UCHL1)	1884±790 (47.10±19.75)	1636±354 (40.89±8.86)	2156±802 (53.90±20.05)	NS
CD4+CD45RA+	172± 57 (4.30±1.45)	177± 24 (4.43±0.62)	417 (10.43)	NS
CD4+CD45RA−	1364±452 (34.10)±11.30)	1705±231 (42.63±5.78)	1055 (26.38)	NS
CD1a epithelial	527±54	none	550±156	NS
CD4/CD8	0.99±0.08	1.24±0.09	0.77±0.04	$p \leq 0.05$

Values are expressed as cells/mm^2 (mean)±SEM (respectively, % of the designated cells±SEM).
NS = not statistically different.
SEM = standard error of the mean.
CD4+CD45RA− number is based on the substraction of CD4+CD45RA+ from CD4+.
[a]Group is not included in the statistical analysis because of the few patients studied.

in DCL patients. HLA-DR+ cells were almost universally present in all three clinical forms of ACL.

Double staining procedures allowed the identification of 'virgin' T cells (CD4+CD45RA+) (Fig. 2) and 'memory' T cells (CD4+CD45RA−). DCL patients had the highest values of 'virgin' T cells (417 cells/mm^2), whereas LCL patients (172±57 cells/mm^2) and MCL patients (177±24 cells/mm^2) had similar values (Fig. 3). Based on these values, 'memory' T cells were calculated by subtracting the number of cells stained by the two antibodies (CD4 and CD45RA) from the total CD4+ cell values. 'Memory' T cells (1705 cells/mm^2) were higher in MCL than in LCL (1364 cells/mm^2) and DCL (1055 cells/mm^2), however these differences were not statistically significant (Fig. 3). The ratio T 'memory'/T 'virgin' cells was higher in MCL lesions (9.6:1) than in LCL (7.9:1) with the lowest values in DCL patients (2.5:1) (Table 1). The comparison of this ratio between LCL and MCL was not statistically significant.

Discussion

In previous work (Modlin et al., 1985) we characterized the immunophenotypes present in the granulomas of LCL and DCL. The most outstanding finding was a

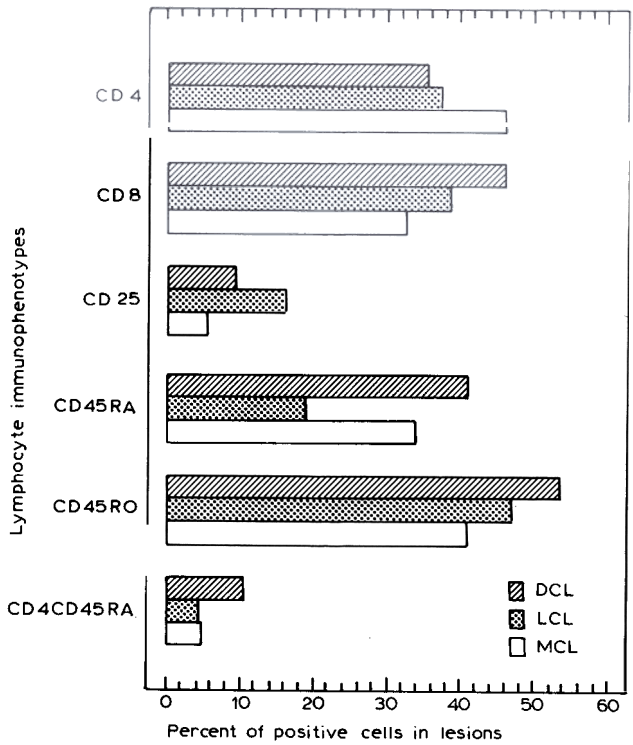

Fig. Percentages of the lymphocyte immunophenotypes in the lesions of localized cutaneous (LCL) muco-cutaneous (MCL) and diffuse cutaneous leishmaniasis (DCL).

marked decrease of the IL-2 positive cells in DCL and a lack of a particular microanatomic location of the immunocompetent cells in ACL lesions, that can, in contrast, be observed in tuberculoid leprosy and other skin disorders (Modlin et al., 1983, 1984, 1985; Gross et al., 1987; Barral et al., 1987).

Since the T helper-inducer CD4 positive cells are IL-2 producing cells, and recently this T cell subset has been divided into two subgroups depending on the expression of isoforms of the CD45R molecule, in the present study, we characterized the distribution of the so-called 'virgin' and 'memory' T cells, as well as other leukocyte immunophenotypes in the lesions of the different clinical forms of ACL. The results corroborated the absence of a particular topographical distribution for T suppressor-cytotoxic CD8+ and T helper-inducer CD4+ cells in LCL granulomas. However, it was found that CD4+CD45RA+ T cells have a tendency to localize in the periphery of the LCL granuloma. The presence of 'virgin' T cells in this type of lesion suggests that the granuloma may be a site of T cell education even though non-specific recruitment of lymphocytes may not be excluded. In addition, we found values lower than unity for the CD4/CD8 ratio in LCL lesions, confirming our previous results (Modlin et al., 1985), but contrasting with those reported for Brazilian ACL patients (Barral et al., 1987). This low ratio is the result of a higher number of T suppressor-cytotoxic CD8+ cells, which may be mainly T cytotoxic cells, controlling the size of the granuloma. This observation may also explain the

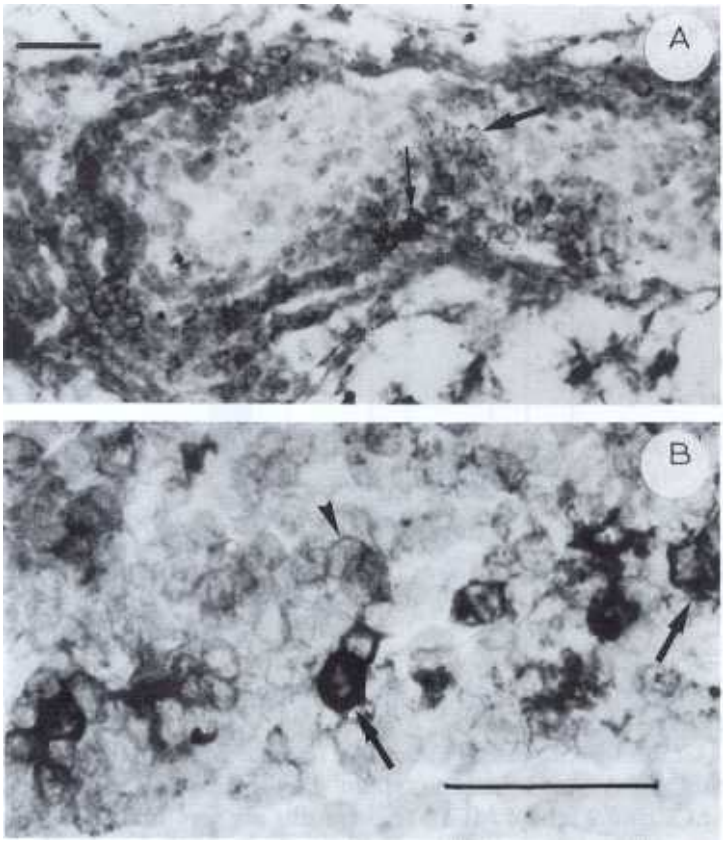

Fig. 2. Virgin T cells (CD4+ CD45RA+) in a granuloma of localized cutaneous leishmaniasis. Double-stained cells are darker (arrows) than CD4+ or CD45RA+ cell (arrow head). Hematoxylin counter-staining. (A) General view of granuloma; (B) Detail of A. Bar = 20 μm.

positive correlation found between the increase of IL-2 receptor positive cells and CD8+ cells in LCL lesions. Recent findings in experimental leishmaniasis have shown a participation of CD8+ cells in the protection against the parasite (Hill et al., 1989).

The high proportion of T helper-inducer CD4+ cells observed in MCL lesions may explain the decreased numbers of this cell group found in peripheral blood by other investigators (Carvalho et al., 1985; Castés et al., 1988) in MCL, T helper-inducer cells may be sequestered from blood to the inflammatory site.

The lack of CD1a+ Langerhans cells in the mucosal epithelium in MCL lesions may reflect the selective migration of antigen-primed Langerhans cells from the epithelium to regional lymph nodes, or may be the result of a direct cytolytic effect of the parasite on these cells. The latter possibility arises from the recent finding that Langerhans cells can be infected by *Leishmania* in vitro (Dompmartin et al., 1988). The absence of these cells has also been observed in mucosal viral lesions (Drijkoningen et al., 1988). The lack of epithelial Langerhans cells in MCL must be

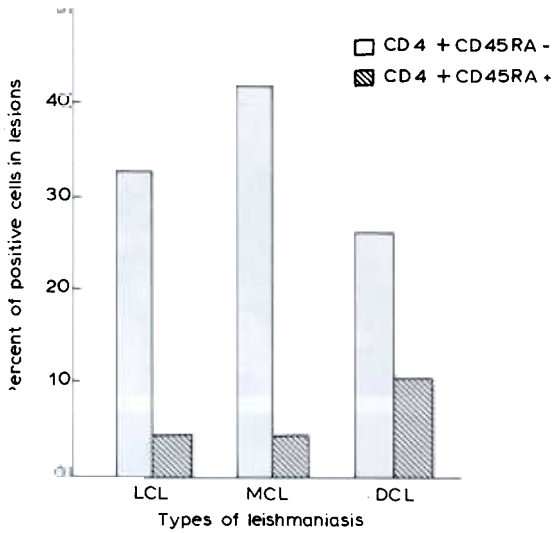

Fig. 3. Percentages of CD4+ CD45RA+ and CD4+ CD45RA− cells in the lesions of localized cutaneous (LCL), muco-cutaneous (MCL) and diffuse cutaneous (DCL) leishmaniasis.

confirmed by ultrastructural analysis, since damage to the CD1a marker could also explain our observations.

In both LCL and MCL cases of ACL we found a predominance of 'memory' T cells CD4+CD45RA− compared to 'virgin' T cells CD4+CD45RA+, with ratios of 7.9:1 for LCL and 9.6:1 for MCL. These results are very similar to those reported by Pirmez et al. (1988) for a smaller number of cases. The numbers of 'virgin' T cells CD4+ CD45RA+ observed in LCL and MCL lesions in the present study are very similar to those reported for normal skin (Bos et al., 1987). However, the number of 'virgin' T cells in DCL are twice those of normal skin.

The analysis of DCL patients is complicated by the small number of untreated individuals available. However, the results demonstrated a higher proportion of T cells with the CD45RO+, CD45RA+, and CD4+CD45RA+ phenotypes, than those observed in LCL and MCL lesions. Similarly, in DCL granulomas the T 'memory'/T 'virgin' cell ratio was 2.5:1, indicating an increase of 'virgin' T cells CD4+CD45RA+ and low numbers of 'memory' T cells CD4+CD45RA−. These results contrast with those observed in LCL and MCL lesions and indicate alterations in the numbers of T cells which are antigen-primed.

The contrasting results in the ratio of T 'memory/T 'virgin' cells between the two polar forms of ACL are very similar to those observed between tuberculoid and lepromatous leprosy by Modlin et al. (1988). They reported significant differences between peripheral blood and the lesions, as determined by the ratio of antigen-reactive cells.

Future studies will include analysis of the ACL lesions after immunotherapeutic treatment of ACL patients with a mixture of heat-killed *Leishmania* and BCG (*Bacillus Calmette-Guerin*), as developed by Convit et al. (1987). These studies will provide insight into the immunocompetent cells required for overcoming unresponsiveness and activating antigen-presenting cells to eliminate the *Leishmania* parasites.

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