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Adhesion molecules in lesions of American cutaneous leishmaniasis

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Abstract: Accessory signals, which include adhesion molecules, MHC-II molecules and cytokines, are necessary to foster the interaction between memory T cells and epidermal cells, that is required to promote cutaneous inflammatory responses. American cutaneous leishmaniasis (ACL) is characterized by a spectrum of immunological manifestations, and is a prototype disease for the study of regulatory mechanisms involved in immune protection against protozoal infection. In the present study, we show that diffuse cutaneous leishmaniasis (DCL) epidermis contains keratinocytes that do not express ICAM-1 and HLA-DR molecules. Langerhans cells (LC) are within normal values or somewhat lower, and a very few cells expressing the HB15 molecule – a new described member of the Ig superfamily – are found in such lesions. Mucocutaneous leishmaniasis (MCL) epithelium shows an increased expression of ICAM-1 and HLA-DR molecules, few HB15$^+$ cells, and an absence of epithelial LC. Localized cutaneous leishmaniasis (LCL) epidermis displays ICAM-1$^+$ keratinocytes organized in patches, a uniform expression of HLA-DR, hyperplasia of LC, and numerous HB15$^+$ cells. In all forms of the disease, infiltrating T cells express more LFA-1β than LFA-1α, but LFA-1β$^+$ T cells are more abundant in LCL granulomas. In contrast, there are more LFA-1α$^+$ T cells in DCL and MCL than in LCL granulomas. LCL lesions also show the highest numbers of HB15$^+$ cells within the granuloma. These results indicate the importance of adhesion molecules in ACL lesions, and open new possibilities for therapeutic schemes oriented towards the control of cell migration.

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Introduction

Leishmaniasisis is a disease caused by flagellated protozoa for the genus *Leishmania,* and is characterized by a number of 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. The disease spectrum of American cutaneous leishmaniasis (ACL) includes immune-responder individuals with the localized cutaneous form (LCL) and non-responder individuals with disseminated or diffuse cutaneous leishmaniasis (DCL) (1, 2). Mucocutaneous leishmaniasis (MCL) is a distinct clinical form characterized by exacerbated cell-mediated immunity and destructive lesions of the oral and nasopharyngeal cavities (3). Parasite species also may be associated with the clinical spectrum, since parasites from the *L. mexicana* and *L. brasiliensis* complexes have exclusively been found in DCL and MCL lesions, respectively (4).

In DCL lesions, we have shown marked decreases of IL-2-positive (IL-2 producing) cells, cells expressing the IL-2 receptor, and memory T cells (5, 6). In addition, we have recently shown that Type 1 cytokines predominate in LCL lesions and Type 2 cytokines in DCL lesions, whereas a mixed cytokine pattern is found in MCL lesions (7). These results confirmed the previously described unresponsiveness of T cells from DCL patients to leishmanial antigens (1, 3, 8, 9).

Lymphocyte migration is regulated by adhesive interactions between T cells and endothelial cells

lining blood vessel walls. These interactions are controlled by adhesion molecules that fall into four main groups: intergrins, the immunoglobulin supergene family, selectins and the Hermes (CD44) group (10). The intergrin leukocyte function-associated antigen 1 (LFA-1) plays an important role in T-cell adhesion to activated endothelial cells expressing the intercellular adhesion molecule 1 (ICAM-1) or ICAM-2 (11–13). LFA-1 is expressed by all T lymphocytes, but at one- to two-fold higher levels by memory $CD4^+$ T cells, compared to naive $CD4^+$ T cells (14). Besides cellular adhesiveness, the interaction between LFA-1 and ICAM-1 provides co-stimulatory signals for T-cell activation (15). Recently, a new member of the Ig superfamily, the HB15 molecule, was characterized on the surface of dendritic cells and activated lymphocytes, suggesting a role in antigen presentation or cellular interactions that follow lymphocyte activation (16).

In the present study we explored the distribution of some adhesion molecules in lesions of the different clinical forms of ACL, using specific monoclonal antibodies, and immunoperoxidase techniques.

Material and methods

Patients

Patients with LCL (n = 19), MCL (n = 14) or DCL (n = 16) forms were studied in the Instituto de Biomedicina. The patients were diagnosed using established clinical, epidemiological and histopathological criteria (2). Parasitological confirmation of the clinical diagnosis was based on Giemsa or hematoxylin-eosin staining of smears from biopsies, culture of minced biopsy material on blood agar slants containing 15% defibrinated rabbit blood and 200 units of penicillin/ml, and the inoculation of hamsters with biopsy macerate, 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, 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 immunostaining.

Monoclonal antibodies

All monoclonal antibodies used were diluted in a modified phosphate-buffered saline (PBS), pH 7.2 (17). These antibodies recognized the following mononuclear cell markers: CD11a (TS1/22, subunit α of LFA-1, LFA-α, diluted at 1:2000), CD18 (TS1/18, subinit β of LFA-1, LFA-1β, diluted at 1:2000), and CD54 (RR1/1, ICAM-1 diluted at 1:3000), and were donated by Dr. T. A. Springer (Harvard Medical School, Boston, U.S.A.); HB15 (diluted at 1:1500) was donated by Dr. T. F. Tedder (Dana-Faber Cancer Institute, Boston, U.S.A.); CD1a (Leu-6, diluted 1:100) was purchased from Becton Dickenson, Inc. (Mountain View, U.S.A.); and HLA-DR (B33.1.1, diluted at 1:500) was donated by Dr. G. Trinchieri (The Wistar Institute, Philadelphia, U.S.A.).

Immunoperoxidase staining

The immunostaining procedure was carried out as previously described (18, 19). Briefly, the samples were hydrated in PBS and sequentially incubated for 30 min with primary mouse monoclonal antibody, biotinylated horse anti-mouse IgG at 50 μg/μl (BRL, Gaithersburg, U.S.A.) for 15 min, and streptavidin-horseradish peroxidase conjugate (BRL, U.A.S.) at 1:300, for 30 min. Five-minute washes with PBS were performed between each incubation. The reactions were developed for 10 min with 90 μM H_2O_2 and 3-amino-9-ethylcarbazole (AEC) at a final concentration of 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 and the use of an antibody of irrelevant specificity at the same concentration.

Leukocyte counting

Cell counting was carried out using a light microscope with a millimetered scale (Carl Zeiss, Germany), calibrated to determine the number of cells/mm^2 in epidermis and dermal granulomas. Only cells with a visible nucleus and showing red immunostaining were counted as positive. To obtain 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 $2\text{-}4 \times 10^4$ cells per section. The percentages of each phenotype were calculated. There are approximately 4500 cells/mm^2 of infiltrate, according to a previous count of the nucleated cells in a hematoxylin and eosin-stained section.

Statistical analysis

The cell counts were presented as means ± standard errors of the mean (SEM). The means were calculated using the values obtained for each individual patient. Comparisons between groups were made

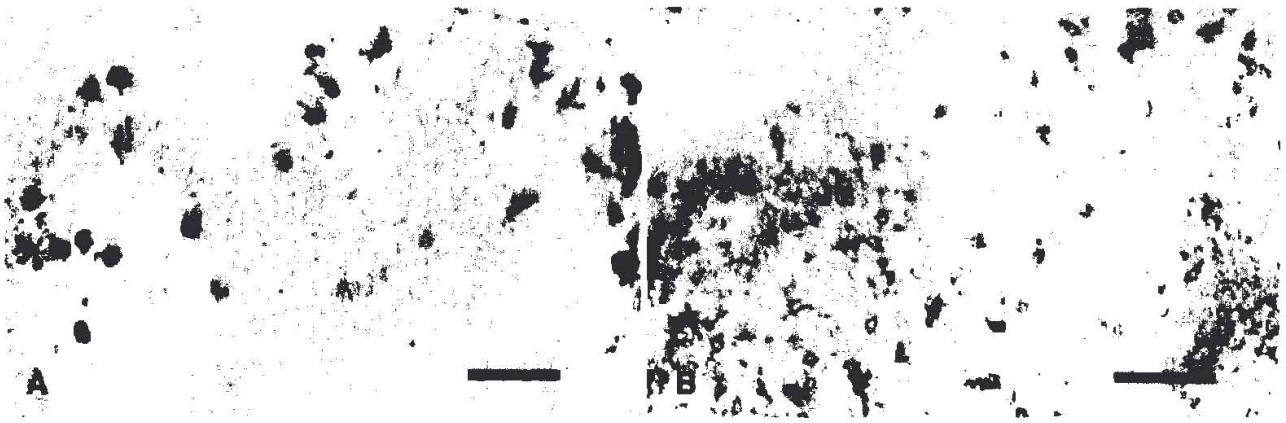

Figure 1. HB15 immunostaining in lesions of localized cutaneous leishmaniasis. Avidin-biotin immunoperoxidase. A. Immunoreactive cells in the epidermis and near the basal membrane; B. $HB15^+$ cells in a dermal granuloma. Bars = 20 μm.

with the nonparametric Mann-Whitney test and Student's t test for unpaired samples. p values less than 0.05 were considered significant.

Results

In situ *characterization of adhesion molecures in the epidermis of American cutaneous leishmaniasis*

We evaluated the presence of HLA-DR, ICAM-1, HB15 and CD1a molecules on epidermal cells. LCL epidermis was characterized by a uniform expression of HLA-DR, ICAM-1^+ keratinocytes distributed in patches, and high numbers of $CD1a^+$ Langerhans cells (854 ± 130 cells/mm^2) and $HB15^+$ cells (349 ± 64 cells/mm^2) (Figs. 1 and 2, Table 1). In contrast, DCL epidermis showed significantly fewer ($p \leq 0.05$) $CD1a^+$ Langerhans cells (580 ± 22 cells/mm^2) and $HB15^+$ cells (79 ± 15 cells/mm^2), and the keratinocytes failed to express HLA-DR or ICAM-1 molecules (Fig. 2, Table 1). The epithelium in MCL lesions lacked $CD1a^+$ cells, showed low numbers of $HB15^+$ cells, but displayed a widespread distribution of HLA-DR and ICAM-1 (Table 1). LCL epidermis also showed many spherical and dendritic LFA-$1\beta^+$ cells (Fig. 3).

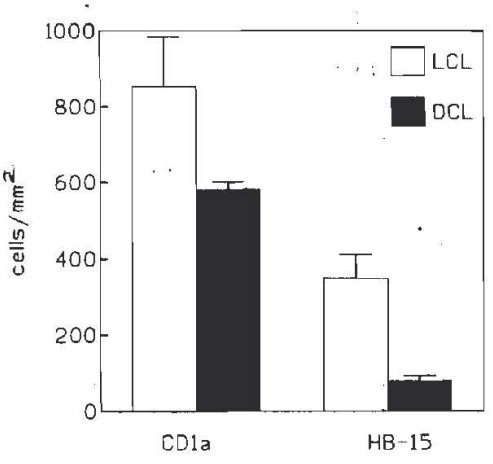

Figure 2. Density of epidermal cells expressing the CD1a and HB15 molecules in localized (LCL) and diffuse cutaneous leishmaniasis (DCL). Values are expressed as mean ± SEM cells / mm^2 of epidermis.

In situ *characterization of adhesion molecules in granulomas of American cutaneous leishmaniasis*

In the dermis, the α and β subunits of LFA-1 were characterized with monoclonal antibodies against the CD11a and CD18 molecules respectively. Infiltrating T cells expressed more LFA-1β than LFA-1α in all ACL lesions (Figs. 3 and 4, Table 1). The

Table 1. Numbers of cells immunoreactive for adhesion molecules in the lesions of American cutaneous leishmaniasis

Adhesion molecule	Localized cutaneous leishmaniasis n=19	Muco-cutaneous leishmaniasis n=14	Diffuse cutaneous leishmaniasis n=16	$p \leq 0.05$
Epidermis:				
CD1a	854±130	none	580±22	LCL-DCL
HB-15	349±64	37–73	79±15	LCL-DCL
HLA-DR	uniform	uniform	366±25 (only LC)	
ICAM-1	patches KC	uniform	none	
Dermis:				
LFA-1α	740±132 (16.44±2.93)	1927±240 (42.82±5.33)	1470±164 (32.66±3.64)	LCL-DCL LCL-MCL
LFA-1β	4229±198 (94.00±4.40)	3244±185 (72.08±4.11)	3444±185 (76.53±4.11)	LCL-DCL LCL-MCL
HB-15	1082±98 (24.04±2.17)	503±59 (11.17±1.31)	539±42 (11.97±0.93)	LCL-DCL LCL-MCL

Values are expressed as the mean±SEM cells/mm^2, except for HB15 in mucocutaneous leishmaniasis expressed as numerical range.
In the dermis, the values in parenthesis are the mean±SEM percentage of the designated cells, with respect to the total cell infiltrate.
LC=Langerhans cells.
KC=keratinocytes.

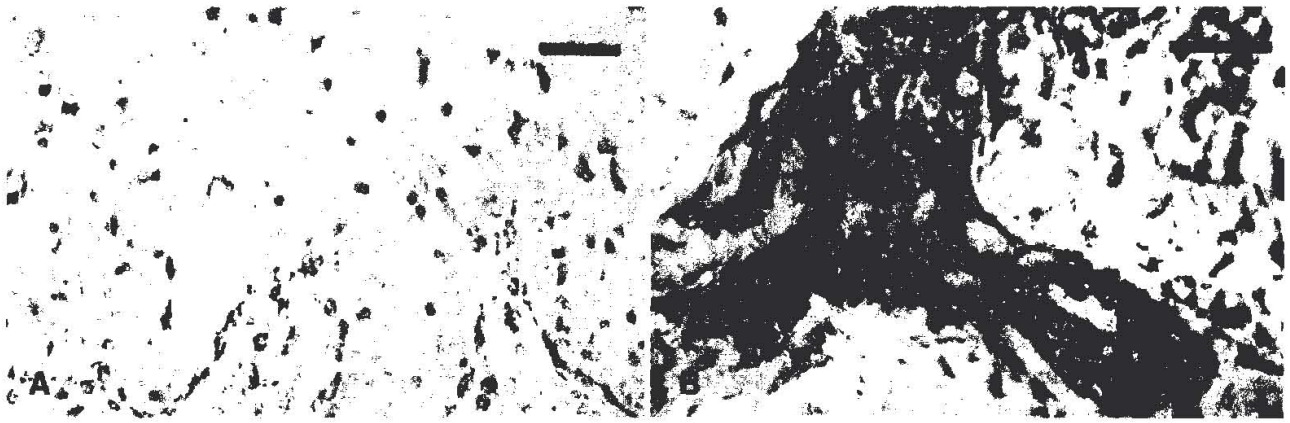

Figure 3. Immunocytochemical analysis of LFA-1β expression in lesions of localized cutaneous leishmaniasis. Avidin-biotin immunoperoxidase. A. Spherical and dendritic cells in the epidermis and basal membrane; B. Abundant LFA-1β$^+$ cells in a dermal granuloma. Bars = 20 μm.

number of LFA-1β$^+$ T cells was significantly higher in LCL lesions (4229 ± 198 cells/mm^2) than in DCL (3444 ± 185 cells/mm^2; $p \leq 0.05$) and MCL (3244 ± 185; $p \leq 0.05$) lesions (Fig. 4, Table 1). In contrast, the number of LFA-1α$^+$ T cells was higher ($p \leq 0.05$) in DCL (1460 ± 164 cells/mm^2) than in LCL (740 ± 132 cells/mm^2), but similar to that of MCL (1927 ± 240 cells/mm^2) (Fig. 4, Table 1). In LCL and MCL, an accumulation of LFA-1β$^+$ T cells was noted towards the epidermal basal membrane. The LFA-1β/LFA-1α ratio was higher in LCL (5.7:1) than in MCL (1.6:1) and DCL (2.3:1). The number of infiltration HB15$^+$ T cells was higher ($p \leq 0.05$) in LCL (1082 ± 98 cells/mm^2) than in DCL (539 ± 42 cells/mm^2) and MCL (503 ± 59 cells/mm^2) (Figs. 1 and 4, Table 1).

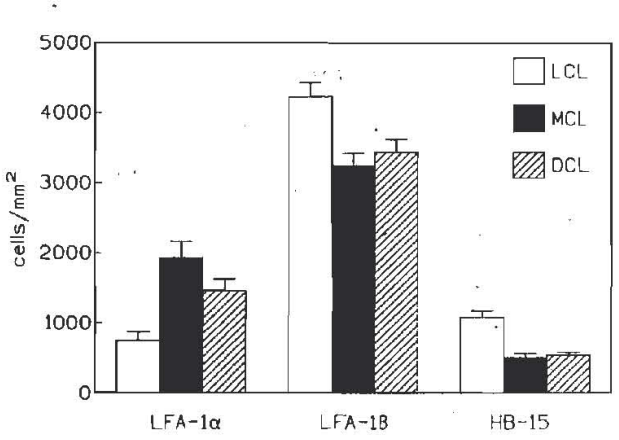

Figure 4. Density of infiltrating cells expressing the LFA-1 and HB15 molecules in the granuloma of localized (LCL), mucocutaneous (MCL) and diffuse cutaneous leishmaniasis (DCL). Values are expressed as mean ± SEM cells/mm^2 of granuloma.

Discussion

Epidermotropic migration of dermal T cells is launched by keratinocyte-derived chemokines and other accessory signals. Subsequent T-cell attachment to the epidermis may be mediated by LFA-1/ICAM-1 interaction, and interferon-τ from activated infiltrating cells may in turn influence chemokine production (20). In the present study we demonstrated significant differences in HLA-DR and ICAM-1 expression by the keratinocytes over the clinical spectrum of ACL, and these results confirm previous findings by our group (21). They are: abundant Langerhans cells, a cohort of keratinocytes expressing ICAM-1, and the uniform expression of HLA-DR in LCL epidermis; keratinocytes failing to express HLA-DR and ICAM-1 molecules in DCL epidermis; and the lack of epithelial Langerhans cells and the uniform expression of HLA-DR and ICAM-1 molecules in MCL epithelium. These results demonstrate an epithelial defect, and suggest the importance of the epidermis as an immunoregulatory site in leishmaniasis.

The disparity observed, in both LCL and DCL, between CD1a and HB15 values may be related to cell triggering, since HB15 expression has been associated with antigen presentation or cellular interactions after lymphocyte activation (16). Our results suggest that the HB15 molecule may distinguish a subset of primed CD1a$^+$ Langerhans cells which can present antigen to memory T cells.

An important finding was the higher expression of LFA-1β over LFA-1α in ACL. Moreover, it may be significant that both DCL and MCL lesions had higher numbers of LFA-1α$^+$ T cells than did LCL lesions, and that most epidermotropic T cells in LCL are LFA-1β$^+$. Recent studies have demon-

strated that only the cytoplasmic domain of the β subunit of LFA-1 is important in the regulation of cellular adhesion (22). The demonstration that the β subunit is necessary for the expression of LFA-1α, and that LFA-1β is highly conserved during evolution, may support the use of the LFA-1β/LFA-1α ratio os a measure of cellular adhesion. In the present study, the results suggest a defect in the regulation of adhesion signals in DCL and MCL granulomas. In DCL, the alteration may be associated with the elevated number of naive T cells present in these lesions (6).

Lymphocyte expression of HB15 is generally restricted to activated proliferating cells (16). The numbers of $HB15^+$ cells in LCL granulomas were similar to those previously observed for T cells expressing the IL-2R (6), an immunoreactivity usually associated with cell division. Thus, HB15 may be another *in situ* marker of cell activation. The low numbers of $HB15^+$ cells in DCL and MCL granulomas suggest a defect in the expression of this Ig superfamily molecule, which may contribute to the chronic proinflammatory infiltrate of MCL and the ineffective granuloma of DCL.

The present study favors the concept of the epidermis as an important homing site for primed T cells. In this system, accessory signals, such as the expression of adhesion and MHC-II molecules and cytokine secretion, may govern the effector phase of the immune response. Failures at this level will cause a defective immune response that is unable to eliminate the pathogen, or produce tissue damage. Here we demonstrate a defect at the level of accessory signals in DCL lesions that may account for the selective anergy observed in these patients. The macrophagic granuloma containing many naive T cells that characterize DCL lesions (6) may be the result of defective signalling from the epidermis which promotes the migration of naive and TH2 cells. Defective signalling at another level may account for the proinflammatory state that causes tissue damage in MCL.

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