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Epidermal Langerhans Cells and Dendritic Epidermal T Cells in Murine Cutaneous Leishmaniasis. Immunocytochemical Study.

Martín A. Sánchez, Gisela Cáceres-Dittmar, Olivia Oriol, Walter Mosca, Georg Kraal*, and Felix J. Tapia.

Instituto de Biomedicina, Universidad Central de Venezuela, Apartado 4043, Caracas 1010A, Venezuela, Fax 58-2-861-1258, and *the Department of Cell Biology, Faculty of Medicine, Free University, 1081 BT Amsterdam, The Netherlands.

ABSTRACT

In the present study, Langerhans cells (LC) and Dendritic Epidermal T Cells (DETC) were studied in ***Leishmania*** susceptible BALB/c and resistant C57BL/6 inbred mice. LC and DETC were characterized immunocytochemically using the monoclonal antibodies NLDC-145 and Thy-1.2 respectively. A positive Pearson's correlation was observed in healthy BALB/c and C57BL/6 mice, where the density of both cell types always drifted in the same direction. In contrast, no correlation was observed in the *L. **mexicana***-infected mice. These results show that the balance between LC and DETC is altered by the parasite insult. The LC/DETC ratio was always higher in healthy mice than in *L. **mexicana***-infected mice. In addition, the differences between healthy and infected animals were greater in BALB/c than in C57BL/6. Even though, the absolute numbers were always higher for LC than for DETC, the cellular increment after the infection was more prominent in the DETC population. The present study showed differences in the epidermal involvement of susceptible and resistant mouse models of leishmaniasis.

KEYWORDS

Cellular immunology, dendritic epidermal T cells, epidermis, Langerhans cells, leishmaniasis.

INTRODUCTION

The epidermis is an active component of the skin immune system. Langerhans cells (LC), keratinocytes and dendritic epidermal T cells (DETC) are the main cellular constituents of this system. LC are unique antigen-presenting cells characterized by a delayed antigen presentation that may promote early systemic immunity [1]. LC participate in up-regulatory mechanisms of the immune response, with an important role in infectious diseases [2]. DETC, formerly referred as Thy-1+ dendritic epidermal cells [3], are murine T lymphocytes associated to γδ+ T cells [4]. The TCR γδ+ cells play a role in the down-regulation of contact hypersensitivity in vivo [5]. The LC/DETC ratio in murine epidermis influences the intensity of contact hypersensitivity [5].

Human 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) has few parasites within well defined lesions, which generally heal after treatment, or spontaneously. In contrast, diffuse cutaneous leishmaniasis (DCL) is characterized by the presence of progressive nonulcerated nodules, rich in parasites. These lesions occur infrequently and are resistant to treatment [6]. In mice, depending on the animal strain, ***Leishmania*** strain and the number of inoculated parasites, it has been possible to reproduce the distinct clinical forms observed in humans. Thus, susceptible BALB/c mice reproduce lesions similar to DCL, and resistant C57BL/6 mice show LCL-like lesions [7,8]. The murine model of cutaneous leishmaniasis is extremely important for the analysis of the cellular response leading to the resolution of lesions induced by ***Leishmania***.

Giannini [9] showed that low doses of UVB applied locally to the inoculation site suppressed the development of skin lesions. UVB affected epidermal cells but did not alter the parasite load, suggesting that the local epidermal perturbation during the initial phase of infection

influence the response to the *Leishmania* parasite and the subsequent development of clinical disease. Recently, Will et al. [10] showed that freshly isolated LC, but not cultured LC, are highly active in presenting *L. major* antigen in vitro to T lymphocytes from primed mice and to parasite-specific T cell clone, thus emphasizing the importance of this epidermal cell in leishmaniasis.

In the present study, we used an immunocytochemical technique to characterize epidermal LC and DETC during *Leishmania* infection in both susceptible and resistant mice. In addition, we have evaluated the LC/DETC ratio as a criterion to determine the epidermal involvement in the immune response to the parasite.

MATERIALS AND METHODS

Animals and infection

BALB/c (n = 24) and C57BL/6 (n = 24) female mice aged 6-8 weeks old were inoculated subcutaneously in the left footpad with 10^3 amastigotes of *Leishmania mexicana* (MHOM/BZ/82/BEL21). The amastigotes were obtained according to Pérez et al. [8]. Briefly, the amastigotes were extracted from nodules of hamsters infected a month earlier with 10^6 amastigotes, which were inoculated subcutaneously into the footpad. The nodules were aseptically dissected out and washed in phosphate-buffered saline (PBS, pH 7.4) with added antibiotics, and finely cut and ground in a Petri dish containing cold PBS. Suspensions were filtered through a sterile sieve to remove large debris, the parasites counted in a hemocytometer and adjusted to $4x10^4$ per ml.

One week after infection and every two weeks until the eleventh week, groups of 4 mice were killed by cervical dislocation and the experimental footpad removed. Control groups included healthy BALB/c (n = 24) and C57BL/6 (n = 24) mice.

Analysis of the cutaneous lesions

The cutaneous lesion was evaluated by measuring the footpad thickness with a dial gauge caliper every two weeks for 11 weeks. The presence of parasites was confirmed by Hematoxylin-eosin and Giemsa staining of smears from longitudinal sections of infected footpad tissues.

Epidermis separation

Footpad skin was taken and cut into 1 mm^2 pieces; about 4 pieces were obtained from each footpad. The skin pieces were immersed in buffered EDTA for 150 min. at 37°C. After washing in PBS, the epidermis was removed from the dermis under a stereomicroscope using wooden toothpicks. Epidermal sheets were placed for 5 min. in PBS at room temperature until immunoperoxidase staining.

Monoclonal antibodies

A rat monoclonal antibody NLDC-145, which recognized murine dendritic cells including epidermal LC [11], was used 1:10 (culture supernatant); a monoclonal antibody against Thy-1.2 (clone 5a8), purchased from Cedarlane Labs, U.S.A., was used 1:20 (culture supernatant). Dilutions and immunostaining were carried out using a modified PBS pH 7.2 [12].

Immunoperoxidase staining

Immunoperoxidase staining was carried out as previously described [13,14] with some modifications for the immunocytochemical characterization of epidermal sheets. Briefly, after fixation in fresh acetone for 5 min, the epidermal pieces were transferred to round-bottom microplates, hydrated in PBS and sequentially incubated for 90 min with primary rat monoclonal antibody, biotinylated sheep anti-rat IgG (Vector Labs., CA, U.S.A.) at 1:60 (50 µg/ml) for 45 min, and streptavidin-horseradish peroxidase conjugate (B.R.L., U.S.A.) at 1:300 for 30 min. Five minute washes with PBS were done 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 0.1M acetate buffer, pH 5.2. The epidermal sheets were then washed and mounted on glass slides with glycerin-gelatin. Controls consisted of omission of the primary antibody or the use of an antibody of irrelevant specificity at the same concentration.

Cell quantification

Cells were counted using a light microscope. Only dendritic cells showing a red immunostaining were counted as positive. All fields were counted in each epidermal sheet at a magnification of 400x. This represents about 20 fields per sheet. To obtain a representative sample, four animals were killed for each analytical point. The experimental footpad of each

animal was cut into at least four pieces, which were then immunostained for each cell marker.

A percentage increment was calculated between the values for healthy and *L.mexicana*-infected mice for each particular cell marker.

Statistical analysis

All the information was expressed as mean ± SEM. 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 LC and DETC in healthy and infected animals for each experimental point was calculated using Pearson's correlation method.

RESULTS

Cutaneous disease in *L.mexicana*-infected mice

L.mexicana-infected BALB/c mice showed a progressive and statistically significant ($p \leq 0.05$) increase of footpad thickness from the third week after infection (2.40 ± 0.016mm) until the 11th week (3.63 ± 0.063mm). The 11th week was the last measured value before the lesions became ulcerated (Fig. 1). In intermediate resistant C57BL/6 mice, all the differences between healthy and *L. mexicana*-infected C57BL/6 mice were statistically significant ($p \leq 0.05$). The lesions of *L.mexicana*-infected C57BL/6 mice were in essence smaller than the lesions of *L.mexicana*-infected BALB/c (Fig. 1). *L.mexicana*-infected C57BL/6 mice also showed a progressive increase in footpad thickness starting on the 3rd week postinfection.

Density of epidermal Langerhans cells NLDC-145+ in healthy and *L.mexicana*-infected mice

L.mexicana-infected BALB/c mice showed an increase in the numbers of epidermal NLDC-145+ LC, starting with values similar to those found in healthy mice (Table 1) and reaching maximal values between the 3rd (Fig. 2) and 5th weeks. These values start to decrease after the 9th week and reach normal values on the 11th week. The differences between both groups showed statistically significant values after the 3rd week (Table 1).

L.mexicana-infected C57BL/6 mice showed a significant increase of LC the first 5 weeks after infection. On the 7th week, these values were lower than those observed in the healthy animals, but remained within normal ranges for the rest of the evaluation.

Figure 2 Epidermal cells in EDTA-separated epidermis of BALB/c *Leishmania*-infected mouse (3 weeks after infection). A. Langerhans cells; B. Dendritic epidermal T cells. Avidin-biotin immunoperoxidase using anti NLDC-145 and Thy-1.2, respectively. Bar= 10μm.

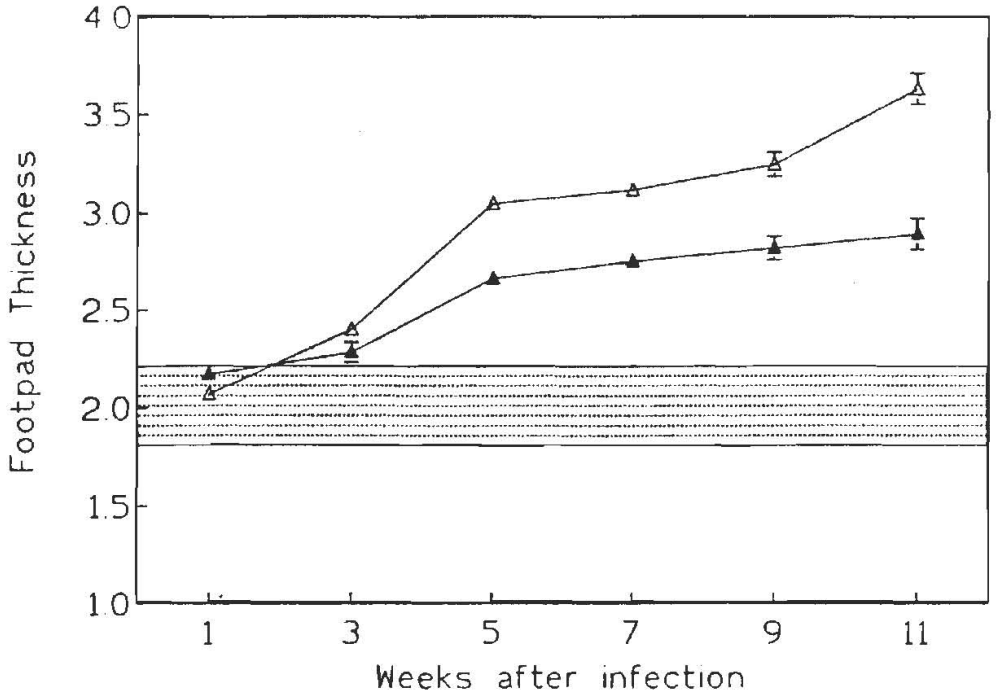

Figure 1 Development of cutaneous disease in *Leishmania*-infected mice. BALB/c (Δ) and C57BL/6 (▲) mice were inoculated in the footpad with 10^3 amastigotes of *L. mexicana mexicana*. Footpads were measured every 2 weeks for 11 weeks, and the lesion size was determined by sustracting the left footpad thickness of healthy animals from the inoculated left footpad thickness. The doted area represents the mean thickness of the footpads from healthy mice ± 2SEM. Each point represents the mean of four mice.

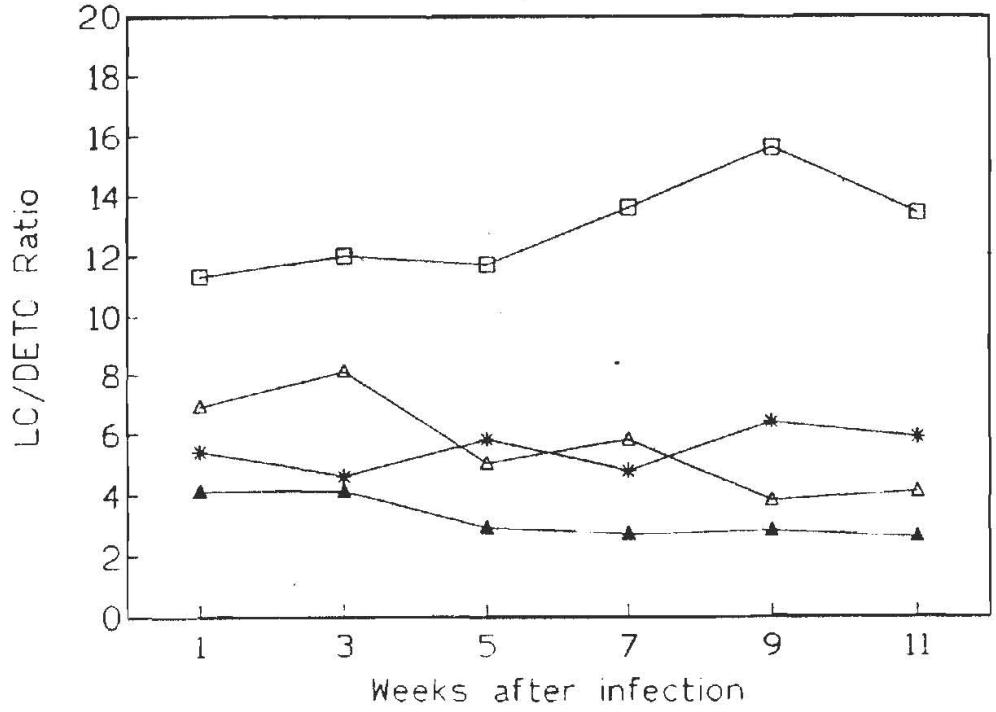

Figure 3 Ratio between epidermal Langerhans cells and Dendritic Epidermal T Cells in *Leishmania*-infected and healthy mice. The experimental groups are: healthy BALB/c (■), healthy C57BL/6 (★), infected BALB/c (Δ) and infected C57BL/6 (▲). Note that the values in healthy animals are always higher than those for infected mice.

TABLE 1: Langerhans cell densities in healthy and *L. mexicana*-infected mice.

	Langerhans cells (NLDC-145+)*ϕ					
Time (weeks)	(A) Healthy BALB/c	(B) Lm-infected BALB/c	% increment	(C) Healthy C57BL/6	(D) Lm-infected C57BL/6	% increment
1	1350 ± 24	1450 ± 56	7.40	794 ± 23	1076 ± 23	35.52
3	1317 ± 19	2106 ± 31	59.90	1067 ± 29	1284 ± 29	20.34
5	1438 ± 32	2196 ± 34	52.71	799 ± 27	1108 ± 22	38.67
7	1237 ± 23	1937 ± 31	56.58	842 ± 24	718 ± 22	14.73
9	1164 ± 24	1408 ± 39	20.96	762 ± 21	951 ± 33	24.80
11	906 ± 32	1163 ± 30	28.36	715 ± 21	852 ± 29	19.16

* Values expressed as mean of cells/mm² ± SEM

Ø Differences among groups = A-B $p<0.05$ (except on week 1); C-D $p<0.05$

Density of Dendritic Epidermal T Cells in healthy and *L.mexicana*-infected mice

L.mexicana-infected BALB/c mice showed an increase of DETC from the 1st week that reach a maximal value on the 5th week (Figs. 2 and 3). After this time, the values fall but remain higher than those found in healthy BALB/c mice. The comparison between both groups showed statistically significant differences in all the points evaluated (Table 2).

L.mexicana-infected C57BL/6 mice showed an increase of DETC with a two-fold increase that reached a maximal value on the 5th week. The differences between healthy and *L.mexicana*-infected mice were always statistically significant ($p \leq 0.05$) (Table 2).

Langerhans cells/dendritic epidermal T cells ratio in healthy and *L.mexicana*-infected mice

Both LC and DETC increase after the parasite infection, but the percentage increment was always higher for DETC (Tables 1 and 2). In addition, the percentage increment of DETC was higher in C57BL/6 mice than in BALB/c mice (Table 2).

The LC/DETC ratio was evaluated in the four experimental groups (Fig.4). Healthy mice showed higher values for the LC/DETC ratio than *L.mexicana*-infected mice. The mean LC/DETC ratio in healthy BALB/c mice was 13:1 whereas in infected mice it was 5.7:1. In healthy C57BL/6 mice the mean ratio was 5.5:1, and 3.3:1 in infected mice.

A Pearson's correlation analysis of both

TABLE 2: Dendritic Epidermal T cell densities in healthy and *L. mexicana*-infected mice.

Time (weeks)	Dendritic epidermal T cells (Thy-1+)*ϕ (A) Healthy BALB/c	(B) Lm-infected BALB/c	% increment	(C) Healthy C57BL/6	(D) Lm-infected C57BL/6	% increment
1	118 ± 6	206 ± 10	74.58	137 ± 11	256 ± 14	86.86
3	109 ± 7	258 ± 14	136.70	232 ± 16	303 ± 13	30.60
5	121 ± 7	428 ± 16	253.72	138 ± 9	369 ± 15	167.39
7	93 ± 7	328 ± 11	252.69	176 ± 9	251 ± 18	42.61
9	74 ± 6	354 ± 17	378.38	120 ± 8	329 ± 15	174.17
11	67 ± 4	280 ± 16	317.19	122 ± 9	315 ± 14	158.20

* Values expressed as mean of cells/mm² ± SEM

Ø Differences among groups = A-B $p<0.05$; C-D $p<0.05$

cell types during the 11 weeks of evaluation showed a positive correlation in healthy BALB/c mice ($r = 0.91$) and healthy C57BL/6 mice ($r = 0.97$). These results meant that for each experimental point LC and DETC densities always changed in the same direction *L.mexicana*-infected BALB/c and C57BL/6 mice showed no correlation.

DISCUSSION

In the present study, we have corroborated our previous finding that epidermal LC are increased after the infection of susceptible and resistant mice with *Leishmania* parasites [2,13]. These results suggested a participation of LC in the immune response to the parasite.

The present results showed a positive Pearson's correlation between LC and DETC densities in healthy mice during an 11-week period of evaluation. These results imply a very close association of the two cell groups under normal conditions. This balance was broken when the animals were infected with *Leishmania*, where both cell groups were increased but a significant correlation was not observed for each experimental point analyzed. It is worth noticing that in L. mexicana-infected mice the high values for LC appeared on the 3rd week, whereas the high values for DETC appeared on the 5th week.

The increase in the density of DETC after the *Leishmania* infection may be the result of epidermal LC presenting the parasite antigen to this cell group. The lack of a significant correlation between the two cell types in the infected animals may suggest that DETC are also involved in other pathways of the inflammatory

process after the infection, or that LC are also priming other cell types. The former may be supported by the findings of Shiohara et al. [15] who observed an increase in the numbers of DETC after the induction of graft-versus-host disease in normal mice. Results that suggest an important role for DETC in protecting the epidermal integrity from a secondary insult. Our results have shown that while the absolute numbers were always higher for LC than for DETC, the cellular increment after the infection was more prominent in the DETC population. This increase may be influenced by epidermal cell-derived cytokines as it has been shown by the daily in vivo administration of recombinant IL-2, which stimulates the proliferation of both LC and DETC [16,17], or could be the result of a selective migration of these cells towards the epidermis.

The proliferation of both cell types after ***Leishmania*** infection is consistent with a delayed-type hypersensitivity immunopathogenesis, where the epidermis plays an important role. These observations may be the result of a direct involvement of LC and DETC, or a consequence of the immune response against the parasite that may be triggered in the granuloma.

Recent findings have shown that LC can be infected by ***Leishmania*** in vitro and in vivo [18,19]. In addition, we have shown a lack of epithelial CD1a+ Langerhans cells in the lesions of human muco-cutaneous leishmaniasis, which may be the result of a selective migration of antigen-primed LC from the epithelium to regional lymph nodes, or the result of direct cytolysis of these cells by the parasites [20]. Our earlier findings and the ones presented here suggest that LC may participate in the pathogenesis of leishmaniasis by being target cells for the parasite.

An important finding was that healthy BALB/c mice have a larger density of epidermal LC than that observed in healthy C57BL/6 mice; the latter is very similar to that found in normal human skin [2]. Since LC are possible target cells, this apparent high density may favour the development of the disease in these animals. In addition, the high LC/DETC ratio observed in healthy BALB/c mice is the result of the elevated numbers of LC, since the density of DETC is very similar between the susceptible and resistant mice.

The present study shows how a parasite stimulus alters the balance of epidermal LC and DETC, and thus suggests the importance of the epidermal compromise in murine cutaneous leishmaniasis. Future studies will further analyze the epidermal participation in this disease after selective depletion and increment of LC and DETC.

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RESUMEN

En el presente estudio analizamos las Células de Langerhans (CL) y los linfocitos T dendríticos epidérmicos (DETC) en ratones susceptibles BALB/c y resistentes C57BL/6 a la infección por ***Leishmania.*** Las CL y los DETC fueron caracterizados inmunocitoquímicamente utilizando los anticuerpos monoclonales NLDC-145 y Thy-1.2 respectivamente. Los resultados mostraron una correlación de Pearson positiva entre los dos grupos celulares en los ratones sanos de ambas cepas, indicando una similitud en los cambios de densidad de ambos tipos celulares. En contraste, no se observó correlación en los ratones infectados con ***L. mexicana.*** Por otra parte, las diferencias entre los animales sanos e infectados fue mayor en BALB/c que en C57BL/6. Sin embargo, los números absolutos fueron siempre mayores para CL que para DETC, con un mayor porcentaje de incremento para los DETC después de la infección. El presente estudio muestra diferencias en el compromiso epidérmico entre los modelos susceptibles y resistentes de leishmaniasis múrida.

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