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CD8+ T cells *in situ* in different clinical forms of human cutaneous leishmaniasis

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Abstract

Introduction—*Leishmania braziliensis* infection induces a large spectrum of lesions that clinically manifest as nodules or papules that progress to ulcers. Although it is already known that T helper cells predominate in the lesions, cytotoxic T cells have also been reported to be present, and their role in leishmaniasis immunopathogenesis is not well known. This study investigated the amounts of CD8+ and granzyme B+ cells in different clinical forms of human cutaneous leishmaniasis (CL).

Methods—Forty tissue fragments from early (E-CL) and late CL (L-CL) lesions and from disseminated leishmaniasis (DL) - papules and ulcers - were characterized. The inflamed area per fragment was calculated, and the CD8 and granzyme B expression levels in the infiltrates were quantified by counting positive cells in 15 fields. The localization of CD8 and granzyme B was graded subjectively.

Results—Inflammation was higher in L-CL and DL ulcers. CD8 expression was increased in late ulcerated lesions compared to recent lesions. The increase in CD8+ cells also correlated with the duration of the lesion. Papules had a higher frequency of granzyme B+ cells than E-CL lesions, although the frequency was similar to those for late and DL ulcers. CD8+ cells were mostly found in the papillary dermis.

Conclusions—CD8+ T and granzyme B+ cells are present in the inflammatory infiltrates of CL and DL and may participate in the immunopathogenesis of *Leishmania* infection.

Keywords

Leishmaniasis; Cytotoxicity; Inflammation; Cutaneous; CD8; Human

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

INTRODUCTION

Leishmaniasis, a disease caused by a protozoan of the genus *Leishmania*, affects millions of individuals in many regions of the world. Infections by *Leishmania braziliensis*, the most prevalent species in Brazil, lead to lesions in the skin or mucosa that become cutaneous leishmaniasis (CL) or mucosal leishmaniasis (ML). Cutaneous leishmaniasis lesions start as small papules in the presence or absence of lymphadenopathy, and they progress into one or more skin ulcers. This localized skin ulceration is characterized by a chronic inflammatory response involving lymphocytes, plasma cells and macrophages, with and an eventual granulomatous reaction and necrosis[1,2].

The tissue response in the initial disease manifestation, including early ulcers (≤ 20 days of lesion appearance) and papules from disseminated leishmaniasis (DL), has not been well studied. DL is an emerging form of leishmaniasis that corresponds to 1.9% of all CL cases; it is characterized by the appearance of multiple pleomorphic lesions in more than two noncontiguous areas of the body, starting from a primary ulcer[2–5]. The mechanisms involved in DL have not been elucidated. It has been suggested that parasite, host and environmental factors may contribute to the dissemination of the parasite throughout the body[5].

Cluster of differentiation 8 (CD8)+ T cells are essential in the response against viruses and malignant cells, but few studies report the role of these cells in the defense against *Leishmania* parasites[6–8]. Although the presence of cluster of differentiation 4 (CD4)+ T cells within the inflammatory infiltrate in leishmaniasis lesions has already been shown, cytotoxic T cells in the lesions of CL patients[6] have also been reported.

The role of CD8+ T cells remains unclear, and contradictory results have been obtained for *Leishmania* infection; these cells may contribute to the killing of *Leishmania* or to the immunopathology of this parasite. A significant proportion of CD8+ cells in human leishmaniasis tissue fragments has been reported[9]. The CD8+ lymphocytes could participate in parasite growth control through different mechanisms. These cells have been associated with cure after treatment through interferon gamma (IFN-γ production, and they can also interact directly with *Leishmania*-infected macrophages by killing them through cytotoxic activity via granzyme granules[10–12]. Thus, CD8+ T lymphocytes expressing granzyme appear to be important in the immunopathology of leishmaniasis but may also be responsible for the characteristic lesions of CL. Characterization of the cell composition and the localization of granzyme B in all types of cutaneous leishmaniasis lesions, including early events in the immune response, may help to elucidate the mechanism of tissue damage.

In the present study, we investigated the proportions of CD8+ T cells and granzyme B+ cells in primary and secondary lesions from DL and in early and late CL lesions to better understand the roles of these cells in the pathogenesis of the disease.

METHODS

Patients

Thirty patients from Corte de Pedra, an area of *Leishmania braziliensis* transmission in the State of Bahia, Brazil, were enrolled in this study. A diagnosis of cutaneous leishmaniasis was initially made based on the presence of a typical lesion and a positive *Leishmania* skin test, and the diagnosis was confirmed by parasite isolation or identification of amastigote forms by histopathological analysis. Disseminated leishmaniasis was clinically diagnosed based on the presence of multiple (more than 10 lesions in at least two different areas of the body) acneiform, papular or ulcerated lesions. Confirmation of the diagnosis was performed as established above for CL. Patients were not under treatment at the time of tissue fragment collection. Cases with durations of more than 20 days were classified as late cutaneous lesions (L-CL), and cases with durations of 20 days or less were grouped as early cutaneous lesions (E-CL). For each patient with disseminated leishmaniasis, one tissue fragment was collected from primary ulcers of DL (U-DL), and one was collected from secondary papular lesions of DL (P-DL). Each group of patients consisted of 10 subjects, with a total of 40 tissue fragments. None of the patients had been previously diagnosed with or treated for leishmaniasis.

Tissue fragment collection

Tissue fragments were collected under local anesthesia using a 4mm punch. The specimens were collected from the border of the ulcer and, in case of a secondary lesion in DL, from papules.

The fragments were fixed in 10% formaldehyde, and the slides were prepared at the histotechnology laboratory at *Fundação Oswaldo Cruz* (FIOCRUZ). The tissue samples were dehydrated and embedded into paraffin blocks, and cryosections (4 to 6μm) were placed on silane-pre-coated slides.

Hematoxylin-eosin and immunohistochemistry staining

Sections obtained from paraffin were routinely deparaffinized and rehydrated. Slides were stained with hematoxylin-eosin (HE) for histological diagnosis, for inflammatory infiltrate analysis and to perform immunohistochemistry (IHC) reactions.

Immunohistochemistry reactions were followed by blocking peroxidase activity with 3% hydrogen peroxide for 5min. The slides were incubated for 30min with anti-CD8 antibody (M3164, Spring Bioscience Corp., Pleasanton, CA, USA) at a dilution of 1:100 at 25°C or overnight with anti-granzyme B (262A-16, Cell Marque Corp., Rocklin, CA, USA) at a dilution of 1:100 at 4°C. Immunostaining was performed using the Peroxidase Mouse & Rabbit Kit - HRP (DBS KP500, US) according to the manufacturer's recommendations. All slides were counter-stained with Harris hematoxylin, dehydrated and mounted with Canadian balsam and glass cover slips.

Morphometric analysis

Images from all HE-stained sections were acquired using an optical microscope (NIKON E-600) coupled to a digital color video camera at 400× magnification. The inflamed area was calculated through measurements of the total fragment and inflammatory infiltrate areas. The CD8 and granzyme B expression levels in the infiltrate were quantified by counting the number of CD8+ T and granzyme B+ cells present in 15 fields per fragment. The frequencies of CD8+ and granzyme B+ cells in the papillary and reticular dermis were graded subjectively, and the values indicate the frequency of positive immunostained cells (0, absent; 1, low frequency; 2, medium frequency; 3, high frequency). ImageJ software (National Institute of Mental Health, USA) was used to measure areas and count cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. The Spearman test was used to analyze correlations of inflammation between both groups of cells. The Kruskal-Wallis non-parametric test was performed to compare L-CL, E-CL, U-DL and P-DL groups according to the number of CD8+ T and granzyme B+ cells. Differences in the frequencies of immunostained cells in the papillary dermis and the reticular dermis between patient groups were analyzed using the Chisquare test. Results were considered statistically significant when $p < 0.05$.

Ethical considerations

Informed consent was obtained before the collection of tissue fragments, and all patients received treatment despite their enrollment in this study. The study was approved by the Human Ethics Committee of the Gonçalo Moniz Research Center, FIOCRUZ protocol number 321–2009.

RESULTS

The clinical profile of the 30 patients is shown in Table 1, and the clinical characteristics of different lesions are shown in Figure 1. Twenty-one men and 9 women were enrolled, with a mean age ± standard deviation (SD) of 31.52 ± 16.4 years (ages ranged from 9 to 76 years). The duration of lesions ranged from 25 to 60 days for L-CL (34 ± 10 days), 15 to 20 days for E-CL (17 ± 2 days), 20 to 90 days for U-DL (43 ± 20 days) and 8 to 90 days for P-DL (30 ± 25 days). Lesion size varied from 56 to 750mm^2 for L-CL, 9 to 200mm^2 for E-CL and 56 to 460mm^2 for U-DL. P-DL lesions were not measured, and the median number of lesions for the 10 patients with DL was 30 (range, 2–200 lesions) for papular lesions and 3 (range, 1–15) for ulcerated lesions. Cutaneous leishmaniasis patients presented only 1 lesion (range, 1–2).

Histopathological examination of tissue fragments from ulcerated or papular lesions revealed chronic inflammatory reactions in the tissue fragment sections from all of the clinical forms. In all stages of CL, inflammation was characterized by the presence of lymphocytes, plasmocytes and macrophages. Other general histopathological patterns were also found in tissue fragments, such as perivascular and papillary edema (45%), necrosis

(43%), granuloma (43%), giant cells (30%), rare neutrophils (18%) and pigmentary incontinence (28%).

The localization of cells expressing CD8 and granzyme B in the skin revealed that these cells are mostly in the papillary dermis, next to the epidermis, though the quantity of granzyme B+ cells was lower (Figure 2); the co-localization of the two types of cells was not analyzed. The majority of L-CL lesions (6/10) and U-DL lesions (6/10) were grade 3 (high frequency) for CD8+ frequency in the papillary dermis. Among the recent lesions (E-CL and P-DL), the intensity of stained cells had a greater variation, but a majority of the tissue fragments presented higher numbers of cells in the papillary dermis (Table 2). The granzyme B+ cell frequency was classified as grade 2 in the majority of the groups. Only 4 tissue fragments in the CD8+ T cell analysis and 2 in the granzyme B+ analysis had a greater grade in the reticular dermis than the papillary dermis. Cells also appeared in a homogenous distribution (10 to 40% of cases per group).

Comparing the inflammatory infiltrate percentage between groups, P-DL lesions had the lowest percentage (median=21%; range 6–38%). A higher percentage of inflammation was present in L-CL (median=37%; range 21–52%) and U-DL (median=34%; range 20–56%) patients (Figure 3A).

Analyses of the frequencies of CD8+ and granzyme B+ cells were performed using ImageJ software, which allows manual counting of cells.

CD8 expression, similar to the infiltrate extension, was increased in late ulcerated lesions compared to recent lesions (papules and lesions present < 20 days), which had low absolute numbers of CD8+ lymphocytes (Figure 3B).

To determine the contribution of CD8+ cells in the inflammatory infiltrate, the correlation of these cells and the inflammation percentage was analyzed for each group. Only E-CL ($R=0.7$; $p=0.02$) and P-DL ($R=0.79$; $p=0.005$) lesions exhibited a correlation between the number of CD8+ T cells and greater inflammation (not shown).

The papular lesions from DL had a higher frequency of granzyme B+ cells than E-CL lesions ($p=0.0302$) and a frequency similar to those of late and DL ulcers (Figure 3C). Interestingly, only E-CL lesions had a positive correlation between granzyme B+ cells and the inflammatory infiltrate ($R=0.7$; $p=0.0216$). The analysis of late ($R=-0.29$; $p<0.05$) and DL ulcers ($R=-0.22$; $p<0.05$) revealed that the higher the inflammation, the lower the amount of granzyme B+ cells (data not shown).

DISCUSSION

Cutaneous leishmaniasis is the most frequent form of leishmaniasis due to *L. braziliensis* infection. It is clinically characterized by the presence of single or multiple ulcers with a well-delimited and raised border that can persist for months or heal spontaneously. DL, similar to CL, presents initially with an ulcer and then disseminates throughout the body with multiple acneiform and papular secondary lesions[5].

CD8+ and granzyme B+ lymphocytes were more frequent in the papillary dermis. Histopathological analysis of tissue fragments showed the presence of spongiosis. The aggregation of both cells in the papillary dermis, sometimes also in the epidermis, seems to lead to injury in the basal layer and may contribute to ulcer formation. Recently, it has been suggested that the movement of T cells through the dermis and epidermis and their permanence in one layer or another correlates with the effector or memory phase of the immune response[13]. This finding suggests another mechanism for these cells, which together with cytotoxic activity, may contribute to leishmaniasis immunopathology.

In our study, chronic inflammation was observed in all four types of lesions. DL-P and E-CL together showed less inflammatory infiltrate than L-CL and U-DL (although statistical analysis showed no significance). This suggests that inflammation increases as the lesion progresses. These results are in agreement with those of another study showing that a higher intensity of inflammation is seen in L-CL lesions than in ulcers from CL in a very initial form (not yet ulcerated)[14].

Recent studies have demonstrated an increased frequency of CD8+ T cells over CD4+ T cells in ulcerated lesions as well as correlations between the frequencies of CD8+ T cells and CD8+ T cells that express granzyme and the intensity of the inflammation, which indicate a role for these cells in the pathogenesis of CL[14–16]. Santos et al[8]. have also shown the participation of CD8+ granzyme B+ T cells in tissue damage in CL caused by *L. braziliensis*, data that reinforce the association of the intensity of necrosis and the percentage of these cells[8].

CD8+ lymphocytes with cytotoxic function seem to execute a cytolytic activity that may be important for tissue destruction with ulcer formation, as is seen in ML, where the same CD8+ T cells seem to be involved in disease exacerbation[17].

Vieira et al[18]. showed that both localized CL and papular DL lesions presented a higher frequency of CD8+ T cells than CD4+ T cells in inflammatory infiltrates, suggesting that CD8+ cells participate directly in the immune response against parasites, principally by IFN-γ production, which induces macrophage activation[18]. However, CD4+ T cells appear to be the main source of IFN-γ and CD8+ T cells have a principal function of cytotoxicity, contributing to lesion formation[8]. In experimental models, CD8+ T cells have already been shown to be necessary for a protective response and also for the immunopathological development of CL[17].

The evaluation of the presence of cytotoxic activity in inflammatory infiltrates revealed that lesions of less than 20 days had lesser quantities of cells expressing granzyme B than late ulcers and ulcers from DL. This result agrees with a previous study that showed granzyme expression was associated with lesion progression[14]. Interestingly, papular lesions from DL showed higher numbers of granzyme B+ cells than early ulcers. The association of DL with mucosal disease due to the rapid dissemination of lesions throughout the body and mucosa and the association of cytotoxic CD8+ T cells to tissue damage in ML have previously been reported[5,19]. Furthermore, granzyme B does not seem to participate in parasite growth control[8]. The presence of granzyme B+ cells in DL papular lesions is indicative of their role

in pathology. Granzyme B seems to participate in lesion progression in E-CL, as the expression of granzyme B and the inflammation percentage show simultaneous increases in E-CL.

In summary, CD8+ T cells and granzyme B+ cells are present in the inflammatory infiltrates of CL and DL. Further studies are necessary to elucidate the contribution of these cells to the killing or dissemination of the parasite in *Leishmania* infections. Their cytotoxic function and specific localization may be responsible for tissue destruction and may explain the mechanism of ulceration. Due to the scarcity of patients with the DL form who simultaneously present papules and ulcers, a low number of patients was recruited. Further characterization of the inflammatory reaction *in situ* in the two clinical forms and the four-lesion spectrum of human leishmaniasis may help elucidate the role of CD8+ cells and their cytotoxic activity in the immunopathogenesis of this disease.

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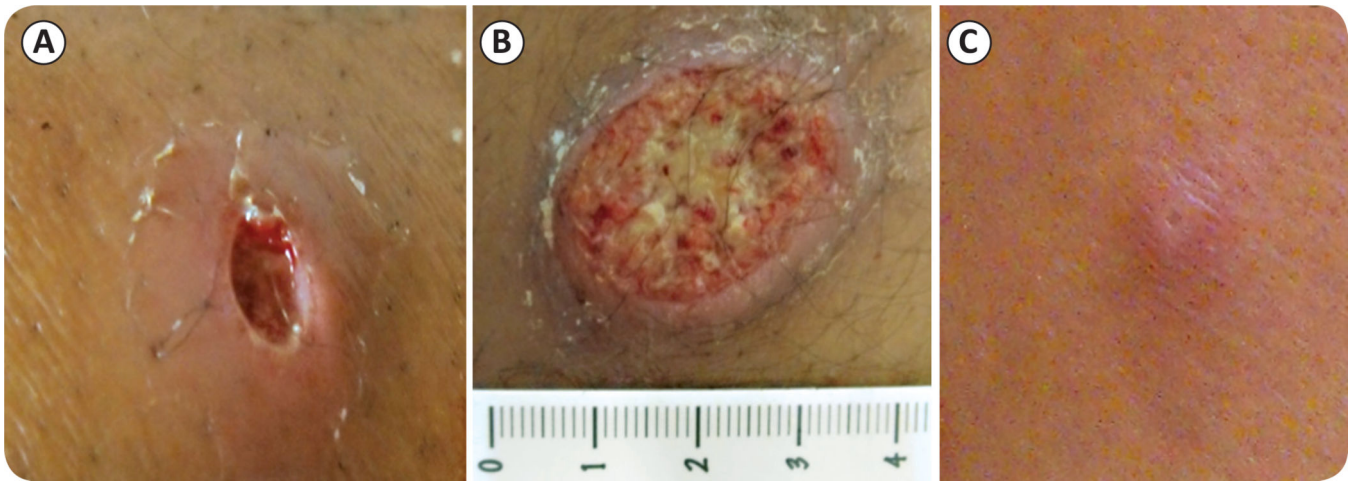

FIGURE 1.

Typical lesions caused by different clinical forms of cutaneous leishmaniasis. A: Early cutaneous leishmaniasis lesion. B: Late cutaneous leishmaniasis lesion. C: Papule lesion, typical non-ulcerated lesion from disseminated leishmaniasis.

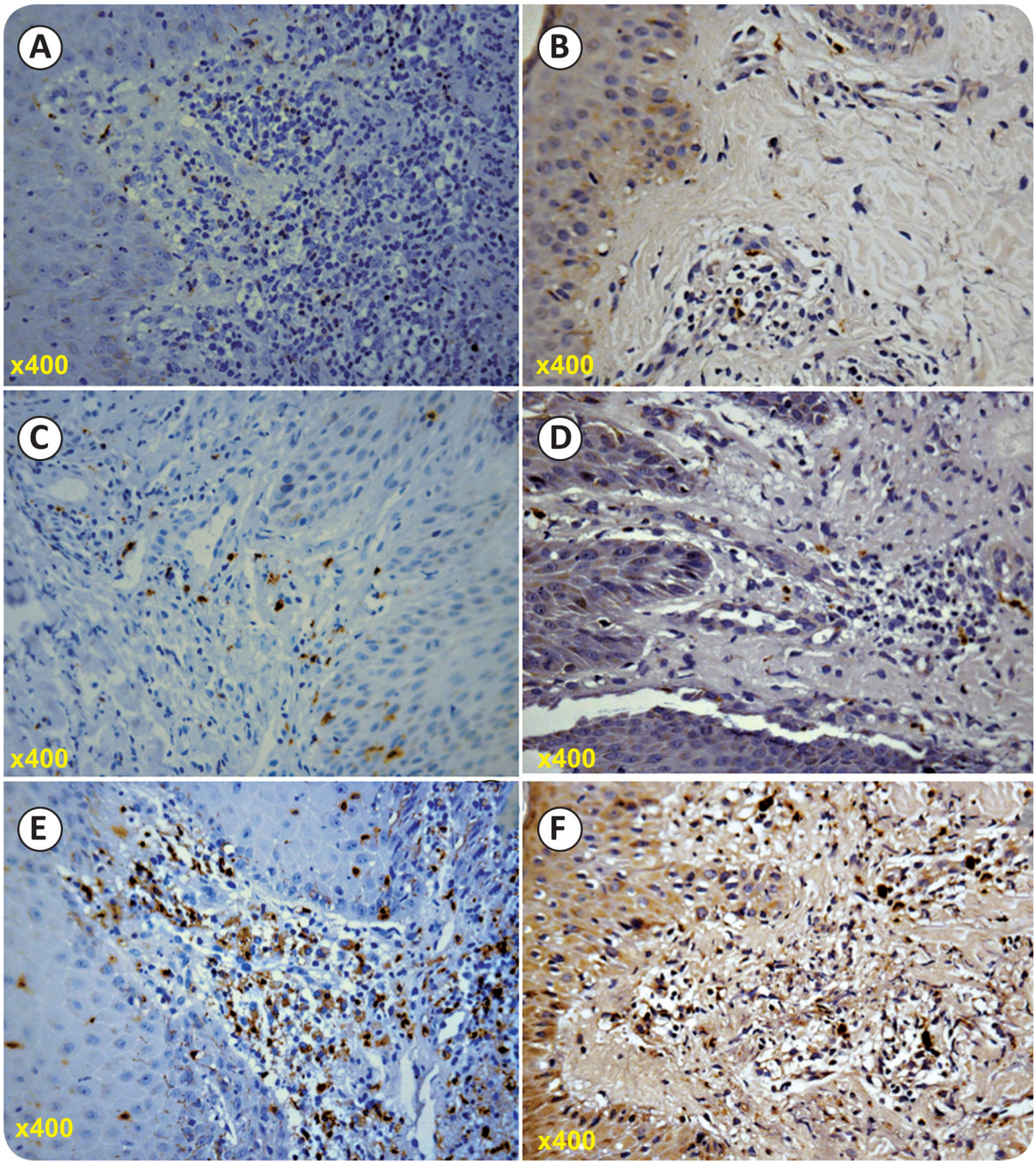

FIGURE 2.

Frequency of CD8+ cells in the papillary dermis: A: Tissue fragment graded as low frequency. B: Tissue fragment graded as medium frequency. C: Tissue fragment graded as high frequency. Frequency of granzyme B+ cells in the papillary dermis. D: Tissue fragment graded as low frequency. E: Tissue fragment graded as medium frequency. F: Tissue fragment graded as high frequency. All images have the same magnification (400×).

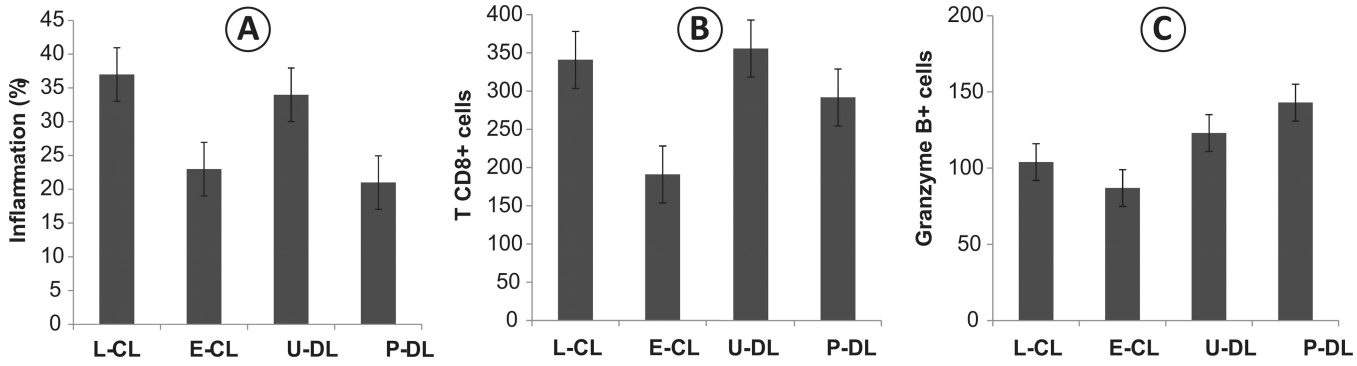

FIGURE 3.

A: Comparison of the inflammation percentage in different lesions from cutaneous leishmaniasis. B: Comparison of the numbers of CD8+ T cells in different lesions from cutaneous leishmaniasis. C: Comparison of the numbers of granzyme B+ cells in different lesions from cutaneous leishmaniasis. Median ± standard error is represented with bars. The Kruskal-Wallis test was performed. L-CL: late cutaneous lesions; E-CL; early cutaneous lesions; U-DL: primary ulcers of DL; P-DL: secondary papular lesions of DL.

TABLE 1

Clinical characteristics of cutaneous leishmaniasis patients.

Patient	Age/gender	Clinical form	Duration of lesions (days)	*Leishmania* infection detection in HE	Location of ulcerated lesion	Ulcer area (mm × mm)	DTH (mm × mm)
1	22/M	L-CL	60	0	IM	30 × 20	15 × 15
2	19/M	L-CL	30	0	IM	30 × 20	8 × 7
3	42/F	L-CL	30	+	IM	30 × 15	14 × 14
4	18/M	L-CL	45	+	IM	30 × 25	20 × 22
5	19/M	L-CL	30	0	IM	10 × 8	19 × 16
6	39/F	L-CL	25	+	IM	20 × 15	15 × 16
7	9/M	L-CL	30	+	IM	10 × 10	14 × 10
8	27/M	L-CL	30	+	trunk	20 × 15	16 × 11
9	27/M	L-CL	30	0	IL	20 × 20	12 × 10
10	37/M	L-CL	30	0	IL	ND	negative
11	15/M	E-CL	20	0	SL	6 × 9	30 × 30
12	18/M	E-CL	15	0	SL	4 × 4	10 × 12
13	9/M	E-CL	15	+	SL	3 × 3	negative
14	36/F	E-CL	15	+	IL	8 × 5	20 × 14
15	26/F	E-CL	15	+	SL	3 × 3	14 × 13
16	25/M	E-CL	15	+	trunk	6 × 5	9 × 9
17	16/F	E-CL	20	+	IL	20 × 10	19 × 16
18	37/M	E-CL	20	+	IL	16 × 9	21 × 19
19	16/F	E-CL	20	+	IL	4 × 8	negative
20	23/M	E-CL	20	0	IL	8 × 6	21 × 15
21	50/F	DL (U/P)	60/15	+/0	IL	7 × 8	16 × 16
22	53/M	DL (U/P)	60/30	+/+	face	20 × 15	negative
23	37/M	DL (U/P)	30/15	+/+	IM	ND	15 × 12
24	37/M	DL (U/P)	30/10	0/+	SM	10 × 9	10 × 9
25	59/F	DL (U/P)	30/30	0/0	SM	20 × 23	5 × 5
26	60/M	DL (U/P)	30/60	+/0	IM	10 × 10	ND
27	17/M	DL (U/P)	90/15	0/0	SM	10 × 15	22 × 18
28	37/F	DL (U/P)	30/30	0/0	SM	10 × 11	18 × 15

Patient	Age/gender	Clinical form	Duration of lesions (days)	*Leishmania* infection detection in HE	Location of ulcerated lesion	Ulcer area (mm × mm)	DTH (mm × mm)
29	30/M	DL (U/P)	30/90	0/+	IM	10 × 10	ND
30	76/M	DL (U/P)	38/8	+/0	IM	10 × 30	10 × 10

DTH: delayed type hypersensitivity; HE: hematoxylin-eosin; M: male; F: female; L-CL: late cutaneous leishmaniasis; E-CL: early cutaneous leishmaniasis; DL: disseminated leishmaniasis; U: ulcer; P: papule; +: presence; 0: absence; IM: inferior limb; SM: superior limb; ND: not determined. Papules were not measured.

TABLE 2

Number of cases with a high frequency of immunostained cells in the papillary dermis or reticular dermis.

	T CD8+		Granzyme B+	
	papillary dermis	**reticular dermis**	**papillary dermis**	**reticular dermis**
L-CL	6/10	0/10	6/10	1/10
E-CL	7/10	2/10	7/10	0/10
U-DL	6/10	0/10	7/10	1/10
P-DL	7/10	2/10	6/10	0/10

There were 10 patients per group. Ten tissue fragments stained for CD8+ T cells and twelve for granzyme B+ cells were found to have a homogenous distribution of cells. p=0.0105 for CD8+ T cells and granzyme B+ cells (Chi-square test). CD8+: *cluster of differentiation 8*; L-CL: late cutaneous lesions; E-CL: early cutaneous lesions; U-DL: primary ulcers of DL; P-DL: secondary papular lesions of DL.