

Histopathological and immunohistochemical profile of the American cutaneous leishmaniasis with emphasis on FXIIIa+ dermal dendrocytes *

*Perfil histopatológico e imuno-histoquímico da leishmaniose tegumentar americana com ênfase nos dendrócitos dérmicos FXIIIa+ **

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Abstract: Background: Mucocutaneous leishmaniasis is a parasitic infectious disease with relevant immunological aspects. Objective: To study the histopathological and immunohistochemical aspects of 21 leishmaniasis tegumentary biopsies. Methods: Polyclonal anti-Leishmania antibody was used to confirm the presence of Leishmania amastigotes. Histopathological classification comprised IV standard groups. The immunopathological pattern was evaluated as to the presence of FXIIIa+ dermal dendrocytes, CD1a+ Langerhans cells, CD68+ macrophages, CD20+ B lymphocytes, and CD3+ T lymphocytes. The FXIIIa+ cells were quantified and compared to specimens of normal skin obtained from unexposed areas. The other cells were counted in a semi-quantitative way. The number of FXIIIa+ cells was statistically analyzed using the Mann-Whitney test. Results: Among the histopathological groups, I and II standards prevailed. The FXIIIa+ cells were observed for different aspects and compared to normal skin, without significant statistical differences ($p = 0.157$). There was no relation between the amount of CD1a+, CD68+, CD20+ and CD3+ cells when compared to each other or to FXIIIa+ cells. Conclusion: There was no difference between the number of FXIIIa+ cells in leishmaniasis and in normal skin. However, more studies are needed in order to understand the role of FXIIIa+ cells in leishmaniasis.

Keywords: Autoimmunity; Cutaneous leishmaniasis; Dendritic cells; Factor XIIIa

Resumo: FUNDAMENTOS: Leishmaniose tegumentar é doença parasitária infecciosa que apresenta aspectos imunológicos relevantes.

OBJETIVO: Estudar a histopatologia e aspectos imuno-histoquímicos de 21 biópsias de leishmaniose tegumentar.

MÉTODOS: Anticorpo policlonal anti-Leishmania foi utilizado para identificação das leishmânias. A classificação histopatológica adotada foi em grupos padrões de I a V. Foram analisados os dendrócitos dérmicos FXIIIa+, células de Langerhans CD1a+, macrófagos CD68+, linfócitos B CD20+ e T CD3+. As células FXIIIa+ foram quantificadas na derme papilar e comparadas a peles normais obtidas de área não exposta à luz solar, sendo o número de células FXIIIa+ avaliado estatisticamente através do teste de Mann-Whitney. As demais células foram contadas semiquantitativamente.

RESULTADOS: Entre os grupos histopatológicos, predominaram os I e II. Não houve diferença estatisticamente significativa ($p=0,157$) entre o número de células FXIIIa+ na leishmaniose e na pele normal. Não foi observada diferença significativa entre a presença das células CD1a+, CD68+, CD20+ e CD3+, quando comparadas entre si ou com as células FXIIIa+.

CONCLUSÃO: Não houve diferença no número de células dendríticas FXIIIa+ entre a leishmaniose e pele normal. No entanto, sugere-se que mais estudos sejam necessários para se entender o papel dessas células na leishmaniose.

Palavras-chave: Auto-imunidade; Células dendríticas; Fator XIIIa; Leishmaniose cutânea

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INTRODUCTION

American cutaneous leishmaniasis (ACL) is a parasitic, infectious but non-contagious disease caused by protozoa of several species of genus *Leishmania* that affects skin and mucosa.

Regardless of the *Leishmania* species that parasites the individual, the first affected organ is the skin. Patients are infected chronically by the parasite and can later on develop *leishmaniasis*.

Moros *et al.*¹ stated that the exact mechanism by which the immune system intervenes in the destruction of the parasite is unknown, but cell immunity is considered fundamental in the resolution of the infection.

In a review of 1982, Grimaldi² observed that the parasites of genus *Leishmania* induce a variety of complex responses in vertebrate hosts, carried out or modulated by their immune system. The author also pointed out that the fate of *Leishmania* inside the macrophages depends on particular parasite/host relations, involving not only the parasite intrinsic properties, but also the genetically determined characteristics of the host cell or those of its interactions with other immunocompetent cells.

In this study, tissue immune reaction was investigated by histopathological examination of ACL lesions and immunohistochemical features of dermal dendrocytes (DD), Langerhans cells (LC), macrophages (MO), and B and T lymphocytes, using markers FXIIIa, CD1a, CD68, CD20, and CD3, respectively, with relevance for the FXIIIa+ dermal dendrocytes. These and the Langerhans cells are considered dendritic cells (dC) with antigens, responsible for the cell immune response, and the first cells to get in contact with the invading microorganism (*Leishmania*).

MATERIAL AND METHODS

Twenty-one ACL biopsies were submitted to this investigation, after patients had been informed about the research and signed the informed consent form, following approval by the Research Ethics Committee.

Histopathological examination

With hematoxylin-eosin (HE) staining, the five histopathological patterns identified by Magalhães *et al.*^{3,4} for the classification of ACL were used and are represented in Table 1.

Immunohistochemical method

We used the streptavidin-biotin-peroxidase method. Four-micron thick histological sections were collected on previously prepared slides (Dako Silanized Slides Code No. S3003), deparaffinated in xylene and rehydrated in ethanol and water. The endogenous peroxidase activity was blocked.

Antigenic retrieval was done in a 96°C water bath with citrate buffer (Dako target retrieval solution), inhibition of the specific bonds with skimmed milk (30ml TRIS + NaCl + 0.3g of bovine albumin Merck + 0.3g Molico skimmed powdered milk). After draining the milk, the primary antibodies were used at the dilutions described below, then placed in a humid chamber at room temperature for 30 minutes (incubation with specific primary antibody). The biotinylated secondary antibody from an LSAB kit was used. The development (antigen-antibody bond) was visualized with diaminobenzidine, Dako DAB+ kit, the slides counterstained with Harris' hematoxylin, and mounted with cover slips and Entellan.

In the anti-*Leishmania* polyclonal antibody protocol, the incubation step was modified, bringing it to a whole night in a humid chamber at room temperature, with the antibody diluted at 1/4000 in diluting solution with a Dako unspecific bond reducer. The *Leishmania* species used was *L.(L.) amazonensis* (strain MHOM/BR/73/M2269), isolated from a cutaneous leishmaniasis case from the state of Pará, classified by monoclonal antibodies and isozymes, at the Evandro Chagas Institute, in Belém, PA.

The dilution protocols for the antibodies were as follows: anti-FXIIIa, dilution 1/800; anti-CD1a, dilution 1/200; anti-CD68, dilution 1/500; anti-CD20, dilution 1/300; anti-CD3, dilution 1/600; and a special protocol for the anti-*Leishmania* antibody with a dilution of 1/4000.

Leishmania analysis

An observation was considered as positive if at least one structure was marked in brown (color given by diaminobenzidine, the chromogen used) and was morphologically compatible with an amastigote. In this sense, the size, shape and localization of the structures inside the macrophage vacuoles were considered. The presence of marks in the cytoplasm of mononuclear inflammatory cells, mainly those with morphological characteristics of macrophages and endothelial cells, was also investigated. Such positivity patterns were respectively named "cellular" and "vascular".^{5,6}

Analysis of FXIIIa+ dermal dendrocytes (DDFXIIIa+)

These cells were analyzed as to their localization, distribution and morphological aspect in the dermis and within the inflammatory infiltrate, using the quantitative method. They were examined under an Olympus CBA (Micronal -Brazil) optical microscope, with a 40x lens and a 10x ocular (final magnifi-

TABLE 1: Histopathological classification of cutaneous leishmaniasis

Groups	Name	Main characteristics	Estimated prognosis
I	Exudative cell reaction	Dermal or mucosal chorion histiolympoplasmocytic infiltrate with cell proportions tending to equivalence	Prognosis: good (cutaneous forms) Prognosis: poor (mucosal forms)
II	Exudative and necrotic reaction	Tissue necrosis in dermis or chorion of mucosa of variable amplitude, rounded or oval, besides the histiolympoplasmocytic infiltrate. Necrosis can present as the granulocytic type, nuclear dust or eosinophilic type, associated to acute vasculitis	Prognosis: good
III	Exudative and necrotic-granulomatous reaction	Disorganized granulomatous reaction around or near the area of tissue necrosis, characterized by the presence of activated macrophages and of giant cells. Presence of histiolympoplasmocytic infiltrate. Less frequent acute vasculitis	Prognosis: good
IV	Exudative and granulomatous reaction	Disorganized granulomatous reaction isolated within the inflammatory infiltrate, without tissue necrosis. Presence of histiolympoplasmocytic infiltrate	Prognosis: excellent
V	Exudative and tuberculoid reaction	Granulomatous reaction composed of macrophages, epithelioid cells and giant cells, disposed in well-delimited tuberculoid displays, besides the histiolympoplasmocytic infiltrate	Prognosis: fair (cutaneous forms) Prognosis: poor (mucosal forms)

SOURCE: MAGALHÃES ET AL.^{3, 4}

cation of 400x) and adding together five sequential high-magnification fields, starting at the dermal-epidermal junction.⁷ In the cases in which this procedure was not possible, such as ulceration, cutting fold, etc., the next field in which such alterations were not present was counted. The result of this count was expressed by the sum of the number of cells in the five high-magnification fields, in each lesion examined.

As controls for the number of DDFXIIIa+, we used six normal skin fragments obtained during necropsy from the suprapubic region (unexposed to sun) of cadavers without any skin disease, less than 12 hours from the time of death.

Analysis of Langerhans cells, macrophages, T and B lymphocytes

After immune marking with their respective antibodies, these cells were analyzed for their presence and distribution in the epidermis and dermis, using the semi-quantitative method, with a 40x lens and a 10x ocular of the same microscope, and classified as: absent (0); small amount, up to 5 cells (+); moderate amount, from 5 to 10 cells (++) and great amount, over 10 cells (+++).⁸

Statistical analysis

The amount of DDFXIIIa+ in the leishmaniasis skins was compared with that of normal skins/controls by means of the Mann-Whitney test.

To check the association of the FXIIIa+ cell count, the classification into histopathological groups, and the normal skin control group, the Kruskal-Wallis test was used.

RESULTS

From the 21 cases with a confirmed clinical and epidemiological diagnosis of ACL confirmed by the anti-*Leishmania* polyclonal antibody, the following results were obtained.

All patients had cutaneous leishmaniasis. The lesions were present predominantly in males, in the third decade of life, on the lower limbs, as shown in Table 2.

Table 3 shows the number of lesions of each patient, the time of progression and the histopathological group to which they belonged. According to the histopathological analysis, the predominant histopathological groups were I and II.

Immunohistochemical test result

Demonstration of *Leishmania* and of parasite antigens in the ACL lesions was represented by the presence of amastigote forms and by "cell marking". As control material, lesions with amastigote forms evidenced by HE and Giemsa staining and a bone marrow biopsy with a great amount of control amastigote forms were used.

The morphology of the DDFXIIIa+ was

TABLE 2 - Site of the lesion, gender and age of patients with cutaneous leishmaniasis

Case	Site of the lesion	Gender	Age(years)
1	Left shoulder	+	36
2	Left arm and forearm	+	50
3	Frontal region	+	37
4	Left arm	+	50
5	Cervical region	+	21
6	Right leg	+	38
7	Not informed	+	27
8	Right wrist	+	29
9	Left leg	+	27
10	Left malleolar region	+	59
11	Dorsal side of the right foot	+	67
12	Left thigh	+	65
13	Right foot	+	74
14	Left clavicular region	+	39
15	Right thigh	+	18
16	Left leg	+	65
17	Multiple lesions on the right hip	+	30
18	Frontal region	+	14
19	Dorsal side of the left foot	+	40
20	Right elbow	+	28
21	Right thigh	+	31

TABLE 3: Number of lesions, time of progression, and histopathological group of patients with cutaneous leishmaniasis

Case	Number of lesions	Time of Progression	Histopathological group
1	01	3 months	II
2	Several	7 months	II
3	01	Not informed	IV
4	01	Not informed	II
5	01	5 months	V
6	01	Not informed	II
7	01	Not informed	I
8	01	1 months	III
9	01	11 months	II
10	01	3 months	I
11	01	2 meses	I
12	02	1 months	I
13	01	Not informed	I
14	01	Not informed	V
15	01	Not informed	III
16	01	Not informed	I
17	Several	Not informed	II
18	02	4 months	II
19	01	Not informed	I
20	01	6 months	II
21	01	Not informed	I

fusiform, their localization at the level of the papillary dermis, distributed horizontally under the dermal-epidermal junction, around the capillaries or superficial vessels in association with mast cells, monocytes and macrophages, and diffusely in the mid and deep reticular dermis, in loose connective tissue, around the skin appendages, sweat glands and pilosebaceous follicles, as seen in Figure 1.

In the normal skin control group (Figure 2), the results showed a maximum number of DDFXIIIa+ equal to 60 and a minimum number of 42.

No significant quantitative relation was found between the cells immunomarked by antibodies FXIIIa+, CD1a (Figure 3), CD68 (Figure 4), CD20 and CD3, in the ACL lesions, as represented in Table 4.

Statistical analysis results

Analysis of the data regarding the variable “amount of DDFXIIIa+” obtained in the samples of normal and leishmaniasis skin, using the Mann-Whitney test (U = 36; p-value = 0.157), showed no evidence of a statistically significant difference.

Comparing the amount of FXIIIa+ cells in ACL and the classification into the five histopathological groups, it was demonstrated that the amount of FXIIIa+ cells in group II was significantly larger than in group I (Mann-Whitney test: U = 12.5; p-value = 0.036).

A comparison between the predominant groups I and II and the control skin regarding the number of DDFXIIIa+, using the Kruskal-Wallis test, showed no difference (p = 0.185).

DISCUSSION

In Leishmania infection, the first target cells of the parasites are dendritic cells – Langerhans cells and dermal dendrocytes – and macrophages.⁹ In view of the relevant aspects of the DDFXIIIa+, still under study according to the literature, we chose to perform this research using this marker, comparing it in cutaneous leishmaniasis and in normal control skin, in order to observe the behavior of this group of dendritic cells. Other cells also cluster to granulomas, such as lymphocytes, plasmocytes, eosinophils and fibroblasts.¹⁰

An analysis of the relation between the progression time of lesions (time of disease) and the histopathological group or immunohistochemical findings was not possible, due to the lack of the first information in 11 cases.

In this study, histiolymploplasmocytic infiltration was present in all histopathological groups, in agreement with the reports of Magalhães *et al.*¹¹, appearing to play the role of an unspecific cell response to the presence of a strange agent. Lymphocytes were found in all lesions. Nuclear dust,

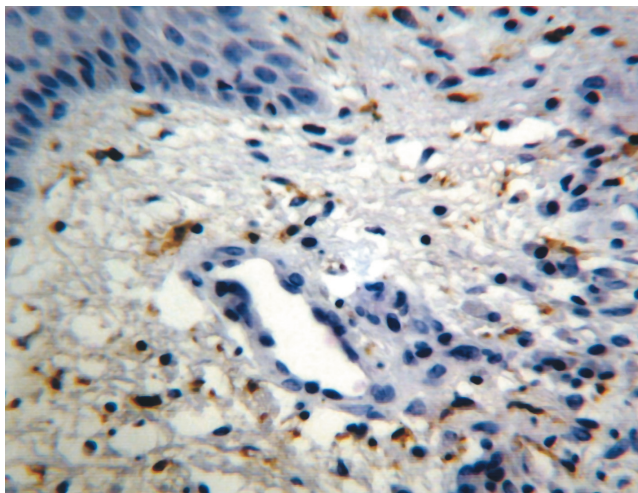


FIGURE 1: FXIIIa+ dermal dendrocytes in the dermis of a cutaneous leishmaniasis lesion (immunoperoxidase with anti-FXIIIa antibody, 400x)

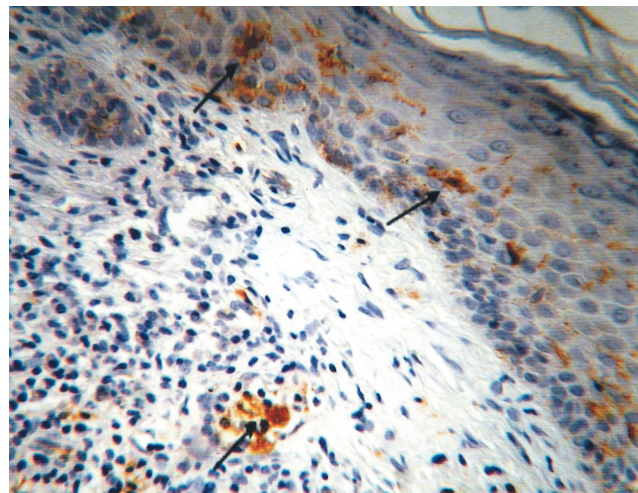


FIGURE 3: Langerhans cells in the epidermis and dermis (arrows) in a cutaneous leishmaniasis lesion (immunoperoxidase with anti-CD1a antibody, 400x)

represented by fragments of neutrophils and eosinophils, was present in most cases examined. In this nuclear dust phase, no plasmocytes were present, as opposed to older lesions. The histiolymphoplasmocytic infiltrate may also play a more aggressive rather than simply reactive role. This assumption should be proven by other studies, using monoclonal antibodies against the various subpopulations of lymphocytes, allowing identification within the tissues of the types of lymphocytes which take part in self-aggression reactions.

In ACL lesions, the FXIIIa+ cells were present at locations similar to those described in the literature.¹²⁻¹⁷ These results suggest that the basic pathogenic mechanism in granuloma formation is similar, regardless of its origin, in either sarcoidosis, tubercu-

losis or leishmaniasis.

DD hypertrophy in inflammatory and granulomatous skin conditions with a clinical and histological aspect of delayed hypersensitivity reaction suggests that they may play the role of skin immune response mediator cells. The localization of these cells, underlying the epidermis, perivascular areas and in the inflammatory cell infiltrate, is compatible with the role of antigen-presenting cells (APC).^{16,18-20}

The examined lesions were from areas exposed to sunlight, and our findings are in contrast with the results of Hoyo *et al.*¹³, who reported an increase in the number of these cells, as well as of several cytokines which are present in ACL, stimulated by the ultraviolet rays. Comparing the number of these cells in ACL with the number of cells in normal skin of the

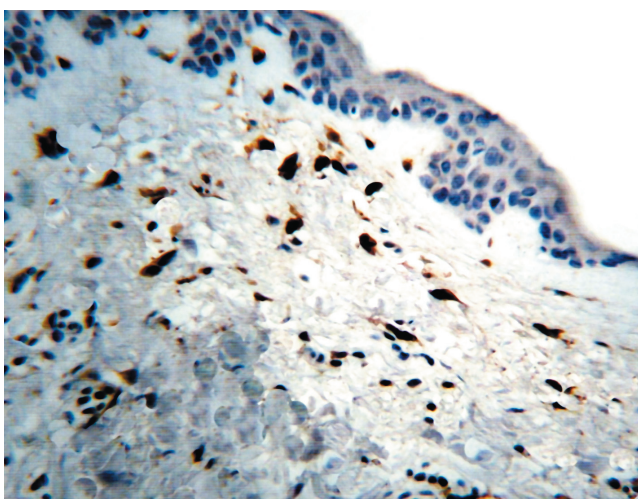


FIGURE 2: FXIIIa+ dermal dendrocytes in the dermis of normal control skin (immunoperoxidase with anti-FXIIIa antibody, 400x)

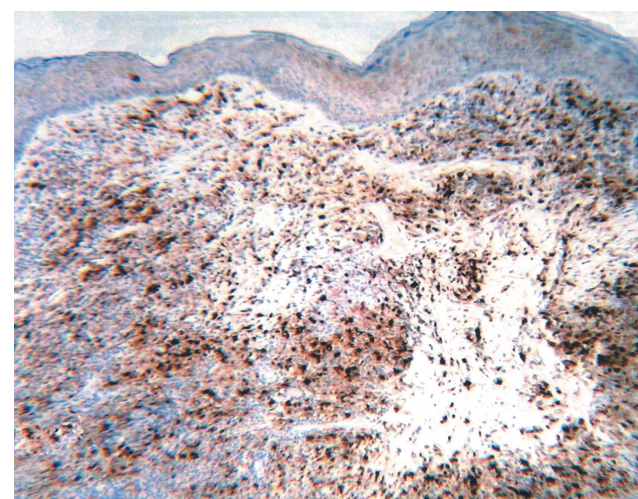


FIGURE 4: Macrophages in the epidermis and amidst the dermal inflammatory infiltrate in a cutaneous leishmaniasis lesion (immunoperoxidase with antiCD68 antibody, 100x)

TABLE 4: Aspects regarding the presence or absence of ulceration, histopathological groups and quantification of the immunomarked cells of patients with cutaneous leishmaniasis (all positive for the anti-*Leishmania* antibody)

Case	Aspects	Histopathological group	FXIIIa+ (*)	CD1a	CD68	CD20	CD3
1	P/ulc	II	32	++	+++	++	+++
2	N/ulc	II	62	+	+++	+	++
3	P/ulc	IV	0	+	+++	++	+++
4	P/ulc	II	51	++	++	+++	++
5	N/ulc	V	63	++	++	+++	++
6	N/ulc	II	47	+++	++	++	+
7	N/ulc	I	30	+++	+++	++	+++
8	N/ulc	III	33	++	++	+++	++
9	Ulceration	II	36	++	+++	+	++
10	P/ulc	I	20	++	++	+	++
11	P/ulc	I	52	+++	++	+++	++
12	N/ulc	I	40	++	++	+	++
13	P/ulc	I	51	++	++	++	+
14	Ulceration	V	NR	++	+++	++	+++
15	P/ulc	III	19	++	+++	++	+
16	N/ulc	I	29	++	++	+	++
17	P/ulc	II	63	++	+++	+	+++
18	Ulceration	II	0	0	++	+	+++
19	P/ulc	I	12	+	+++	+	++
20	P/ulc	II	38	++	+++	++	+++
21	P/ulc	I	31	++	+++	+	+++

(*) number of cells in five sequential high-magnification fields (400x);

P/ulc: partially ulcerated; N/ulc: not ulcerated epidermis; Ulceration:

totally ulcerated; 0: absence of cell marks; NR: count not performed for technical reasons;

+ small amount of immunopositive cells; ++ moderate amount of immunopositive cells;

+++ : great amount of immunopositive cells

suprapubic region, that is unexposed to sunlight and in which the number of DD should be decreased, no statistically significant evidence was found.

Comparing the amount of FXIIIa+ cells in the normal skin control group and in the predominant histopathological groups of ACL (I and II), there was no statistically significant difference. This finding should be analyzed in further studies, correlating the inflammatory cell infiltrate and time of lesion.

Pagliari & Sotto¹⁴ investigated the DDFXIIIa+, analyzing their morphological aspects and quantifying them in skin with paracoccidioidomycosis and in normal skin. In the skin with the fungus, the FXIIIa+ cells were observed at locations similar to those found in this study, and the reduced number of these cells in the normal skin was used as control.

In a study on DDFXIIIa+ in 47 ACL cases, Sotto¹⁶ also found hypertrophy and an increased number of these cells, as compared to the normal skin control group (five specimens from the thoracic region – an area of little sun exposure – and five from the knee region – an area of relatively greater sun exposure). This DDFXIIIa+ population did not pre-

sent any difference when compared with the granulomatous or unspecific tissue response. The author further observed that these cells have the capacity of internalizing amastigote forms of *Leishmania*. Based on these results, he concluded that the DDFXIIIa+ take part in the pathogenesis mechanisms of ACL, probably also acting as APC's, conclusions which are similar to those of a research on chromoblastomycosis.^{16,18-20}

Natah et al.¹⁸ did not rule out that a subgroup of FXIIIa+ cells might represent epithelial Langerhans cells, which do not yet express the CD1a marker, that is determinant in their path to the epithelium.

The locations of the LC observed in this research are consistent with the reports of the literature, since, being located in the epidermis, they would be infected by *Leishmanias* and would then, some cells in the superficial and deep dermis and amidst the inflammatory infiltrate present in the dermis, be on the migration route towards areas of T cells.²¹ After skin infection by *Leishmania*, the LC are responsible for transporting the parasites from the

epidermis to the dermis.²² On the other hand, these cells have the unique ability of ingesting the parasites from the infection site in the skin and taking them to T-cell areas drained by the lymph nodes.²²

Cerio et al.²³ suggested that the dermal dC (DDFXIIIa+) could be a pluripotent cell population capable, under several circumstances, of acting as macrophages, as APC and probably differentiating themselves into Langerhans cells and migrating to the epidermis.

Macrophages, immunomarked with the anti-CD68 antibody, were the cells observed in the greatest amounts as compared to others, both in the epidermis, isolated as exocytoses or in microabscesses, and in the dermis, amidst the inflammatory infiltrate, in focal or diffuse concentrations. Semi-quantitative analysis of these cells did not show any significant quantitative correlation between the FXIIIa+, CD1a and CD20 or CD3 cells.

The B lymphocytes immunomarked with the anti-CD20 antibody varied in marking intensity and semi-quantitative analysis in relation to the histopathological groups. They were not observed in the epidermis, and were present in nests or clusters in the dermis amidst the inflammatory infiltrate. We observed that there is a reverse semi-quantitative relation between the CD20 and the CD3 cells, i.e., the greater the amount of CD20 cells, the smaller or more moderate the amount of CD3 cells, and vice-versa.

In relation to the B lymphocytes, the T lymphocytes immunomarked with anti-CD3 antibody were always in a location different from those, or amidst them.

The presence of dermal dendrocytes is related, according to the literature, to antigen presentation to T lymphocytes.^{24,25} The analysis of T and B lymphocytes, although done in a global manner, had the objective of assessing their relation with dendrocytes.

The similarity of the number of DDFXIIIa+ found in this study with the amount found in normal skin could be a consequence of the alterations observed in the DDFXIIIa+ population, resulting from factors present in the skin environment, related to characteristics of the immune response mediated by ACL cells. It is speculated whether the finding of a non-increased number of DDFXIIIa+, as in other research^{16,19,20}, could result from the migration of LC/DD FXIIIa+ from skin to lymph nodes. A greater number of ACL cases and a comparison of the amount of DDFXIIIa+ with normal skin from sun-exposed and unexposed areas are believed to be needed to achieve a statistically significant result.

CONCLUSIONS

1) The histopathological groups proposed by Magalhães et al.^{3,4} that predominate were I (exudative cell reaction) and II (exudative and necrotic reaction), with a similar number of cases. The number of DDFXIIIa+ was statistically higher in group II than in group I.

2) The number of FXIIIa+ dermal dendrocytes showed no statistically significant difference in the ACL lesions when compared to that of normal controls.

3) The CD1a+, CD68+, CD20+ and CD3+ cells did not show any relevant relation, either between each other or with regard to the FXIIIa+ cells. □

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