Inflammation in disseminated lesions: an analysis of CD4+, CD20+, CD68+, CD31+ and vW+ cells in non-ulcerated lesions of disseminated leishmaniasis

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Disseminated leishmaniasis (DL) differs from other clinical forms of the disease due to the presence of many non-ulcerated lesions (papules and nodules) in non-contiguous areas of the body. We describe the histopathology of DL non-ulcerated lesions and the presence of CD4-, CD20-, CD68-, CD31- and von Willebrand factor (vW)-positive cells in the inflamed area. We analysed eighteen biopsies from non-ulcerated lesions and quantified the inflamed areas and the expression of CD4, CD20, CD68, CD31 and vW using Image-Pro software (Media Cybernetics). Diffuse lymphoplasmacytic perivascular infiltrates were found in dermal skin. Inflammation was observed in 3-73% of the total biopsy area and showed a significant linear correlation with the number of vW+ vessels. The most common cells were CD68+ macrophages, CD20+ B-cells and CD4+ T-cells. A significant linear correlation between CD4+ and CD20+ cells and the size of the inflamed area was also found. Our findings show chronic inflammation in all DL non-ulcerated lesions predominantly formed by macrophages, plasmacytes and T and B-cells. As the inflamed area expanded, the number of granulomas and extent of the vascular framework increased. Thus, we demonstrate that vessels may have an important role in the clinical evolution of DL lesions.

Key words: disseminated leishmaniasis - histopathology - immunohistochemistry

Skin infection by *Leishmania braziliensis* promastigotes results in different forms of cutaneous lesions that include, but are not limited to localised (CL), disseminated (DL) and mucocutaneous forms. DL was first described clinically in Costa et al. (1986) and immunologically described in Turetz et al. (2002). DL can be distinguished from other forms of leishmaniasis by the clinical manifestation of multiple acneiform, papules and nodular lesions in two or more non-contiguous areas of the body (Bittencourt & Barral 1991). The ulcer, likely in the same place as the haematophagous insect bite, is the primary lesion of CL and DL (Bittencourt & Barral 1991). These ulcerated lesions share the same histopathological aspects in CL and DL (Carvalho et al. 1994). Although the dissemination mechanism remains unclear, the ulcerated lesions in CL and DL are indistinguishable.

Tissue analyses of typical CL are required to diagnose CL or DL. Histopathological analysis of the CL ulcer reveals a chronic perivascular infiltrate of lymphocytes, plasmocytes, macrophages, epithelioid and giant cells, all of which eventually arrange in granulomas with or without necrosis (Bittencourt & Barral 1991, Machado et al. 2002). Prior to ulcer formation, patients with early CL present with a non-ulcerated lesion (Magalhães et al. 1986).

However, biopsies of early CL are difficult to obtain because patients usually seek out for medical attention only when they have an ulcerated lesion. Therefore, in this study we have analysed non-ulcerated lesions from DL patients in attempting to better understand the non-ulcerated cutaneous lesion present in DL and in early CL. These non-ulcerated lesions were biopsied, processed and analysed. Inflammatory infiltration and vessels expressed by CD31 and von Willebrand's factor (vW) quantified. In parallel, we correlated quantified inflammation with the expression of CD4, CD20 and CD68 cell markers.

PATIENTS, MATERIALS AND METHODS

DL patient selection - All patients included in this study were recruited from Corte de Pedra, a village located southwest of Salvador in the state of Bahia, Brazil. Informed consent was obtained from all patients and the project was approved by the Human Ethical Committee of the Gonçalo Moniz Research Centre, Oswaldo Cruz Foundation, protocol 221/2010. Patients under 18 years of age were included with the approval of their parents or other guardian. Patients who were on treatment and children under four years old were excluded from the study.

DL diagnostic criteria - Patients were clinically diagnosed for DL. The diagnosis was confirmed by positive delayed-type hypersensitivity (DTH) to leishmanial an-

Financial support: NIH (AI-30639)
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Received 7 February 2012
Accepted 5 October 2012

tigens via the Montenegro skin test and histopathological identification of *Leishmania* amastigotes. The DTH test was considered positive when the skin induration measurement was > 5 mm after 48-72 h, after the injection of 25 µg of *Leishmania* antigen prepared as previously described in 0.1 mL of saline solution intradermally into the internal side of the left forearm.

Biopsies - Only non-ulcerated, papular and nodular lesions were biopsied and included in this study. A 4-mm-diameter punch was used after application of local anesthaesia as routinely done at the health care facility for posterior diagnosis. All biopsies were maintained in formalin (10%) for a period of 24 h or less. The tissue samples were dehydrated and embedded in paraffin blocks. Five-micron slices were stained by haematoxylin and eosin (HE) and periodic acid-Schiff to exclude the possibility of infection by fungi.

Histopathological analysis - The histological analysis included objective and subjective reasoning as well as morphometry and immunohistochemistry (IHC) procedures. Conventional methodology (paraffin embedding and HE staining) was also used.

IHC - Four-micrometer sections were obtained from paraffin-embedded tissue and mounted onto 3-aminopropyltriethoxysilane-coated glass slides. Sections were deparaffinised with xylene and rehydrated with descending graded alcohols and distilled water. Peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. Sections were antigen-retrieved in a 96ºC bath with citrate buffer (DAKO target retrieval solution) as needed and nonspecific reactions were blocked with 3% powder milk for 20 min. The slides were incubated for 1 h with anti-CD4, anti-CD20, anti-CD31, anti-CD68, anti-vW and rabbit anti-*L. braziliensis* antibodies with respective dilutions of 1:10, 1:20, 1:15, 1:500, 1:50 and 1:1600 at 25ºC as suggested by fabricant (DAKO). The immunostaining was performed using an Envision TM+Dual Link System-HRP (DAB) (DAKO K4065-1). All slides were counterstained with Harris haematoxylin, dehydrated and mounted with Canadian balsam and glass cover slips. Sections were analysed by optic microscopy (NIKON E-600).

Morphometric analysis - All stained sections were captured using an optical microscope coupled to a digital colour video camera at 400X magnification. The resulting images were morphologically analysed semi-automatically with the Image-Pro Plus (Media Cybernetics, USA) software. Inflammation percentage was possible to be determined in all sections with the use of the software mentioned above. Each skin fragment extent was measured by circling its total area and compared with the inflamed area measured using the same method. Positivity was defined as the identification of structures marked in brown as visualised with the chromogen diaminobenzidine. For amastigotes, the size, shape and location of structures within vacuoles of macrophages were also considered.

Quantitative analysis of cell populations of $CD4^+$ T-lymphocytes, $CD20^+$ B-lymphocytes, $CD68^+$ macrophages, $CD31^+$ and vW factor$^+$ endothelial cells was determined by measuring the positive areas in captured images. Positive cells were manually counted on a computer monitor by circling stained cells. Vessel areas marked with anti-CD31 and anti-vW factor were quantified by circling all of the marked vessel lumen. The number of positive cells was expressed in cells/μm^2.

Representation and statistical analysis of results - The data were presented in tables and graphics in which the numbers represent absolute values or proportions. The data was validated by applying the linear correlation of Pearson. Significance was accepted at $p < 0.05$.

RESULTS

Secondary skin lesions emerged after *L. braziliensis* dissemination. These secondary lesions were in general acneiform, papular or nodular and non-contiguous with the primary ulcer. In this study, we biopsied and analysed non-ulcerated lesions that occurred in 18 patients with DL (Fig. 1A).

DL was not equally distributed between males (65%) and females (35%). The mean age ± standard deviation was 29 ± 14 years (median: 27; ranges: 5-50 years). Patients presented more than 10 nodular lesions (Table). Histopathological analysis revealed a chronic inflammatory reaction in all 18 biopsies (Fig. 1B).

All fragments of non-ulcerated lesions displayed a lymphoplasmacytic infiltrate, every fragment presented inflammation which varied from 3-73% from the entire area of each respective fragment extension; a rich vascularised framework was also observed (Fig. 1B). Other general histopathological findings aside of vessels in papillary dermis in all 18 biopsies: perivascular and papillary oedema (16), vasculitis (6), necrosis (9), granulomas (9), giant cells (4), rare neutrophils (6) and pigmentary incontinence (14). Amastigotes were observed frequently in HE stained slide (9) biopsies; by IHC, we were able to detect amastigotes in 10 of 18 biopsies (Fig. 1C).

The inflammatory cells in non-ulcerated biopsies were quantified by number of cells per μm^2. The following results were calculated: $CD68^+$ macrophages, 103.4 ± 64.1 cells/μm^2, $CD20^+$ B-cells, 100.2 ± 72.2 cells/μm^2 and $CD4^+$ T-cells, 62.7 ± 32.8 cells/μm^2 (Figs 1D, 2A). $CD20^+$ B-cells and $CD4^+$ T-cells were significantly associated with the extent of inflammation (Figs 2B, 3A, B). Other cells at the site of inflammation were not identified with the three cell markers used. It is likely that $CD8^+$ T-cells, plasmacytes and undifferentiated cells could also be associated with inflammation in skin lesions after *Leishmania* dissemination.

Vessels and activated endothelial cells were prominent in the skin lesions. Small capillaries were seen at the papillary dermis as well as in the superior dermis. Perivascular oedema and a lymphoplasmacytic infiltrate were also seen even in the less inflamed biopsies (Fig. 3C).

Vessels were identified by immunostaining using anti-CD31 and anti-vW factor (Fig. 4A, B). Both endothelial cell markers reacted similarly (data not shown). There is a significant linear correlation between vessels and the inflammation extent ($r^2 = 0.2859$; $p = 0.027$) (Fig. 4C).

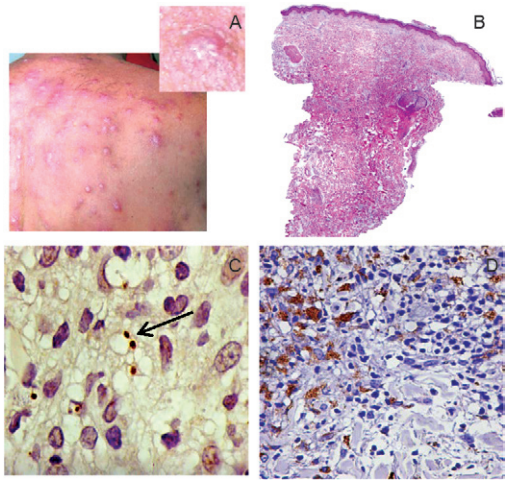

Fig. 1A: clinical characteristics of patient diagnosed with disseminated leishmaniasis due to *Leishmania braziliensis* dissemination. The insert shows a detail of a typical non-ulcerated lesion; B: haematoxylin and eosin (HE) stained section of a non-ulcerated lesion biopsy with 3% of inflammation; C: immunostained *Leishmania* amastigotes (1,000X) (arrow); D: immunostained CD68$^+$ macrophages (400X).

TABLE

Patients clinical characteristics

Variable	n = 18
Age (mean in years ± SD)	29 ± 14
Sex (female/male)	06/12
Duration of disease (mean in days ± SD)	44 ± 25
Number of secondary lesions	10 < 1,000

SD: standard deviation.

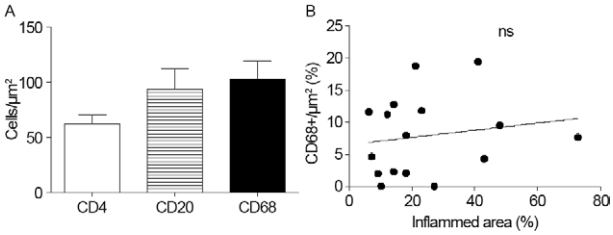

Fig. 2A: CD4$^+$ T-cells, CD20$^+$ B-cells and CD68$^+$ macrophages per µm^2 increase with the extent of inflammation; B: Pearson's correlation [not significant (ns)] coefficient between CD68$^+$ cells and the inflammation extent of skin lesions after *L. braziliensis* dissemination.

DISCUSSION

DL lesions differ clinically from CL lesions, which are characterised by ulcerated skin lesions. DL leads to the formation of multiple non-ulcerated lesions with a greater number of pleomorphic cells than CL lesions. This indicates the participation of inflammation during early infection.

However, our data pointed out two aspects not previously emphasised in the histological studies of CL. First, the high frequency of B-cells and plasmocytes in non-ulcerated lesions points to a role of antibodies in the pathological process or in the immune response to *Leishmania*. Secondly, this is the first time attention has been given to the perivascular inflammation and associated frequency of endothelial cells within the inflamed area in non-ulcerated lesions.

Inflammation ranged from minimal to extensive infiltration in the analysed tissues that demonstrated evidence of pathological changes. Thus, the varying extent of chronic inflammatory infiltrates may suggest that the development of lesions is a continuous process.

Dermal inflammation reaches the epidermis, causing acanthosis, spongiosis and exocitosis (data not shown). Perivascular oedema was commonly found and is probably associated with the progression of pathology. Lymphoplasmacytic perivascular infiltrations in the dermis were observed in all non-ulcerated lesions. We observed amastigotes by IHC in 10 out of 18 tissue samples. These results may indicate that tissue inflammation results from *Leishmania* antigen-specific response.

A great number of CD20$^+$ B-cells were found in ulcerated lesions and in diffuse cutaneous leishmaniasis is a rare manifestation of leishmaniasis, characterised by multiple, slowly progressive nodules or plaques without ulcers (Magalhães et al. 1986). The presence of oedema in all biopsies analysed for this study may reinforce this assumption. Parasites may take advantage of the antigen-specific response by penetrating into phagocytes (Schurr et al. 1986). Oedema containing IgG may help opsonalization facilitating phagocytosis.

Inflammation in CL has been associated with T-cells, as demonstrated by a positive correlation between the frequency of CD4 T-cells producing tumour necrosis factor (TNF)-a or interferon (IFN)-g (Schurr et al. 1986). Here we show that B-cells are also involved in the pathology because there is a correlation between the presence of CD20$^+$ B-cells and tissue inflammation. Further studies exploring the role of B-cells in the pathogenesis of *L. braziliensis* should be performed.

CD4$^+$ T-cells are well implicated in the immune response against *Leishmania* and are the main source of IFN- g in CL (Faria et al. 2009). With the evolution of the disease, a higher number of CD4$^+$ T-cells become correlated with the presence of IFN- g (Vieira et al. 2002, Faria et al. 2009). As the disease evolves, a growing number of CD4$^+$ T-cells produce IFN- g. Our analysis of CD4$^+$ T-cells is consistent with a mechanism of inflammation progression in which CD4$^+$ T-cells activate macrophages against amastigotes. Our results on CD4$^+$ T-cells were expected given the growing chronic inflammation seen in *L. braziliensis* tissue (Antonelli et al. 2005, Carneiro et al 2009).

When hosting *Leishmania* amastigotes, macrophages activated by T-helper (Th)1 and IFN- g may control parasite's growth. Th1 lymphocytes generate interleukin (IL)-10, which may down-regulate IFN- g (Wana-

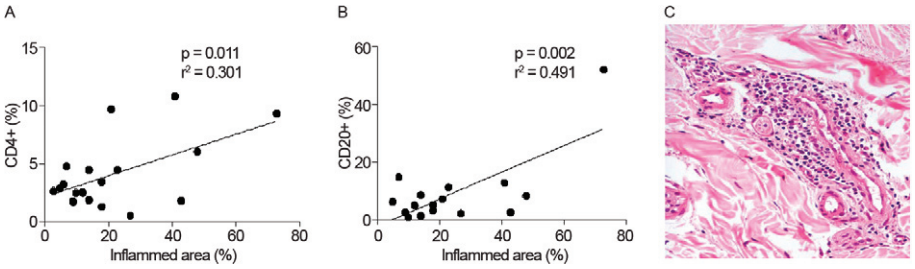

Fig. 3A: linear correlations of Pearson between $CD4^+$T and inflamed area; B: linear correlations of Pearson between $CD20^+$ B-cells and inflamed area; C: chronic perivascular inflammation stained with haematoxylin and eosin (200X).

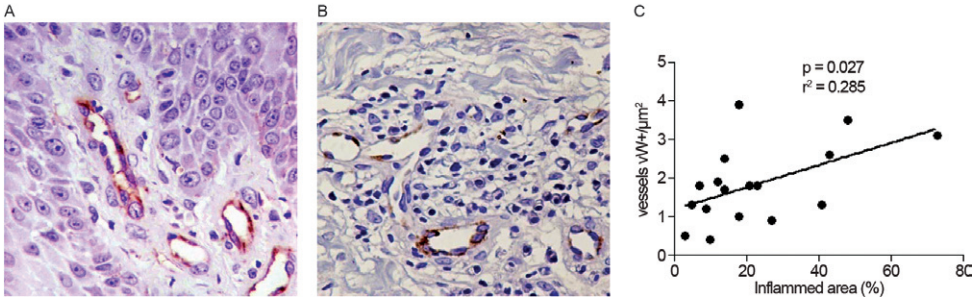

Fig. 4A: immunostained $CD31^+$ small vessels (400X); B: immunostained von Willebrand factor (vW) positive in the dermis (400X); C: linear correlation of Pearson between vW^+ vessels and inflammation in the 18 non-ulcerated lesions.

sen et al. 2008). Great expression of IL-10 at inflamed sites may regulate the host response to tissue damage and lead to posterior healing (Vieira et al. 2002, Wanasen et al. 2008).

Our study highlights a possible correlation between the presence of granulomas and inflammation in *L. braziliensis* infected tissue. This finding may be an indication of the immune modulation of tissue inflammation. IFN-g and TNF-a in the inflamed tissue may lead to an inflammatory response that forms granulomas (Silveira et al. 2005). Granulomas were present in nine out of 18 the biopsies analysed; these biopsies were also observed to be among the ones with more inflammation area. The total amount of cells that were immune-marked in tissue samples composed 30% of the entire fragment measured. All other cells composing the fragment inflammatory infiltrate may be plasmocytes, $CD8^+$ T-cells, natural killer or mast cells, among other cells not analysed in this study.

Furthermore, in less inflamed tissue, a diffuse cellular infiltration remained, surrounding small venules. Our data demonstrates a relevant correlation between vW^+ cells and inflammatory infiltrates. Therefore, our analysis presents the possibility that vessels are present and may proliferate from an early stage of the disease. The amount of $CD31^+$ cells were similar to vW factor when vessels were enumerated (data not shown).

We hypothesise that vessels play a crucial role in the early events after *L. braziliensis* dissemination. Thus, the increasing expression of vW in the vasculature of DL non-ulcerated lesions is a potential regulator of lesion growth (Low & Di Pietro 2003, Pusztaszeri et al. 2006).

As our results indicate, vessel proliferation may allow parasite survival and growth. This phenomenon could lead and sustain the evolution of cutaneous lesions.

Non-ulcerated lesions apparently have a variety of inflammation intensities, ranging from moderate to intense with cells organised in granulomas. Our findings suggest that chronic inflammation was present in all non-ulcerated lesions and was predominantly formed by macrophages, plasmocytes, T and B-cells.

REFERENCES

Antonelli LR, Dutra WO, Almeida RP, Bacellar O, Carvalho EM, Gollob KJ 2005. Activated inflammatory T-cells correlate with lesion size in human cutaneous leishmaniasis. *Immunol Lett 101*: 226-230.

Bittencourt AL, Barral A 1991. Evaluation of the histopathological classifications of American cutaneous and mucocutaneous leishmaniasis. *Mem Inst Oswaldo Cruz 86*: 51-56.

Carneiro FP, de Magalhães AV, Couto AJALA, Bocca Al, Muniz-Junqueira MI, Sampaio RNR 2009. Foxp3 expression in lesions of the different clinical forms of American tegumentary leishmaniasis. *Parasite Immunol 31*: 646-651.

Carvalho EM, Barral A, Costa JML, Bittencourt A, Marsden P 1994. Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis. *Acta Trop 56*: 315-325.

Costa JM, Marsden PD, Llanos-Cuentas EA, Netto EM, Carvalho EM, Barral A, Rosa AC, Cuba CC, Magalhães AV, Barreto AC 1986.

Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. *J Trop Med Hyg 89*: 319-321.

Faria DR, Souza PE, Durães FV, Carvalho EM, Gollob KJ, Machado PR, Dutra WO 2009. Recruitment of $CD8^+$ T-cells expressing granzyme A is associated with lesion progression in human cutaneous leishmaniasis. *Parasite Immunol 31*: 432-439.

Low EQ, Di Pietro LA 2003. Quantification of wound angiogenesis. *Methods Mol Med 78*: 319-327.

Machado P, Araujo C, Silva AT 2002. Failure of early treatment of cutaneous leishmaniasis in preventing the development of an ulcer. *Clin Infect Dis 34*: 69-73.

Magalhães AV, Moraes MAP, Raick AN, Llanos-Cuentas A, Costa JML, Cuba-Cuba C, Marsden PD 1986. Histopatologia da leishmaniose tegumentar por *Leishmania braziliensis braziliensis*. 1. Padrões histopatológicos e estudo evolutivo das lesões. *Rev Inst Med Trop 28*: 253-262.

Pusztaszeri MP, Seelentag W, Bosman FT 2006. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor and Fli-1 in normal human tissues. *J Histochem Cytochem 54*: 385-395.

Schurr E, Kidane K, Yemaneberhan T, Wunderlich F 1986. Cutaneous leishmaniasis in Ethiopia: I. Lymphocyte transformation and antibody titre. *Trop Med Parasitol 37*: 403-408.

Silveira FT, Lainson R, Corbett CEP 2005. Further observations on clinical, histopathological and immunological features of borderline disseminated cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. *Mem Inst Oswaldo Cruz 100*: 525-534.

Turetz ML, Machado PR, Ko AI, Alves F, Bittencourt A, Almeida RP, Mobashery N, Johnson Jr WD, Carvalho EM 2002. Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. *J Infect Dis 186*: 1829-1834.

Vieira MGS, Oliveira F, Arruda S, Bittencourt AL, Barbosa Jr AA, Barral-Netto M, Barral A 2002. B-cell infiltration and frequency of cytokine producing cells differ between localized and disseminated human cutaneous leishmaniases. *Mem Inst Oswaldo Cruz 97*: 979-983.

Wanasen N, Xin L, Soong L 2008. Pathogenic role of B-cells and antibodies in murine *Leishmania amazonensis* infection. *Int J Parasitol 38*: 417-429.