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Full Length Research Paper

Quantitative evaluation of the mast cell population in border of ulcers in America Cutaneous Leishmaniasis – ACL

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Abstract

American Cutaneous Leishmaniasis (ACL) is an infectious disease with a broad spectrum of presentation, has two clinical poles, one anergic and other hyperergic. The inflammatory response to the etiologic agent is complex and involves several cell lines, especially lymphocytes, plasma cells, histiocytes, and antigen-presenting cells. Other cells are observed in inflammatory exudate in a lesser degree, such as eosinophil and mast cells – MC. Several studies have sought to investigate the role of mast cells in cutaneous Leishmaniasis. This study evaluated 64 skin biopsies of leishmanial ulcer edge. The number of mast cells and leishmania were observed in the timeline. It was found that the number of mast cells and leishmania decline with age of the lesion, there is a negative correlation $p<0.05$. In the late stages of injury (more than eight weeks) the number of mast cell sin the leishmanial lesion sis almost nil. The authors question whether there is really involvement of the mast cells in the chronic phase of Cutaneous Leishmaniasis.

Keywords: Mast Cell, Leishmaniasis, Cutaneous Leishmaniasis.

INTRODUCTION

American Cutaneous Leishmaniasis (ACL) is a group of infectious diseases with the clinical spectrum of presentation similar to that seen in leprosy, ranging from one pole hyperergic to another anergic (Herath et al., 2010). The inflammatory response to the etiologic agents complex and involves several cell lines, especially lymphocytes, plasma cells, histiocytes, and antigen-presenting cells (Gaafar et al., 1995; Elhassan et al., 1994). Other cells are observed in inflammatory exudate in a lesser degree, such as eosinophil and mast cells - (Katakura et al., 1993; Marovt et al., 2010; Saito et al., 1996). Knowledge about the histophysiology of mast cells and eosinophil in recent years, has gained attention from researchers. The role of these cells in the body homeostasis, inflammatory response and infections has been described (Saito et al., 1996; SILVA, 2008; Oliani and Gil, 2006; Jeziorska et al., 1995; Yazdanbakhsh, 1996). Studies has shown a complex machinery, present especially in mast cell involved in the production of complex molecules that regulate the vascular diameter, leukocyte migration, as well as intervening in the modulation of immune response in the skin and mucous membranes (Theoharides and Cochrane, 2004; Raposo et al., 1997; Metz and Maurer, 2007). This knowledge has allowed mast cells to be added as part of inflammatory response, including the inflammatory phenomena determined by biological agents. This study evaluates the mast cells population in the early and late

phase of the ACL, discussing the results based on the current literature, speculating what would be the role of this cell in the afore said morbidity.

MATERIALS AND METHODS

Ethical approval for this study was obtained from the relevant local ethics committees (Ethics committee of the Medical School of the Federal University of Ceará in Barbalha).

Tissue specimens and patients

Data relating to patients were obtained from Outpatient Tropical Medicine at the Federal University of Ceará, Campus Cariri, Ceará, Brazil. The health records of patients were retrieved and sociodemographic and clinical data were analyzed (n=64, male: female ratio =1.56; group mean age =37.46875 ±19.84381). The diagnoses of patients presenting ACL were confirmed by clinical examination, Montenegro intradermoreaction (IDRM), culture with NNN media and histopathological analysis in the Laboratory of Experimental Pathology (LAPEX) from College of Medicine at the Federal University of Ceará, Campus Cariri, Ceará, Brazil. The archived tissue blocks from surgically resected samples (n=64) at LAPEX were evaluated in this study.

Histological, histochemical staining and Immunohistochemistry for identification of mast cells and Leishmania

For the purposes of morphological and histochemical analysis, samples were fixed in formalin, embedded in paraffin, serially sectioned at 05 microns and evaluated under a conventional light microscope. Sections were stained with hematoxylin and eosin (H&E) for routine examine. In order to visualize mast cells, two sections of each sample were stained with 1% toluidine blue and counter stained with 5% methanol yellow for 5 min, following which the sections were dehydrated, cleared and mounted with synthetic balsam. The sections were stained with Giemsa for Leishmania visualization and count (Armed Forces Institute of Pathology, 1960).

For the Immunohistochemical evaluation of Mast cell, in order to compare the histochemical count, sections were mounted on organosilane-coated slides (Dako Silanized Slides Code No. S3003). The primary mouse monoclonal antibody against Tryptase Antigen (Clone AA1 - Isotype IgG1, kappa, to formalin use of the DAKO CORPORATION) was detected with the aid of an ELITE ABC KIT (product # K0690, Vector Laboratories, Inc. 30-Ingold Road, Burlingame, CA 94010 – USA), employing chromogen diaminobenzidine for colour development. Slides were then counter stained with Mayer's hematoxylin and mounted. Negative controls were obtained by substituting normal whole rabbit serum (product # X0902; Dako) for the primary antibodies. A sample of the normal skin (shown previously to be strongly positive to MAST CELL) was used as a positive control.

For the Immunohistochemical evaluation of Leishmania, in order to compare the count of histochemical (Giemsa); four-micron thick histological sections were collected on previously prepared slides (Dako Silanized Slides Code No. S3003); then deparaffinated in xylene and rehydrated in ethanol and water were performed. 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 (30mlTRIS + NaCl + 0.3g of bovine albumin Merck + 0.3gMolico skimmed powdered milk). After draining the milk, the primary antibodies Anti-Leishmania polyclonal antibody (diluted at 1/4000) in diluting solution with a Dako unspecific bond reducer were used, then placed in a humid chamber at room temperature for 30 minutes. The biotinylated secondary antibody from an LSAB kit DAKO was used. The development (antigen-antibody bond) was visualized with diaminobenzidine, Dako DAB+ kit, the slides counter stained with Harris' hematoxylin, and mounted. Anti-*Leishmania* serum was produced at the Leishmaniasis Surveillance Laboratory of Evandro Chagas Clinical Research Institute (IPEC), FIOCRUZ, by immunization of albino rabbits inoculated with four doses of soluble protein extracts of promastigotes forms of *Leishmania (Leishmania) chagasi* (MHOM/BR/1974/PP75).

Determination of mast cells

Mast cells was identified on the basis of cytoplasm with intense purple granules and nuclei presenting a bluish morphological aspect with Toluidine method (figure 1), and cytoplasm with brown colour at Immunohistochemistry (figure 2). For quantitative analysis, an Optical microscope Olympus -BH2 (model CX31, RTSF, Miami, FL, USA), with • 10 ocular and • 40 objective lenses, was employed, and an ocular lattice (area 0.092 mm2) with 100 points composed of 10 horizontal and 10 vertical test lines was superimposed on the test field to be measured. A total area of 1.84 mm2 was evaluated for each of the samples, and this corresponded to 20 randomly selected high-power microscopic fields located in regions of high mast cells densities.

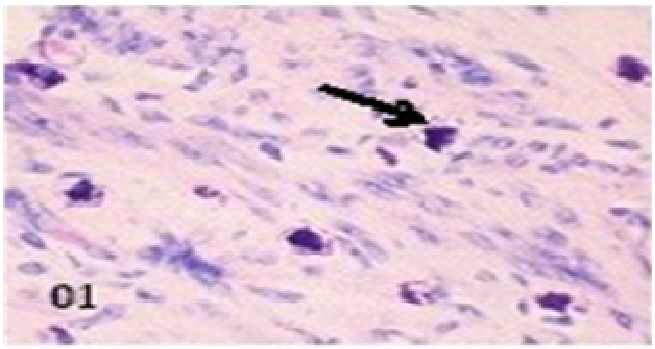

Figure 1. Mast cell stained by toluidine.

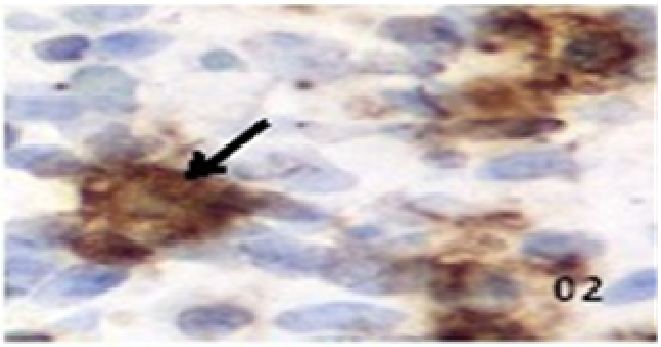

Figure 2. Mast cell by immunohistochemistry.

Figure 3. Leishmania by immunohistochemistry.

Determination of Leishmania

The Leishmania stained with Giemsa were identified as small body vacuolated with blue kinetoplast, observed within macrophages or scattered in tissue (figure 4). In the immunohistochemistry small brown point were observed (figure 3). For quantitative analysis, an Optical microscope Olympus - BH2 (model CX31, RTSF, Miami, FL, USA), with • 10 ocular and • 40 objective lenses, was employed, and an ocular lattice (area 0.092 mm2) with 100 points composed of 10 horizontal and 10 vertical test lines was superimposed on the test field to be measured. A total area of 1.84 mm2 was evaluated for each of the samples, and this corresponded to 20 randomly selected high-power microscopic fields.

Statistical analysis

Data were tabulated in Microsoft Excel spread sheet (2010). A data base was performed with the Epi Info Version 3.5.3 release by CDC in January 26, 2011 and Graph Pad Prism, version 3.00, mar 25, 1999. The comparison between counts made by histochemistry and

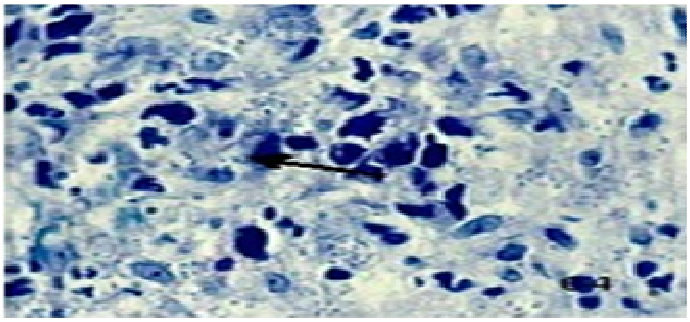

Figure 4. Leishmania stained by Giemsa.

Table 1. Epidemiological variables of the population.

	Epidemiological variables of the population	
	N(64)	N(100%)
Male	42	66
Female	22	34
Rural	60	94
Urban	04	06

Table 2. Evolution in weeks at diagnosis.

	Evolution in weeks at diagnosis					
Weeks	**N(64)**	**N(100%H)**	**MAST1***	**LEISH1***	**MAST2****	**LEISH2****
02	04	06,25%	255	192	253	133
03	05	07,90%	276	169	275	129
04	10	15,42%	439	272	438	190
05	05	07,90%	172	96	172	57
06	04	06,25%	113	58	112	38
08	06	09,40%	142	74	142	48
Recent	34	53,12%	1397	861	1392	595
09	08	12,42%	150	66	148	29
12	06	09,40%	91	27	90	09
16	05	07,90%	59	14	59	04
20	03	04,68%	31	03	31	00
22	03	04,68%	20	03	20	00
25	02	03,12%	04	00	04	00
28	01	01,56%	01	00	01	00
30	01	01,56%	00	00	00	00
32	01	01,56%	00	00	00	00
Late	30	46,88%	356	113	353	42

*Histochemical staining. ** Immunohistochemistry.

immunohistochemistry were performed using paired sample t-test, considering ap<0.05. The comparative analysis between the results obtained for the count of mast cells and Leishmania was performed using the Pearson's coefficient considering ap< 0,05.

RESULTS

The study population was predominantly male and living in rural areas as shown in Table 1. The youngest patient was 09 years old, the oldest 85. The average age of cases is around 37.4 years. The study population is made up of brown (n = 43, 67.20%), blacks, (n=04, 6.20%) and whites (n=17, 26.60%). Farmers (n = 37) account for half the population (50%). The progression of the lesions was divided into recent and late. Recent injuries are considered the lesions with down to 08 week sat diagnosis, late lesions with more than 08 week sat diagnosis. The distribution of the time evolution can be observed in Table 2.

The number of the Mast Cells in the lesions decreased over the weeks, as show in figure 5. This decreased observed has a negative linear correlation (Pearson's

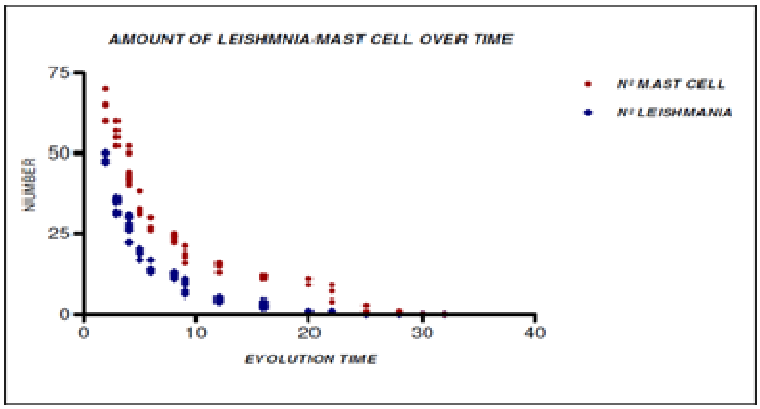

Figure 5: Amount of Leishmania/Mast cell over time

Coefficient = -0, 85061, 95% confidence interval: -0.9068 to -0.7647 with P<0.0001; Strong Negative Correlation), (Carvalho et al., 1994). A similar aspect was seen with the number of the Leishmania. The Leishmania reduce in number over time, figure 5.This reduction has a negative correlation (Pearson's Coefficient = -0, 78745, 95% confidence interval: -0.8657 to -0.6716 with P<0.0001; Strong Negative Correlation), (Carvalho et al., 1994).

The data collected show that the number of Mast Cells and Leishmania decrease with time of infection and there's a strong correlation between the reduction of Leishmania and the number of mast cell (Pearson's Coefficient = 0,9831, 95% confidence interval: 0.9068 to -0.7647 with P<0.0001).

There was no statistical difference between the count of mast cells in histochemistry and immunohistochemistry. On the other hand count for Leishmania the immunohistochemistry was more sensitive than histochemistry.

DISCUSSION

The experimental model of the American Cutaneous Leishmaniasis caused by *Leishmania Mexican*, determines a progressive disease which rapidly spreads in the susceptible BALB/c mouse, whereas in C57BL/6 mice the disease tends to be self-healing. The first model it has been established that Th1 cytokines lead to disease resistance. On the other hand in the BALB/c mice early presence of IL-4 and IL-10 determine a Th2 response and disease progression (Sacks and Noben-Trauth, 2002). The cells responsible of producing these IL-4 and IL-10 cytokines in the early phases of the disease remain controversial, although cells of the innate immune system are a likely source. The mast cells are possible candidates that produce these cytokines (Vonstebut, 2007; Villaseñor-Cardoso et al., 2008). Besides, due to their location in the skin and mucosa, mast cells are one of the first cells to encounter invading pathogens. They are found in association with blood vessels, as well as in tissues and surface exposed to external environment such as skin and mucosal linings, which are common portals of infection (Galli et al., 2005). This fact would have an important role in the early stages of Leishmaniasis, say some authors (Villaseñor-Cardoso et al., 2008; Romão et al., 2009). In experimental model of Visceral Leishmaniasis an interleukin-3-dependent augmentation in mast cell committed progenitors is observed in BALB/c but not in C57BL/6 mice during *Leishmania* infection. The mast cell supernatants inhibit IFNg -dependent restriction of *Leishmania* growth in macrophages in BALB/c mice whereas the reverse phenomenon occurs in C57BL/6 mice. Most of this data point to a possible involvement of mast cells in cutaneous infection by Leishmania, although KATAKURA, 1993, demonstrated that the evolution of the Cutaneous Leishmaniasis is independent of the Mast cell (Katakura et al., 1993).

In this work we observed the presence of mast cells in regular issue in the early stages of skin lesions. The number of mast cells decreased over time. The old lesions showed a smaller number of mast cells. Reducing the number of mast cells over time showed a direct correlation with the decrease in the number of Leishmania in the wound.

The reduction of mast cells over time and maintenance of the disease creates a question: It would really be the mast cells one the initiators and maintainers of the lesion in Cutaneous Leishmaniasis? (Saha, 2004), demonstrates that *Leishmania donovani*is responsible for the modulation of the mast cells which would explain the reduction of these cells in the timeline (Saha, 2004). In the same way *Leishmnaia (Viannia) braziliensis "invitro"* may activate the Mast Cell and also modulate the cytokines production, especially IL-4 (Oliveira et al., 2004). In present study none of the cases of the recent lesion (injury less than eight weeks) was observed absence or reduced number of mast cell. The normal number of mast cells in the skin was used the data provided by Janssens and associates, (2005). On the other hand, the number of mast cells gradually reduces

with age of the lesion. This observation suggests that even though the mast cell is a cell involved n the early stages of ACL, probably should not being the late phase of the skin lesion. Who would be responsible for the maintenance of the lesion where as mast cells and Leishmania reducer significantly in the late phase of Cutaneous Leishmaniasis?

Sakaguchi and associates (Sakaguchi et al., 1995; Asano et al., 1996) reactivated interest in the concept of T-cell-mediated suppression around the year 1990 by showing that a minor population (<10%) of CD4+ T cells, which co-expresses the interleukin-2 receptor (IL-2R) α-chain (CD25), is primordial for the control of auto reactive T cells *in vivo.* Subsequent *in vitro* studies by several groups showed that CD4+CD25+ T cells are both hyporesponsive and suppressive (Thornton and Shevach, 1998; Read et al., 1998). CD4+CD25+ T cells were discovered originally in mice, but a population with identical phenotypic and functional properties has been defined recently in humans (Levings et al., 2001; Baecher-Allen et al., 2001).Studies have related the presence of this cell as the main stay in the maintenance of some diseases, especially Leishmaniasis. Considering that it produces IL4 and especially IL10. Thus, the presence of mast cells in the maintenance of the lesion would not be essential in the late phase of the Cutaneous Leishmaniasis, corroborating the findings of this study (Campanelli et al., 2006; Holaday et al., 1993; Holaday, 1999).

In the present study also found that the use of Giemsa stain to identify Leishmaniain late lesions (more than eight weeks) is not a good method. The immunohistochemistry was more sensitive and statistically representative.

CONCLUSIONS

1. The number of mast cells in Leishmanial infection reduces numerically overtime;
2. The number of Leishmania in skin lesion reduces numerically with the passage of time;
3. There is a positive and statistically significant correlation with the simultaneous reduction of mast cells and Leishmania in skin lesions leishmaniotic, whose cause is unknown;
4. The search for Leishmania in cutaneous lesions of Leishmanias is with more than 8 weeks should be discouraged.

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