B-cell Infiltration and Frequency of Cytokine Producing Cells Differ between Localized and Disseminated Human Cutaneous Leishmaniases

MGS Vieira, F Oliveira/, S Arruda, AL Bittencourt*, AA Barbosa Jr, M Barral-Netto/**/***, A Barral /**/***/+**

Centro de Pesquisas Gonçalo Moniz-Fiocruz, Rua Valdemar Falcão 121, 40295-001 Salvador, BA, Brasil *Serviço de Anatomia Patológica, Hospital Professor Edgard Santos **Faculdade de Medicina, Universidade Federal da Bahia, Salvador, BA, Brasil ***Instituto de Investigação em Imunologia, Instituto do Milênio, São Paulo, SP, Brasil

Biopsies from human localized cutaneous lesions (LCL n = 7) or disseminated lesions (DL n = 8) cases were characterized according to cellular infiltration, frequency of cytokine (IFN-γ, TNF-α) or iNOS enzyme producing cells. LCL, the most usual form of the disease with usually one or two lesions, exhibits extensive tissue damage. DL is a rare form with widespread lesions throughout the body; exhibiting poor parasite containment but less tissue damage. We demonstrated that LCL lesions exhibit higher frequency of B lymphocytes and a higher intensity of IFN-γ expression. In both forms of the disease $CD8^+$ were found in higher frequency than $CD4^+$ T cells. Frequency of TNF-α and iNOS producing cells, as well as the frequency of $CD68^+$ macrophages, did not differ between LCL and DL. Our findings reinforce the link between an efficient control of parasite and tissue damage, implicating higher frequency of IFN-γ producing cells, as well as its possible counteraction by infiltrated B cells and hence possible humoral immune response in situ.

Key words: leishmaniasis - B lymphocytes - cytokines - $CD8^+$ T cells - IFN-γ

Cutaneous leishmaniasis is a worldwide disease with severe deformating potential in new world. It affects preferentially young economically active patients representing a large burden to the public health system in developing countries. Protection against all forms of leishmaniasis is dependent on cell-mediated immunity (CMI), but the contribution of some cells and cytokines in human disease deserves further scrutiny.

$CD8^+$T cells have been implicated in protection (Muller et al. 1991) being high IFN-γ producers in a murine model of leishmaniasis (Chan 1993). Their role seems to be more in the secondary than in the primary immune response (Muller et al. 1993, 1994). On the other hand, the course of leishmaniasis in mice lacking beta 2-microglobulin (beta 2-m) gene did not differ from their wild-type counterparts (Overath & Harbecke 1993, Wang et al. 1993, Huber et al. 1998) lessening a role of antigen presentation by major histocompatibility complex class I (MHC I) molecules. In man, a higher percentage of $CD8^+$ over $CD4^+$ T cells was found in mucocutaneous leishmaniasis (MCL) lesions (Castes & Tapia 1998), compared to localized cutaneous lesions (LCL), although similar distributions of $CD4^+$ and $CD8^+$ in LCL have been reported (Barral et al. 1987, Esterre et al. 1992, Lima et al. 1994). The presence of cytotoxic $CD8^+$ T cells has been reported in peripheral blood of MCL but not in LCL patients (Brodskyn et al. 1997). Expansion of $CD8^+$ T cells occurs in the peripheral blood of individuals vaccinated against leishmaniasis (Mendonça et al. 1995, Gurunathan et al. 2000). Especially, the percentage of activated $CD8^+$ T cells was higher in fast responding than in slow responding volunteers to vaccination (Pompeu et al. 2001).

The role of B cells in leishmaniasis is also not clear. High antibody levels are present in the more severe clinical form of the cutaneous disease, namely diffuse cutaneous leishmaniasis (DCL) (Schurr et al. 1986, Mengistu et al. 1990), but B cell depletion does not alter the susceptibility or resistance pattern to *Leishmania* infection in mice (Babai et al. 1999, Brown & Reiner 1999). It seems that B cells are important to induce anti-*Leishmania* $CD4^+$ Th1 cells and DTH reaction, in the resistant mouse strain, and take part in the humoral response development in susceptible animals (Scott & Farrell 1982, Scott et al. 1986).

Predominance of Th1 cytokines like IFN-γ, IL-12, IL-2 and TNF-α over Th2 cytokines, IL-4, IL-5 IL-10 and TGF-β, is correlated in mice to the resistance profile against *Leishmania* infection (Belosevic et al. 1989, Chatelain et al. 1992, Lezama-Davila et al. 1992, Barral et al. 1993). Imunological studies in humans demonstrated a combination of Th1 and Th2 cytokines with predominance of Th1 in MCL, Th2 predominate in DCL and predominance of Th1 profile in LCL patients (Caceres-Dittmar et al. 1993, Castes et al. 1993, Tapia et al. 1993).

Human tegumentary leishmaniasis has a diversity of clinical presentations. Evaluating the in situ immune response in different presentations of human leishmaniasis

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+Corresponding author. Fax: +55-71-356.4320. E-mail: abarral@cpqgm.fiocruz.br
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may help in defining the role of cells and cytokines in the course of disease. Herein we report on a comparison of LCL to DL. LCL is the typical presentation, where the majority of the patients have one or two ulcerated lesions, elevated borders and necrotic center, preferentially localized at the lower limbs. LCL has a clinical course of several months, but may exhibit spontaneous healing and clinical cure. Disseminated leishmaniasis (DL) is a rare condition (1% of cutaneous leishmaniasis patients) and is characterized by the presence of multiple (> 20 lesions) ulcerated lesions in several parts of the body. Despite the larger number of lesions, DL patients respond promptly to antimonial treatment and heal faster than LCL patients. The mechanism of dissemination is not clear but the rapid onset, lack of lymph node enlargement and the presence of fever and chills suggest hematogenic dissemination (Costa et al. 1986, Carvalho et al. 1994). Both LCL and DL patients exhibit anti-*Leishmania* CMI (Carvalho et al. 1994). Differences in the in situ immune reactions of these two forms of human leishmaniasis may help us elucidating the participating mechanisms in the effective response against the parasite.

MATERIALS AND METHODS

Biopsy - Biopsies were obtained from seven patients with LCL and eight cases of DL, all from the endemic area Corte de Pedra, Bahia, Brazil with predominance of *L. braziliensis* (Barretto et al. 1981). LCL patients presented unique lesions with a necrotic center and elevated borders. All biopsies were taken from the borders of ulcers. DL patients showed multiple lesions, varying from papules, acneiform lesions and few ulcers. Only acneiform lesions were biopsy from DL patients. Biopsies in these, cases, involved the whole lesion. Characteristics of the patient population are summarized in the Table. Diagnosis of leishmaniasis were based on clinical and pathological observations and confirmed both parasitologically (presence of *Leishmania* amastigotes in tissue sections) and immunologically (a delayed type hypersensitivity – test larger than 5 mm and/or anti-*Leishmania* IgG antibody titers above 1/16).

Immunohistochemical reactions - Formaline-fixed and paraffin-embedded 4 µm sections were incubated in hot humid vapor of pH 6.0 citrate buffer for 5 min. An additional incubation with 10% non-fat milk for 20 min at room temperature was used to block unspecific reactions. The primary antibodies and dilutions used were: CD3 (1:100; DAKO Corporation, Carpinteria, CA, USA), CD4 (1:10; DAKO), CD8 (1:20; DAKO), CD20 (1:100; KAKO) and CD68 (1:20; DAKO). Following primary antibody incubation (40 min, at 37°C), the sections were reacted with biotinilated anti-rabbit antibody (1:300; DAKO) or pork anti-rabbit antibody (1:600; DAKO), followed by peroxidase-conjugated streptavidin (1:50; DAKO) for 30 min at 37°C. Diaminebenzidine (Vector Inc., Burlingame, CA, USA) was used as chromogen. For cytokine staining, endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 20 min at room temperature, followed by an incubation with 3% of trypsin (Sigma-Aldrich, St. Louis, MO, USA) and an additional incubation with 0.1% saponin (Sigma-Aldrich) for 15 min for permeabilizatiom. Blocking of unspecific reactions was performed with 2% normal goat serum for 20 min. The primary antibodies used were anti-IFN-γ(1:200; Genzyme Corporate Offices, Cambridge, England), anti-TNF-α (1:200; Genzyme Corporate Offices) and anti-iNOS (1:500, Genzyme Corporate Offices). Tonsil sections were used as positive controls for cell characterization and sections from pulmonary tuberculosis granulomatous lesions as controls for cytokines and iNOS staining. Monoclonal antibodies were substituted for non-immune rabbit immunoglobulins or irrelevant mouse antibodies as negative controls.

Evaluation criteria - The numbers of positive cells for each marker was counted in five 400X fields comprising a total dermic sectional area of 1.4 mm^2, using an image analysis and processing system (Quantimet Q500MC; Leica, Cambridge, UK). Reactivity to cytokines or iNOS was graded based on the number of positive cells out of 100 cells observed, and classified as negative – no cells positive; low – up to 20% of positive cells; moderate – 21% to 50% of positive cells; or high if more than 50% of the cells were positive.

Statistical analysis - Comparisons of cell numbers ($CD4^+$and $CD8^+$cells) in each disease presentation were made by Wilcoxon paired non-parametric test. For the comparison between leishmaniasis presentations (LCL x DL for each parameter), the unpaired nonparametric Mann-Whitney test was used. Significance was determined as $p < 0.05$. All statistical tests and graphs are done with Prism-GraphPad version 3 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cellular infiltration - T cells were the most frequent cell in both LCL and DL lesion infiltrate comprising approximately 34.8% of the total number of cells. A large number of $CD68^+$ macrophages were also observed (21.6%) followed by $CD20^+$ B cells on the range of 10.3% (Fig. 1). The numbers of total T cells and macrophages did not differ between LCL and DL lesions but there was a statistically significant higher frequency of B lymphocytes in LDL lesions (Fig. 1).

TABLE

Characteristics of patients with localized cutaneous leishmaniasis (LCL) or disseminated cutaneous leishmaniasis (DL)

	Male:Female	Age	No. of lesions	Lesion size (mm)	Disease duration	Positive DTH [a]	Positive serology [b]
LCL	6:1	10-68	1	20-60	15d-12m	5/7	4/5
DL	8:0	20-39	> 20	5-10	1-8 m	4/4	6/6

a/b: number of positive tests over the total number of cases analyzed.

T cells subsets - In order to explore the predominant T cell subset present in the lesion we evaluated CD4 and CD8 expression at LCL and DL lesions. There was a predominance of $CD8^+$ T cells in both disease presentations. $CD8^+$ T cells represented 68.9% of the T cells in LCL whereas $CD4^+$ T cells comprised 31.1% (Fig. 1). Similarly in DL there were 69.4% $CD8^+$ cells versus 30.6% $CD4^+$ T cells (Fig. 1).

Frequency of cell producing cytokines and iNOS enzyme at the lesion site - Expression of IFN-γ was significantly more intense in LCL samples than in DL (Fig. 2). In the 8 samples examined in LCL, IFN-γ expressing cells represented 30% of the total cells, whereas in DL less than 20% of the total cells expressing IFN-γ. No differences were observed between LCL and DL regarding iNOS and TNF-α expression.

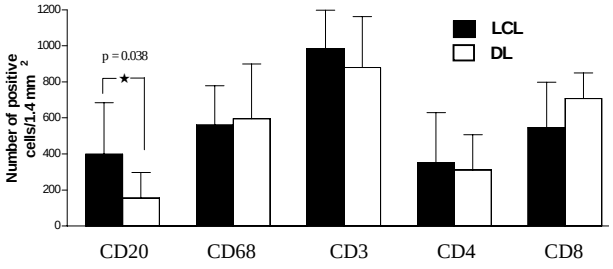

Fig. 1: increased B-cell infiltration in localized cutaneous leishmaniasis (LCL) lesions. Biopsies were taken from skin lesions of LCL and disseminated cutaneous leishmaniasis (DL) patients. Cellular subsets were identified by immunohistochemistry using anti-CD20 (B lymphocytes), anti-CD68 (macrophages), anti-CD3, anti-CD4, and anti-CD8 (T lymphocytes). Each point represents the number of positive cells in the lesion of a patient with LCL or DL lesions. Significance levels are indicated.

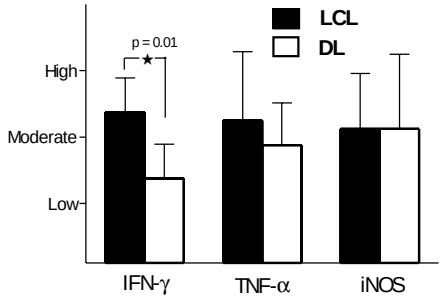

Fig. 2: frequency of cytokine and iNOS producing cells in lesions from localized cutaneous leishmaniasis (LCL) and disseminated cutaneous leishmaniasis (DL) patients. Expression of IFN-γ, TNF-α or iNOS was quantified by immunohistochemistry using anti-IFN-γ, anti-TNF-α or anti-iNOS antibodies. Negative – no positive cells; low – up to 20% of positive cells; moderate – 21% to 50% of positive cells; or high if more than 50% of positive cells. Mean is indicated by bars and standard deviation by lines above bars. Significance levels are indicated.

DISCUSSION

Lesions from LCL patients exhibited a higher frequency of IFN-γ producing cells than DL, which may suggest more efficient protection since it is the main cytokine implicated in *Leishmania* killing and host protection in all forms of leishmaniasis (Sadick et al. 1986, Belosevic et al. 1989, Sadick et al. 1990). The higher expression of IFN-γ in LCL may result in parasite containment at the site of the sand fly bite, preventing parasite dissemination. Such higher expression is not due to a larger cellular infiltration since the numbers of T lymphocytes as well as their $CD4^+$ and $CD8^+$ subsets, were similar between LCL and DL lesions. It is possible that the frequency of activated T cells differs between these two conditions, and other unexplored elements such as IL-10 or TGF-β expression may be contributing to a lesser frequency of IFN-γ producing cells in DL patients.

Despite differences in IFN-γ, there was no difference in the frequency of iNOS enzyme expressing cells between LCL and DL lesions. It is possible that other factors also able of inducing iNOS expression, like TNF-α (Liew et al. 1990a,b), compensate for the diminished IFN-γ in DL, resulting in a similar expression of iNOS. NO is considered a key element to Leishmania killing in the murine models of leishmaniasis (Green, Crawford et al. 1990, Green, Meltzer et al. 1990, Liew et al. 1990), but the NO involvement in human protection against leishmaniasis has never been clearly demonstrated. Our finding of a similar frequency of iNOS enzyme producing cells in two largely diverse presentations of human leishmaniasis with different levels of parasite control gives support to the idea that NO is not elemental in *Leishmania* killing in man.

$CD8^+$ T cells were more frequent than $CD4^+$ in both LCL and DL lesions at our observations. The role these cells play in human leishmaniasis is unclear. They might participate directly in the immune response against the parasite, and secrete Th1 cytokines, principally IFN-γ. However, our results do not lend support to a prominent role of $CD8^+$ T cells in human leishmaniasis, as two diverse forms displayed similar levels of these cells. Future studies may elucidate the role of these cells by double staining of activated cells and intracellular cytokine in situ.

Our results show B cell infiltration higher in LCL than in DL lesions. Besides antibody production, these cells have been implicated in driving Th response, antigen presentation, and expression of costimulator molecules (Liu et al. 1995, van Essen et al. 1995, Amigorena & Bonnerot 1998, Brown & Reiner 1999). In LCL lesions a higher IFN-γ expression could be stimulating B cells to secrete antibodies like IgG1, that could be involved in opsonization and more efficient phagocitosis, leading to better parasite containment. However parasites may use this way to gain entrance into phagocytes, helping in perpetuating the lesion. At present we have no sufficient elements to clarify the complex role of B cells in human cutaneous leishmaniasis.

Immune response in leishmaniasis is implicated in both protection and immunopathology. The predominant IFN-γ and B cell infiltration at LCL, which may be operative in competent containment of the parasites, may also contribute to tissue injury, which might lead to the larger and persistent lesions observed in LCL as compared to DL patients. Additionally, tissue damage may also impair access of the drugs to lesion site, which might be related to a less efficient response to drug treatment observed in LCL patients.

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