ANEXO-8

Interleukin-2 activated natural killer cells may have a direct role in the control of *Leishmania (Leishmania) amazonensis* promastigotes and macrophage infection

Aranha, F.C.S.; Ribeiro Jr, U.; Basse, P.; Corbett, C.E.P.; **Laurenti, M.D.**

Scandinavian Journal of Immunology 2005; 62: 334-341

doi: 10.1111/j.1365-3083.2005.01681.x

Interleukin-2-Activated Natural Killer Cells May Have a Direct Role in the Control of *Leishmania* (*Leishmania*) *amazonensis* Promastigote and Macrophage Infection

F. C. S. Aranha*, U. Ribeiro Jr*, P. Basse†, C. E. P. Corbett* & M. D. Laurenti*

*Infectious Diseases Laboratory (LIM-50), Department of Pathology, University of São Paulo Medical School, São Paulo, Brazil; and †University of Pittsburgh, PA, USA

Received 22 April 2005; Accepted in revised form 16 August 2005

Correspondence to: Dr M. D. Laurenti, Faculdade de Medicina da USP, Depto. de Patologia, Av. Dr. Arnaldo, 455, 1° andar, sala 1209, 01246-903, Cerqueira César, São Paulo, Brazil. E-mail: mdlauren@usp.br

Abstract

To study the role of Natural Killer (NK) cells in *Leishmania* infection, peritoneal macrophages from BALB/*c* mice were infected with *Leishmania* (*Leishmania*) *amazonensis* promastigotes and incubated with interleukin-2 (IL-2)-activated NK (A-NK) cells at different ratios of A-NK cells to infected macrophages (5:1, 1:1, 0.2:1). The A-NK cells were added either together with the parasites (0-h group) or 24 h later (24-h group). Morphological studies of the cultures revealed predominance of parasitic debris within macrophages that were in close contact with A-NK cells and the decrease in parasite recovery was directly proportional to the A-NK cell concentration used. Interferon-γ (IFN-γ) and IL-12 were detected in the supernatant at levels proportional to the A-NK cell concentration used. No significant difference was observed between the groups with respect to NO levels in the culture supernatant. When A-NK cells were added directly to the *L.* (*L.*) *amazonensis* promastigote cultures, the parasite recovery decreased proportional to the number of A-NK cells added. *In vivo* studies demonstrated smaller lesion sizes in animals inoculated with both parasites and A-NK cells compared with parasites alone. Histopathology of the skin lesions from animals receiving A-NK cells together with the parasites showed moderate parasitism and a nodular inflammatory infiltrate formed by mononuclear cells and a few vacuolized macrophages. In contrast, animals inoculated only with the parasites showed a highly parasitized dermis with infiltration of intensely vacuolized macrophages. These results demonstrate the role of A-NK cells in parasite lysis and in resistance of macrophages to *L.* (*L.*) *amazonensis* in the early phase of infection.

Introduction

Leishmania parasites are intracellular protozoa that are transmitted to vertebrate hosts via sandfly bites in the form of flagellated promastigotes [1]. The promastigotes transform into aflagellated amastigotes inside the vertebrate host and multiply by binary division inside macrophages [2]. Depending upon parasite species and host immune response, *Leishmania* infection can cause several forms of disease with different clinical manifestations [3]. Cutaneous leishmaniasis caused by *Leishmania* (*Leishmania*) *amazonensis* is responsible for a spectrum of clinical features in Brazil including localized, disseminated and diffuse cutaneous lesions; the latter, representing an anergic pole of the host response, is a severe form of the disease in Brazil [4].

Natural Killer (NK) cells participate in the innate immune response against leishmaniasis infection, but the exact role of these cells in the host defence is still a matter of discussion. These cells are an important source of interferon-γ (IFN-γ) with the potential to activate leishmanicidal mechanisms in infected macrophages and to trigger the Th-1 type of immune response [5–7]. There are also reports suggesting a direct role of NK cells on the control of the number of parasites and on lesion size in experimental cutaneous leishmaniasis [8, 9]. Despite the abundance of reports showing the protective role of NK cells in the defence against infection caused by leishmaniasis, there are only a few reports that discuss the role of NK cells in the control of the disease. Sartori *et al.* 1999 [10] showed an increase in NK cytotoxic activity in the early phase of

experimental visceral leishmaniasis; however, this increase was not able to inhibit the progression of the disease. On the other hand, Satoskar *et al.* 1999 [11] showed that mice genetically depleted of NK cells and infected with *Leishmania major* were able to produce significant amounts of interleukin-12 (IL-12) and IFN-γ and to control the progression of cutaneous leishmaniasis.

Certain subpopulations of NK cells, after incubation with IL-2, develop the ability to adhere quickly to plastic [12]. These adherent cells can be separated from the nonadherent cells and maintained in culture with IL-2. NK cells activated by IL-2 (i.e. A-NK) display high cytotoxicity *in vitro* and can kill both NK-sensitive and -resistant targets. Following the stimulation with IL-2, these cells begin to proliferate vigorously, and the size and number of their cytoplasmic granules increase [13]. The phenotype of A-NK cells has been shown to be: >95% large granular lymphocytes, >98% asialo-GM1$^+$, >60% NK1.1+ and only 4–9% LyT2+ and <2% L3T4$^+$ [12, 14–16].

In order to better characterize the role of NK cells in the early period of *L.* (*L.*) *amazonensis* infection, we evaluated the effect of A-NK cells on BALB/*c* mice peritoneal macrophage cultures infected with promastigote forms of the parasite. We analysed parasite recovery after A-NK-cell exposure and measured IFN-γ, IL-12 and NO levels in the supernatant of the cultures. We also studied the direct role of A-NK cells on promastigote forms of the parasite and on the evolution of the infection *in vivo*.

Materials and methods

Animals. BALB/*c* mice, males, between 8 and 10 weeks of age, were obtained from General Colony of University of São Paulo Medical School. The animals were kept in the laboratory during the experiments with diet and water *ad libitum* and subjected to alternating light and darkness for 12 h. The protocol was approved by the Institutional Animal Care and Use Committee of the University of São Paulo.

Parasites. L. (L.) amazonensis promastigotes (M17207 strain) were isolated from a patient with cutaneous leishmaniasis in Pará, Brazil, and classified by monoclonal antibodies and isoenzymes in the Instituto Evandro Chagas, Belém (PA), Brazil. The parasites, maintained in BALB/c mice, were isolated and expanded in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FCS (Gibco), 0.25 mM HEPES (Sigma, St. Louis, MO, USA), 10 mg/ml of gentamicin and 100 IU/ml of penicillin (complete RPMI medium) for the experiments. Parasites in the stationary phase of growth, between two and three passages in the culture, were used for macrophage infection. The parasites were washed three times in sterile phosphate saline buffer 0.01 M, pH 7.2 (PBS), and mixed with macrophages to reach a final concentration of five parasites to one macrophage.

IL-2 A-NK cells. BALB/c mice spleens were asceptically harvested, and single-cell suspensions were prepared in complete RPMI medium. After passage through nylon wool, the red blood cells were lysed by ammonium chloride (NH_4Cl 8.29 *g*; K_2HCO_3 1.0 g; EDTA 0.0372 g for 1 l of bidistilled water). Nylon wool nonadherent spleen cells were transferred into culture flasks T75 (Corning, NY, USA) with 30 ml of complete RPMI medium supplemented with 1000 U/ml of r-IL2 (Cetus Corporation, Emeryville, CA, USA). The culture flasks were maintained in a 5% CO_2 incubator at 37 °C. After 2 days, nonadherent spleen cells were removed, and plastic adherents cells were maintained in culture with 30 ml of complete RPMI medium supplemented with r-IL2 for 5 more days. A-NK cells were harvested after a short treatment with 0.02% EDTA [14, 17].

Flow cytometric analysis. The A-NK cells' phenotype was analysed. The cells were washed twice in complete RPMI medium and resuspended at 2×10^5 per tube in staining buffer (PBS 1% bovine albumin). Direct staining with conjugate antibodies (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) was performed. Four microlitre of r-phycoerythrin-conjugated rat antimouse Pan-NK cell (DX5) antibody, 4 µl of FITC-conjugated rat antimouse CD3e (CD3e chain) antibody, 4 µl of FITC-conjugated rat antimouse CD4 (RM4-5) antibody and 4 µl of FITC-conjugated rat antimouse CD8a (53-6.7) antibody were added to tubes with cell suspension and incubated for 30 min at 4 °C. Flow cytometric analyses were performed using a fluorescence-activated cell sorter with logarithmic amplification (Coulter, Fullerton, CA, USA).

Cytotoxicity assay. The cytotoxicity assay was performed according to Kay and Horwitz 1980 [18]. Briefly, the concentration of target cells, YAC-1 (murine lymphosarcoma cells), was adjusted to 10^6 cells/ml in 20 ml of RPMI-1640 and was labelled with sodium chromate $NA_2{}^{51}CrO_4$ (IPEN-CNEN/SP, São Paulo, SP, Brazil). After incubation at 37 °C in 5% CO_2 atmosphere for 60 min, the cells were washed in RPMI 1640 to remove the unlabelled sodium chromate, and the concentration was adjusted to 5×10^6 cells/ml. Hundred microlitre of target cells was added to 100 µl of effectors cells (A-NK cells) in 96-well flat-bottomed culture plates (Costar, Rochester, NY, USA) in a ratio of 1:5, 1:1 and 1:0.2, respectively. The plate was incubated at 37 °C in 5% CO_2 atmosphere for 18 h in a humid chamber. Then, the plate was centrifuged at $200 \times g$ for 5 min, and 100 µl of the supernatant of each sample was read in a Gamma Counter (Pharmacia®, Uppsala, Sweden).

Macrophage culture and infection. About 1×10^5 BALB/c mice peritoneal macrophages were allowed to attach in sterile 13-mm round coverslips (GlassTécnica, São Paulo, SP, Brazil) in complete RPMI medium and placed in

24-well plates (Costar). *Leishmania (Leishmania) amazonensis* promastigotes in stationary phase of culture growth were added to the macrophage monolayers at a ratio of five parasites to one macrophage, and the culture was incubated at 5% CO_2 at 35 °C for 24 h. Three ratios of A-NK : macrophage (5:1, 1:1 and 0.2:1) were studied, using only infected macrophages as control. Two experiments were performed: 0-h group – A-NK cells were added together with parasites; 24-h group – A-NK cells were added after 24 h of macrophage infection with or without the maintenance of r-IL-2. Morphological aspects of the cultures after A-NK exposure were analysed by light and electronic transmission microscopy.

Parasite recovery. The viable parasites were evaluated in macrophage cultures infected with *L. (L.) amazonensis* promastigotes after 24 h of A-NK cell exposure. The RPMI medium was changed with Schneider medium supplemented with 10% fetal bovine serum, 2% human urine, 10 mg/ml of gentamicin and 100 IU/ml of penicillin, and the culture plate was maintained in a 25 °C incubator for 7 days. The promastigote forms of the parasite were quantified using a Neubauer chamber.

NO determination. The NO production was determined through the concentration of nitrites present in the supernatants of macrophage cultures infected with promastigotes of *L. (L.) amazonensis*, as described by Green *et al.* 1990 [19]. Briefly, 300 µl of culture supernatants was incubated with the same volume of Griess reagent (1% sulfonilamide, 0.1% naftilenodiamin dihidrocloret and 2.5% orthophosphoric acid) for 10 min at room temperature. The absorbance was determined in a spectrophotometer using a 550-nm filter. The results were expressed in NO_2^-µM, according to a standard curve.

IFN-γ and IL-12 determination. The IFN-γ and IL-12 levels in the culture supernatants were evaluated by Capture ELISA using mouse standard kits for cytokine measurements (BioSource Europe SA, Fleurus, Belgium). Hundred microlitre of BALB/*c* mice peritoneal macrophages in complete RPMI medium at 2×10^5 cells/ml was placed in 96-well plates (Costar), and the culture was kept at 5% CO_2 incubator at 35 °C for 24 h. Then, 50 µl of parasite suspension was added to the macrophages at the concentration of five parasites to one macrophage. Three ratios of A-NK : macrophage (5:1, 1:1 and 0.2:1) were studied, using only infected macrophages as control. Fifty microlitre of A-NK cells was added together with parasites (0-h group) and after 24 h of macrophage infection (24-h group) with or without r-IL-2. After 24 h of A-NK-cell exposure, the supernatants were collected, and 100 µl was used for IFN-γ (Mouse IFN-γ Immunoassay Kit, KMC4021) and 50 µl for IL-12 (Mouse IL-12 + p40 Immunoassay Kit, KMC0121) measurements.

The role of A-NK cells in promastigote lysis. About 2×10^5 promastigotes of *L. (L.) amazonensis* and A-NK cells were incubated in complete RPMI medium for 4 h at 35 °C with 5% CO_2 and humid atmosphere at concentrations of 5:1, 1:1 and 0.2:1 A-NK : promastigotes. After this period, RPMI medium was changed with Schneider's medium and the culture incubated at 25 °C to measure parasite recovery after A-NK cell exposure. On day 7, the promastigotes were quantified in a Neubauer chamber.

In vivo study. BALB/*c* mice were inoculated subcutaneously with 10^5 *L. (L.) amazonensis* promastigotes into the hind footpads. After 24 h, 3×10^5 A-NK cells were inoculated at the parasite inoculation site. The lesion sizes were measured weekly, and the histopathological changes were studied at day 120 of infection when the animals were killed.

Statistical analysis. The computer program SIGMASTAT 2.0, 1997, was used. Each event was analysed in block, and the difference between the two groups was evaluated by the Paired *t*-test method, with $P < 0.05$ considered statistically significant.

Results

Characterization of A-NK cells

The A-NK cells used in our experiments were characterized morphologically by transmission electronic microscopy as large lymphocytes with multiple intracytoplasmatic granules. The surface phenotype of the A-NK cells on the day 7 in culture analysed by flow cytometry showed: Pan NK (DX5) 97.7%, CD3e 16.6%, CD4 1.9% and CD8a 8.4%. The ^{51}Cr-release microcytotoxicity assay showed that the percentage of lysis of targets cells (YAC-1) was directly proportional to the ratio of effectors cells (A-NK) to target cell used, 49.17% for 5:1, 29.76% for 1:1 and 12.53% for 0.2:1, A-NK : YAC-1, respectively.

Light and electronic microscopy studies

By light microscopy, cells with typical macrophage morphology containing amastigotes were seen in the controls. When A-NK cells were added to the cultures, macrophages and parasites with signs of degeneration were observed, proportional to the concentration of A-NK cells used (Fig. 1). The transmission electron microscopy studies confirmed the results observed in light microscopy. Cells with typical macrophage morphology and intact parasites inside the vacuoles were observed in the controls (Fig. 2A). When incubated with A-NK cells, a predominance of parasitic debris inside the vacuoles of infected macrophages in close contact with A-NK cells and signs of macrophage cellular membrane rupture were observed mainly in the groups with the largest concentration of A-NK cells used (Fig. 2B). Macrophages with intact and degenerated parasites surrounded by the A-NK cells were seen in 1:1 and 0.2:1, A-NK cells : macrophage groups (Fig. 2C,D).

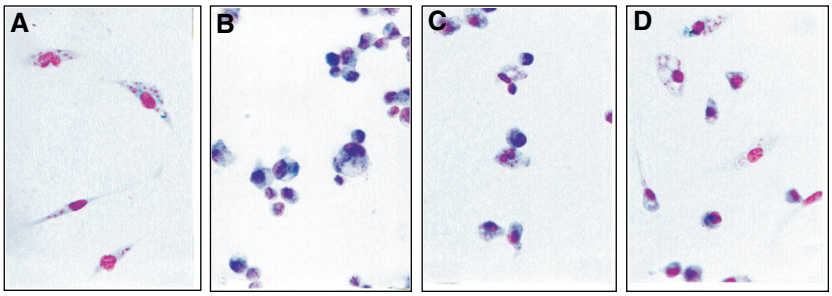

Figure 1 Morphological aspects of BALB/*c* peritoneal macrophage cultures infected with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* and exposed to different concentrations of activated-natural killer (A-NK) cells during 24 h (Giemsa, magnification ×100). (A) control; (B) five A-NK : one macrophage; (C) one A-NK : one macrophage; (D) 0.2 A-NK : one macrophage.

Parasite recovery after A-NK exposition

The parasite recovery in Schneider medium showed a reduced number of viable parasites in the presence of A-NK cells compared with controls after 24 h of exposure to A-NK cells (Fig. 3A). There were significant differences when A-NK cells and macrophage were cocultivated at ratios of 5:1 and 1:1 ($P < 0.05$). While the presence of rIL-2 in the cultures did not cause any difference in cultures containing A-NK cells, the parasite recovery was significantly smaller in the control groups with rIL-2 stimulation than in control groups without rIL-2 stimulation (Fig. 3B).

When the A-NK cells were added to the macrophages simultaneously with the parasites (0-h group), the parasite recovery after 24 h of A-NK cells exposure was also significantly reduced compared with the control ($P < 0.05$). It was directly proportional to the A-NK-cell concentration used (Fig. 3C). In the 0.2:1 A-NK : macrophage group, the presence of rIL-2 in the cultures led to a significant decrease in parasite recovery ($P < 0.05$) (Fig. 3D).

Determination of nitrite concentration

No significant differences in nitrite levels in the supernatants from control and experimental groups were found in any groups, neither at 0-h nor at 24-h (Fig. 4).

Determination of the IFN-γ and IL-12 levels

Both at 0 and 24 h, the IFN-γ levels in the supernatant were high in cultures of infected macrophages that were exposed to A-NK cells compared with the controls (only infected macrophages) ($P < 0.05$). The IFN-γ levels were directly proportional to the concentration of A-NK cells used. The presence of rIL-2 in the cultures further increased IFN-γ levels at A-NK cell : macrophage ratios of 1:1 and 0.2:1 (Fig. 5). No differences were observed between 0-h and 24-h group.

In general, low levels of IL-12 were present in the supernatant of the cultures. There was a slight, but not significant, increase in IL-12 levels in the cultures containing the highest concentrations of A-NK cells in both the 0- and 24-h groups ($P < 0.05$). The presence of rIL-2 in the cultures did not lead to differences in IL-12 levels in any group (Fig. 6).

The role of A-NK cells in the elimination of promastigotes

The parasite recovery after exposing the promastigotes to different concentrations of A-NK cells was smaller than the control and was directly proportional to the A-NK-cell concentrations used. There were significant differences when 5:1 and 1:1 ratios of A-NK cells : promastigotes were used compared with the control (Fig. 7).

In vivo studies

The hind footpad swelling increased with the duration of infection in both groups, but it was smaller in the animals inoculated with both the parasites and A-NK cells compared with the animals inoculated only with the parasites at 45 days of infection with significant difference at 90 days of infection (Fig. 8). Histopathological analysis at day 120 of infection of animals inoculated only with parasites

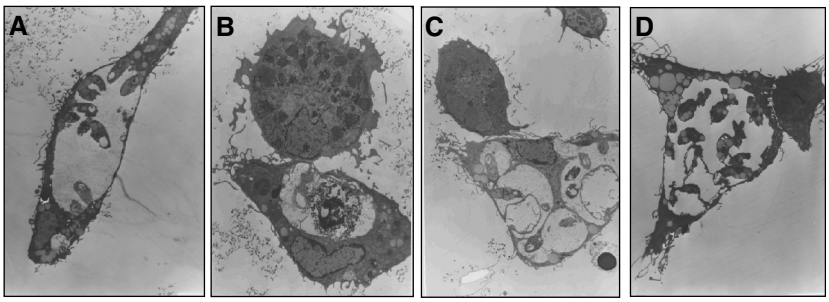

Figure 2 Ultrastructural aspects of BALB/*c* peritoneal macrophage cultures infected with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* and exposed to different concentrations of activated-natural killer (A-NK) cells during 24 h (Magnification ×3000). (A) control; (B) five A-NK : one macrophage; (C) one A-NK : one macrophage; (D) 0.2 A-NK : one macrophage.

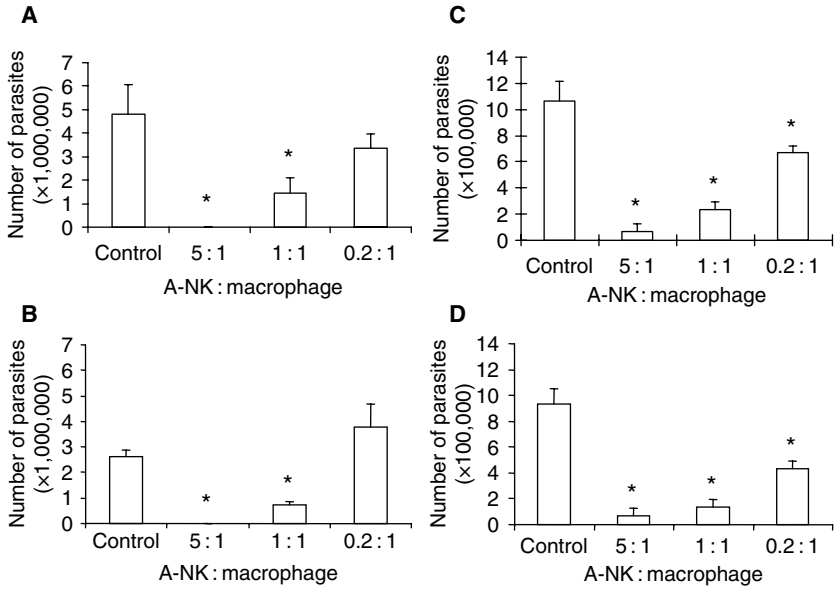

Figure 3 Number of viable parasites from the BALB/*c* peritoneal macrophage cultures infected with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* and exposed to different concentrations of activated-natural killer (A-NK) cells. (A and B) 24-h group without and with presence of rIL-2, respectively; (C and D) 0-h group without and with presence of rIL-2, respectively. ($^*P < 0.05$).

showed foamy cells heavily infected with parasites. In contrast, animals inoculated with parasites and A-NK cells showed a nodular mononuclear inflammatory infiltrate in the dermis characterized by lymphocytes, plasma cells and macrophages with moderate parasitism (Fig. 9A,B).

Discussion

The main function of NK cells, so far identified, is the lysis of cells that are altered from normal self, including tumoural cells and cells infected with intracellular pathogens [20]. NK cells may have an important role in the defence against infection caused by *Leishmania*. In the initial phase of the infection by *Leishmania*, NK cells play a relevant part in controlling the number of parasites and the size of the injury through the activation of leishmanicidal mechanisms [8]. In a model of selective depletion of NK cells with an unbroken repertoire of T and B cells and preservation of the ability to produce IFN-α and IL-2, Laurenti *et al.* 1999 [9] have shown an increase in the number of parasites in injured skin of BALB/*c* mice inoculated with *L.* (*L.*) *amazonensis* after 7 days of infection, supporting the hypothesis of a direct role of these cells in the early phases of the infection.

In this study, the morphologic analysis of the cultures by transmission electron microscopy showed infected macrophages surrounded by A-NK cells. After 24 h of exposition to A-NK cells, some macrophage demonstrated lysis of the cellular membrane, and degenerated parasites (chromatin condensation, extraction of cytosol, absence of microtubules and often loss of integrity of the parasitic membrane) could be seen. The intensity of the cellular and

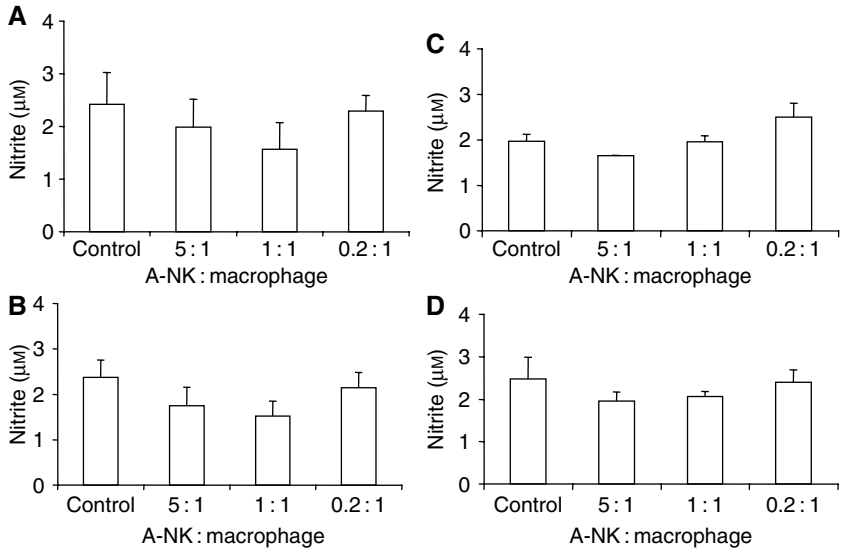

Figure 4 Measurement of nitrite by Greiss method in the supernatant of the BALB/*c* peritoneal macrophage cultures infected with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* and exposed to different concentration of activated-natural killer cells. (A and B) 24-h group without and with presence of rIL-2, respectively; (C and D) 0-h group without and with presence of rIL-2, respectively.

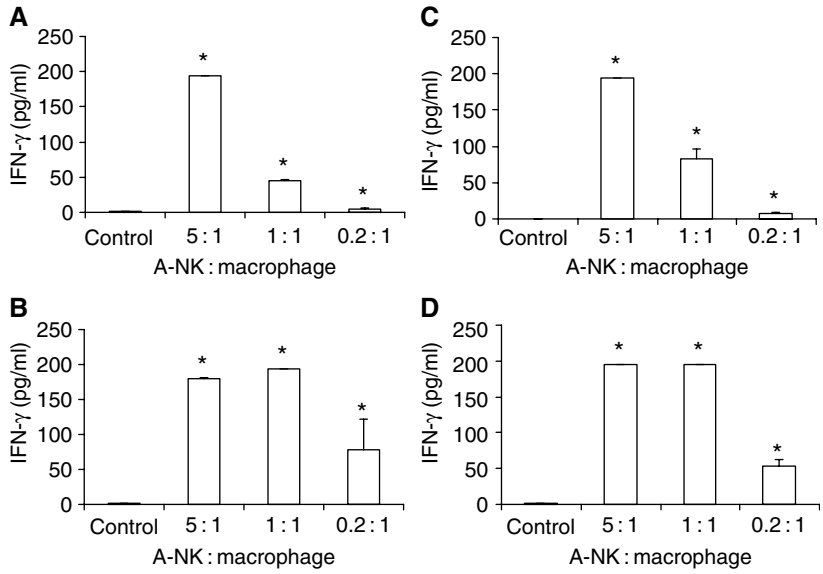

Figure 5 Measurement of interferon-γ by ELISA in the supernatant of the BALB/*c* peritoneal macrophage cultures infected with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* and exposed to different concentrations of activated-natural killer (A-NK) cells. (A and B) 24-h group without and with rIL-2, respectively; (C and D) 0-h group without and with rIL-2, respectively. (*$P < 0.05$).

parasitic degeneration was related directly to the number of A-NK cells used in the cultures. These data support the findings reported by Resnick *et al.* 1988 [20] and suggest a direct role of NK cells in the lysis of macrophages infected by *Leishmania*. In order to determine whether exposure to A-NK cells led to the destruction of parasites outside or inside of the macrophage, a technique of parasite recovery was developed. In this method, the number of viable parasites recovered after A-NK-cell exposure diminished proportionally to the concentration of effector cells used, confirming the data described above. Importantly, in the experiments where the A-NK cells had been added to the macrophage cultures simultaneously with the parasites (i.e. the 0-h group), the recovery of parasites was lower compared with the group where the A-NK cells had been added 24 h after the parasites. This suggests a direct role of the A-NK cells in extracellular lysis of *Leishmania* promastigotes. While the presence of rIL-2 did not modify significantly the effect of the A-NK cells in the cultures of infected macrophages, the presence of rIL-2 in the culture media lowered the recovery of parasites in the control groups. This observation may be related to the activation of macrophages by the rIL-2 causing lysis of intracellular parasites *in vitro*.

The direct role of A-NK cells in control of the promastigote forms of *L.* (*L.*) *amazonensis* was confirmed in the experiment where A-NK cells had been added to the cultures of promastigotes for 4 h. The number of viable parasites was diminished proportional to the number of A-NK cells added. These results confirm the ability of the A-NK cells to kill promastigotes of *Leishmania* before they can enter the macrophages.

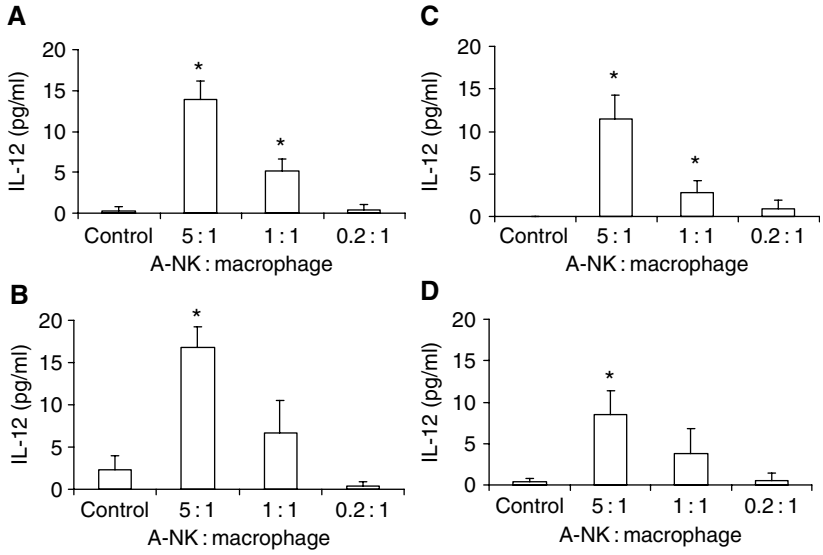

Figure 6 Measurement of IL-12 by ELISA in the supernatant of the BALB/*c* peritoneal macrophage cultures infected with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* and exposed to different concentrations of activated-natural killer (A-NK) cells. (A and B) 24-h group without and with presence of rIL-2, respectively; (C and D) 0-h group without and with presence of rIL-2, respectively. (*$P < 0.05$).

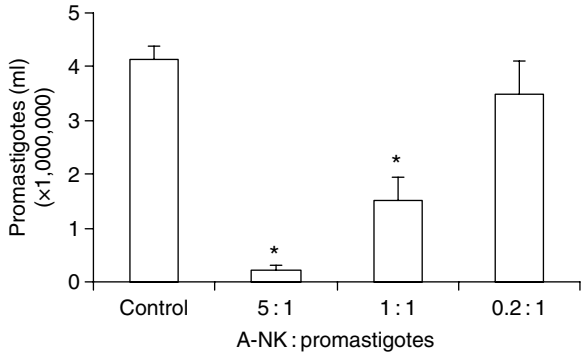

Figure 7 Number of viable parasites after 4 h of activated-natural killer (A-NK)-cell exposure in three different concentrations of A-NK cells to *Leishmania* (*Leishmania*) *amazonensis* promastigotes: 5:1, 1:1 and 0.2:1. (*$P < 0.05$).

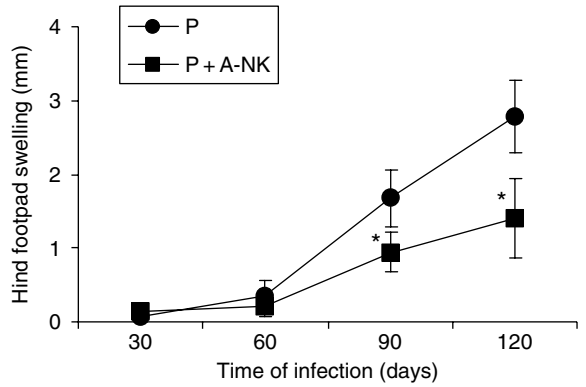

Figure 8 Evolution of the hind footpad swelling of BALB/*c* mice inoculated with promastigotes of *Leishmania* (*Leishmania*) *amazonensis* (P) and inoculated with promastigotes of *L.* (*L.*) *amazonensis and* activated-natural killer (A-NK) cells (P + A-NK). (*$P < 0.05$).

The *Leishmania*-controlling effect mediated by the A-NK cells both in the context of infected macrophages and directly on the extracellular promastigotes can be explained by the high cytotoxic activity of the A-NK cells. In cytotoxic assays, lysis of more than 50% of the target cells was demonstrated when A-NK cells were mixed with YAC-1 cells in 5:1 ratio. In the literature, it has been described that lysis of infected cells by NK cells depends on the action of perforin which induces the formation of pores in the target cell membrane [21, 22].

Beyond the mechanism of direct lysis of infected cells by intracellular parasites, NK cells can produce cytokines which may activate the microbicidal machinery of infected cells needed for intracellular destruction of pathogens. In *Leishmania* infections, NK cells may play an important role in the initial phase of the infection, by producing IFN-γ which activates leishmanicidal mechanisms in infected macrophages. Moreover, the production of IFN-γ by NK cells in the early phase of the infection may induce host $CD4^+$ Th1 responses needed for the resistance to cutaneous leishmaniasis. Thus, mice depleted of NK cells show a reduced production of IFN-γ but increased production of IL-4 by splenic and lymph node cells, and they harbor increased numbers of parasites in the infected tissues [6, 7]. The production of IFN-γ in the supernatants of the macrophage cultures that were infected and exposed to the different concentrations of A-NK cells was directly proportional to the concentrations of A-NK cells used and presented higher levels than infected macrophages cultured without A-NK cells. The presence of rIL-2 in the cultures further increased the production of IFN-γ.

IL-12 stimulates NK cells to produce IFN-γ and may in this way help to activate infected macrophages during early phases of the infection. Mice susceptible to *Leishmania* produce low amounts of IL-12, while resistant mice produce higher amounts of this cytokine [5, 23]. The administration of IL-12 to susceptible mice leads to a reduction in parasite burden and to increased IFN-γ production and reduced IL-4 production. Furthermore, administration of IL-12 antibody leads to an increase in the parasite burden even in resistant mice presumably via a reduction in the production of IFN-γ [24]. Thus, NK cells may be a crucial component to drive the cellular immune response of the Th1 type through IFN-γ secretion in response to IL-12 stimulation [25].

We found that the levels of IL-12 in the supernatant of the macrophages, infected and exposed to different concentrations of A-NK cells, were increased in the presence of A-NK cells (however, to a lower degree than IFN-γ) but did not show a well-defined correlation with the concentration of A-NK cells used. Significant differences in IL-12 production were observed between the controls (macrophages only) and the cultures with 5:1 mixtures of A-NK cells and macrophages. As the presence of rIL-2 in the

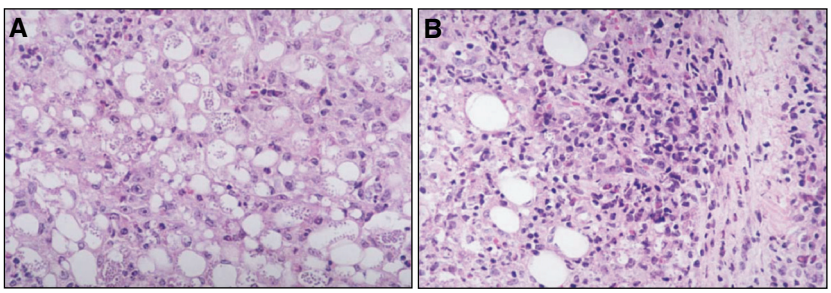

Figure 9 Histopathology of *Leishmania* (*Leishmania*) *amazonensis* subcutaneous inoculation site at day 120 postinfection (H&E, magnification ×40). (A) BALB/*c* mice inoculated with parasite showing foamy cells heavily parasitized and (B) BALB/*c* mice inoculated with parasite and activated-natural killer cells showing nodular mononuclear inflammatory infiltrate with moderate parasitism.

cultures did not modify the production of IL-12, it does not appear that rIL-2 plays an important role in the modulation of the IL-12 production by infected macrophages.

Microbicidal mechanisms leading to the death of *Leishmania* parasites inside macrophages can be activated by the NO which originates through the metabolism of the L-arginine [26]. Studies of macrophages from iNOS-deficient mice confirm the role of NO as an important effector molecule in the death of intracellular parasites such as *Leishmania*. On the other hand, it has been described that *Leishmania* can directly inhibit the synthesis of NO and block the development of Th1 cellular immune responses, by inhibition of IL-12 synthesis [27, 28]. Our experiments showed a general low level of NO with no significant differences between groups of infected macrophages whether these were exposed to A-NK cells or not. This indicates that NO-dependent leishmanicidal mechanisms may not play an important role in our experimental conditions. Other microbicidal mechanisms such as production of oxygen radicals could be involved in the control of the *Leishmania* infection in our model.

The data obtained in our studies indicate that A-NK cells play a major role in the control of the parasitism of infected macrophages via direct lysis of the host cell and/or parasites. This is supported by the reduction in the number of viable parasites, parasitic degeneration and cellular lysis occurring in the cultures of infected macrophages exposed to different concentrations of A-NK cells. Our experiments also confirmed a direct role of the A-NK cells on the control of the number of parasites in the *Leishmania* infection *in vivo*.

Acknowledgments

The study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP 00/07054-9 and 01/00240-4) and HC-FMUSP (LIM-50).

References

1 Sacks D, Perkins PV. Identification of an infective stage of leishmania promastigotes. Science 1984;223:1417–9.
2 Alexander J, Russel DG. The interaction of *Leishmania* species with macrophages. Adv Parasitol 1992;31:175–254.
3 Gontijo B, Carvalho ML. American Cutaneous Leishmanias. Rev Soc Bras Med Trop 2003;36:71–80.
4 Silveira FT, Lainson R, Corbett CEP. Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil – a review. Mem Inst Oswaldo Cruz Rio Janeiro 2004;99:239–51.
5 Solback W, Laskay T. *Leishmania major* infection: the overture. Parasitol Today 1995;11:394–7.
6 Scharton TM, Scott P. Natural Killer cells are a source of Interferon γ that drives differentiation of CD4+ T cell subsets and induces resistence to *Leishmania major* in mice. J Exp Med 1993;178:567–77.
7 Scott P. IFN-γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J Immunol 1991;147:3149–55.
8 Laskay T, Rollinghoff M, Solbach W. Natural Killer cells participate in the early defense against *Leishmania major* in mice. Eur J Immunol 1993;23:2237–41.
9 Laurenti MD, Gidlund MA, Ura DM *et al.* The role of natural killer cells in the early period of infection in murine cutaneous leishmaniasis. Braz J Med Biol Res 1999;32:323–5.
10 Sartori A, Kaneno R, Baruzzi N *et al.* Increased natural killer activity does not prevent progression of experimental Kala-Azar. Rev Inst Med Trop São Paulo 1999;41:215–9.
11 Satoskar AR, Stamm LM, Zhang X *et al.* Mice lacking NK cells develop an efficient Th1 response and control cutaneous *Leishmania major* infection. J Immunol 1999;162:6747–54.
12 Vujanovic NL, Herberman RB, Maghazachi AA *et al.* Lymphokine-activated killer cells in rats. III. A simple method for the purification of large granular lymphocytes and their rapid expansion and conversion into lymphokine-activated killer cells. J Exp Med 1988;167:15–25.
13 Trichieri G, Matsumoto-Kobayashi M, Clark S *et al.* Response of resting human peripheral blood natural killer cells to interleukin 2. J Exp Med 1984;160:1147–69.
14 Gunji Y, Vujanovic NL, Hiserodt JC *et al.* Generation and characterization of purified adherent lymphokine-activated killer cells in mice. J Immunol 1989;142:1749–54.
15 Schwarz RE, Vujanovic NL, Hiserodt JC. Enhanced antimetastatic activity of lymphokine-activated killer cells purified and expanded by their adherence to plastic. Cancer Res 1989;49:1441–9.
16 Ribeiro U Jr, Basse PH, Rosenstein M *et al.* Retention of adoptively transferred Interleukin-2-activated Natural Killer cells in tumor tissue. Anticancer Res 1997;17:1115–24.
17 Ribeiro U Jr, Cecconelo I, Safatle-Ribeiro AV *et al.* Squamous cell carcinoma of the esophagus and multiple primary tumors of the upper aerodigestive tract. Arq Gastroenterol 1999;36:195–200.
18 Kay DH, Horwitz DA. Natural and antibody dependent cytotoxicity of lymphocytes and monocytes. In: Rose NR, Bigazzi PE, eds. Methods in Immunodiagnosis, Kay & Horwitz 1980: 24–5.
19 Green SJ, Meltzer MS, Hibbs JB Jr *et al.* Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J Immunol 1990;144:278–83.
20 Resnick M, Roguel N, Bercovier H *et al.* Lysis of murine macrophages infected with intracellular pathogens by Interleukin2-Activated Killer (LAK) cells *in vitro*. Cell Immunol 1988;113:214–9.
21 Kagi D, Ledermann B, Burki K *et al.* Cytotoxicity mediated by T cells and Natural Killer cells is greatly impaired in perforin-deficient mice. Nature 1994;369:31–7.
22 Konjevic G, Schlesinger B, Cheng L *et al.* Analysis of perforin expression in human peripheral blood lymphocytes, CD 56+ Natural Killer cells subsets and its induction by Interleukin-2. Immunol Invest 1995;24:499–507.
23 D'Andrea A, Rengaraju M, Valiante NM *et al.* Production of natural killer cell stimulatory (interleukin-12) by peripheral blood mononuclear cells. J Exp Med 1992;76:1387–98.
24 Sypek JP, Chung CL, Mayor SE *et al.* Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J Exp Med 1993;177:1797–802.
25 Locksley RM. Interleukin 12 in host defense against microbial pathogens. Proc Natl Acad Sci 1990;90:5879–80.
26 Liew FY. Regulation of cell-mediated immunity in leishmaniasis. Curr Top Microbiol Immunol 1990;155:54–64.
27 Liew FY, Xu D, Chan WL. Immune effector in parasitic infections. Immunol Lett 1999;65:101–4.
28 Balestieri FMP, Queiroz ARP, Scavone C, Costa VMA, Barral-Neto M, Abrahamsohn IA. *Leishmania* (*L.*) *amazonensis*-iduced inhibition of nitric oxide synthesis in host macrophages. Microbes Infect 2002;4:23–9.

ANEXO-9

Reviewing the role of the dendritic Langerhans cells in the immunopathogenesis of American cutaneous leishmaniasis

Silveira, F.T.; Lainson, R.; Gomes, C.M.C.; **Laurenti, M.D.;** Corbett, C.E.P.

Transactions of the Royal Society of Tropical Medicine and Hygiene 2008; 102: 1075-1080

Transactions of the Royal Society of Tropical Medicine and Hygiene (2008) **102**, 1075—1080

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevierhealth.com/journals/trst

REVIEW

Reviewing the role of the dendritic Langerhans cells in the immunopathogenesis of American cutaneous leishmaniasis

Fernando T. Silveira[a,b,*], Ralph Lainson[a], Claudia M.C. Gomes[c], Márcia D. Laurenti[c], Carlos E.P. Corbett[c]

[a] *Parasitology Department, Evandro Chagas Institute (Surveillance Secretary of Health, Ministry of Health), Avenida Almirante Barroso, 492, 66090-000, Belém, Pará State, Brazil*
[b] *Tropical Medicine Institute, Federal University of Pará, Belém, Pará State, Brazil*
[c] *Pathology Department, Medical School of São Paulo University, São Paulo, São Paulo State, Brazil*

Received 15 January 2008; received in revised form 23 May 2008; accepted 23 May 2008
Available online 3 July 2008

KEYWORDS
Dendritic Langerhans cells;
American cutaneous leishmaniasis;
Leishmania (*Viannia*) *braziliensis*;
Leishmania (*Leishmania*) *amazonensis*;
Immunopathogenesis;
Brazil

Summary The role of dendritic Langerhans cells (LCs) in the immunopathogenesis of American cutaneous leishmaniasis (ACL) was reviewed in the light of more recent clinical and immunological features of ACL, caused by the principal human pathogenic leishmanial parasites found in Brazil: *Leishmania* (*Viannia*) *braziliensis* and *L.* (*L.*) *amazonensis*. The report shows a species-specific correlation between the LC density and the CD4+ and CD8+ T-cell profiles in the cellular infiltrate of skin lesions of ACL patients, providing the conclusion that LCs might be influencing the dichotomy of interaction between *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* with the human T-cell immune response. While *L.* (*V.*) *braziliensis* shows a clear tendency to direct infection to the hypersensitivity pole of the ACL clinical—immunological spectrum marked by a strong Th1-type immune response, *L.* (*L.*) *amazonensis* shows the opposite, directing infection to the hyposensitivity pole associated with a marked Th2-type immune response. These are probably the main immunological mechanisms of LCs regarding the immune response dichotomy that modulates infection outcome by these *Leishmania* parasites.

1. Introduction

American cutaneous leishmaniasis (ACL) is a parasitic protozoal disease that is widespread in most Latin American countries, with different species of the genus *Leishmania* serving as etiological agents. At least 14 recognized species of *Leishmania* currently exist within the subgenera *Viannia* and *Leishmania* that may produce ACL (Lainson and Shaw, 1987, 2005). However, in Brazil, where half of these species may be found, *L.* (*Viannia*) *braziliensis* and *L.* (*Leishmania*) *amazonensis* are the principal species that are pathogenic to humans (Silveira et al., 1997, 2004), which justifies the

* Corresponding author. Tel.: +55 91 3081 7799.
E-mail address: silveiraft@lim50.fm.usp.br (F.T. Silveira).

0035-9203/$ — see front matter © 2008 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.trstmh.2008.05.020

focus of this review on these two *Leishmania* parasites. Following infection by these species, some naturally resistant individuals (asymptomatic) will occur, along with others presenting different degrees of susceptibility to infection (symptomatic). Thus, depending upon the species of the infecting *Leishmania* and the infected individual's T-cell immune response, a spectrum of clinical and immunological forms of disease develops.

At the center of the clinical—immunological spectrum is localized cutaneous leishmaniasis (LCL), the most frequent form of ACL, which may be caused by both parasitic species. Generally, LCL consists of one or more ulcerated skin lesions, supported by an efficient Th1-type immune response (Cáceres-Dittmar et al., 1993; Carvalho et al., 1995; Pirmez et al., 1993); this T-cell response mechanism elicits a high degree of resistance against infection by LCL, as well as favorable results from traditional antimony therapy.

From the center of the spectrum, some infections not entirely under the control of T-cell immune mechanisms may evolve to one of the two polar forms of disease: either the T-cell hypersensitivity pole, represented by mucocutaneous leishmaniasis (MCL), for which *L.* (*V.*) *braziliensis* is the main causative agent of this form of ACL, or the T-cell hyposensitivity pole, consisting of anergic diffuse cutaneous leishmaniasis (ADCL), mainly caused by *L.* (*L.*) *amazonensis* in South America or by *L.* (*L.*) *mexicana* in Central America. It has now been determined that this deviation of T-cell immune response during infection to either MCL (hypersensitivity) or ADCL (hyposensitivity) is highly influenced by the specific antigen stimulating the host immune response: i.e. the *Leishmania* species involved. Thus, it would be a misunderstanding to regard *L.* (*V.*) *braziliensis* as potentially responsible for inducing the T-cell response hyposensitivity found in ADCL and *L.* (*L.*) *amazonensis* as responsible for the T-cell response hypersensitivity shown in MCL. This permitted the conclusion that *L.* (*V.*) *braziliensis* is without doubt the most specialized American leishmanial parasite, presenting the ability to stimulate T-cell response, and, in contrast, that *L.* (*L.*) *amazonensis* is a poor stimulator. These immunopathological differences may be regarded as the principal immunomodulatory mechanisms responsible for MCL and ADCL; while the former results from a vigorous T-cell response induced by *L.* (*V.*) *braziliensis*, ADCL comes from a typically deficient T-cell response associated with *L.* (*L.*) *amazonensis*.

Clinically, there are also certain differences that distinguish these diseases: while MCL comprises necrosis of nasopharyngeal mucosal tissues in strong association with high serum production of IFN-γ and TNF-α (Blackwell, 1999; Castes et al., 1993; Da-Cruz et al., 1996; Ribeiro-de-Jesus et al., 1998), ADCL is definitively only a dermal disease, presenting nodular skin lesions rich in amastigote forms of the parasite disseminated all over the body; this suggests a specific failure of T-cell mechanisms in controlling the infection, resulting in a very weak response to traditional antimony treatment (Barral et al., 1995; Bonfim et al., 1996; Convit et al., 1993; Petersen et al., 1982). Thus, when nasopharyngeal mucosal tissue compromise is associated with *L.* (*L.*) *amazonensis*, it has only been found in rare cases of ADCL of prolonged duration and presents almost complete absence of T-cell response. Unlike *L.* (*V.*) *braziliensis*, which disseminates through the bloodstream, *L.* (*L.*) *amazonensis* seems to use a contiguous mechanism for disseminating from the skin to the mucosal tissue. However, recent evidence indicates that, besides the specific immunomodulatory mechanism of *L.* (*V.*) *braziliensis* antigen, a host genetic factor (IL-6-174 G/C promoter polymorphism) may also influence MCL pathogenesis (Castellucci et al., 2007).

Between the central LCL form and the two extreme pathogenicity poles MCL and ADCL, an intermediate form exists, known as borderline disseminated cutaneous leishmaniasis (BDCL), which may be caused by either *L.* (*V.*) *braziliensis* or *L.* (*L.*) *amazonensis*. However, even this condition presents certain differences that should be considered: in BDCL caused by *L.* (*V.*) *braziliensis*, the process of dissemination is relatively rapid, requiring only 2—3 months to produce around 100 ulcerated skin lesions; by contrast, in BDCL caused by *L.* (*L.*) *amazonensis*, the dissemination process is slower, requiring 1—2 years to produce around 5—10 infiltrated skin lesions. The term 'borderline' was introduced to characterize clinical evidence, namely the incomplete T-cell immune suppression found in these patients, principally in those infected by *L.* (*L.*) *amazonensis*, who present greater T-cell response inhibition than those infected by *L.* (*V.*) *braziliensis*. Despite this, sufficient observations have been reported to conclude that T-cell response functions well enough to facilitate recovery in BDCL patients following successful treatment with two to three LCL-therapeutic antimony regimes (Carvalho et al., 1994; Costa et al., 1986; Silveira et al., 2004, 2005; Turetz et al., 2002).

2. T-cell immune suppression in human *Leishmania* (*L.*) *amazonensis* infection

For a relatively long period, ever since the initial evidence involving T-cell immune suppression was found in *L.* (*L.*) *amazonensis* LCL patients (Lainson et al., 1986), attempts to improve understanding regarding the mechanisms of action of *L.* (*L.*) *amazonensis* have been made. For instance, initial comparisons concentrated on the delayed-type hypersensitivity (DTH) reactions between LCL patients infected by *L.* (*V.*) spp. [e.g. *L.* (*V.*) *braziliensis*] with individuals infected by *L.* (*L.*) *amazonensis*; the conclusion reached described a higher prevalence rate ($P < 0.05$) of positive DTH reactions in LCL *L.* (*V.*) *braziliensis* patients than in individuals infected by *L.* (*L.*) *amazonensis* (Silveira et al., 1991). This then permitted researchers to speculate concerning the possibility that *L.* (*L.*) *amazonensis* proteins, such as heat-shock protein 70 (hsp 70), might be partially suppressing the T-cell response of *L.* (*L.*) *amazonensis* LCL patients.

Interestingly, similar results were also observed in *Cebus apella* monkeys (Primates: Cebidae) experimentally infected with *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis*; no positive DTH reaction was found in the five monkeys inoculated with *L.* (*L.*) *amazonensis* during the 4 month disease evolution period. By contrast, monkeys experimentally infected with *L.* (*V.*) *braziliensis* presented positive DTH-reactions in all five cases for at least 2 or 3 months out of the 7 month period of disease duration (Silveira et al., 1990).

Moreover, in a subsequent experiment using the lymphocyte proliferation assay for all clinical forms of ACL

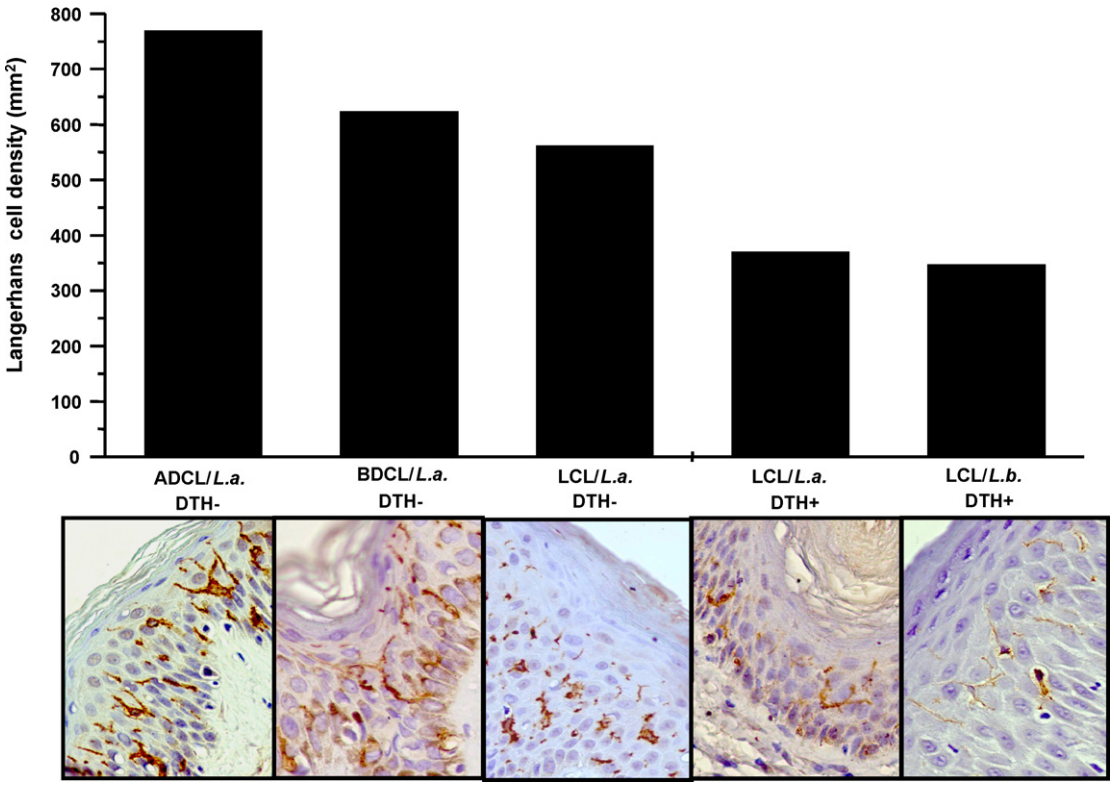

Figure 1 Immunocytochemistry analysis of dendritic Langerhans cell (LC) density in the cellular infiltrate of cutaneous lesions of the following clinical—immunological spectrum of American cutaneous leishmaniasis (ACL) in Amazonian Brazil, consisting of the anergic diffuse cutaneous leishmaniasis (ADCL) and borderline disseminated cutaneous leishmaniasis (BDCL), both due to *Leishmania* (*L.*) *amazonensis* and with the delayed-type hypersensitivity (DTH) negative$^{(-)}$; localized cutaneous leishmaniasis (LCL) due to *L.* (*L.*) *amazonensis* with two groups, DTH^{-} and DTH positive$^{(+)}$, and LCL due to *L.* (*Viannia*) *braziliensis* with DTH^{+}. These clinical—immunological forms of ACL were preformed with five to eight patients; the ADCL and BDCL forms had five cases each. *L.a.* = *L.* (*L.*) *amazonensis*; *L.b.* = *L.* (*V.*) *braziliensis*. For recognition of LC, the specific monoclonal antibody Anti-Human CD1a was used (clone 010) (DAKO-Denmark) and the technical proceedings were as used by Xavier et al. (2005).

caused by *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis*, lower prevalence rates ($P < 0.05$) for lymphocyte proliferation reactions were determined for forms caused by *L.* (*L.*) *amazonensis* ($ADCL^{DTH-} < BDCL^{DTH-} < LCL^{DTH-} < LCL^{DTH+}$) than those caused by *L.* (*V.*) *braziliensis* ($LCL^{DTH+} > BDCL^{DTH-} < MCL^{DTH+}$), principally in cases of LCL and MCL (Silveira et al., 1998).

These findings have encouraged the conclusion that human *L.* (*L.*) *amazonensis* infection leads to a specific T-cell immune suppression. In this way, it was also interesting to show a progressive decrease of both CD4+ and CD8+ T-cell profiles in the cellular infiltrate of cutaneous lesions from the reactive (DTH^{+}) LCL central form to the non-reactive (DTH^{-}) extremity forms, BDCL and ADCL, due to *L.* (*L.*) *amazonensis* ($ADCL^{DTH-} < BDCL^{DTH-} < LCL^{DTH+}$), confirming the ability of this parasite to suppress the T-cell immune response (Silveira et al., 2004).

In the same instance, it was also evidenced by a semi-quantitative reverse transcription PCR (RT-PCR) that revealed that only *L.* (*L.*) *amazonensis*-infected patients showed increased mRNA expression for IL-4 in biopsies of LCL and ADCL cutaneous lesions, principally in ADCL, whereas no expression was observed for this cytokine in biopsies of any *L.* (*V.*) *braziliensis* LCL and MCL cases. This finding strongly suggests that human *L.* (*L.*) *amazonensis* infection probably involves a high antigen-specific Th2-type immune response activation at the draining lymph nodes, which results in a proliferation of T-cells primed to operate as Th2-type cytokine-producing cells, mainly IL-4, in the peripheral blood and at the focus of cutaneous infection (Silveira et al., 2004).

3. General background on the dendritic Langerhans cell functions

Dendritic Langerhans cells (LCs) are the principal epidermal dendritic antigen-presenting cells (APCs), MHCII^{+}, which comprise 1—3% of total epidermal cells and are derived from cells originating in the bone marrow (Katz et al., 1979). More specifically, they are monocyte precursors that have migrated to the skin and differentiated into these important immune surveillance cells (Palucka and Banchereau, 2006). They also represent a subset of dendritic cells (DCs) characterized by the presence of cytoplasmatic organelles called Birbeck granules, associated with Lag antigens and Langerin (CD207) (Valladeau et al., 2000). LCs are prominent DCs in epithelia, but their role concerning immunity and tolerance remains poorly understood (Kissenpfennig and Malissen, 2006).

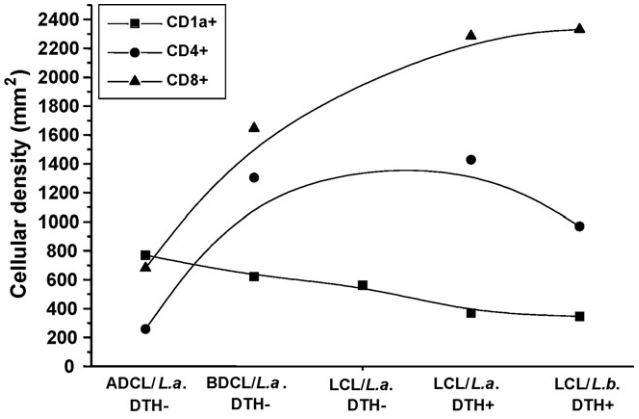

Figure 2 Cellular density of dendritic Langerhans cell (LC; CD1a+) and CD4 (CD4+) and CD8 (CD8+) T-lymphocytes in the cellular infiltrate of cutaneous lesions of the following clinical—immunological spectrum of American cutaneous leishmaniasis (ACL) in Amazonian Brazil, consisting of the anergic diffuse cutaneous leishmaniasis (ADCL) and borderline disseminated cutaneous leishmaniasis (BDCL), both due to *Leishmania* (*L.*) *amazonensis* and with the delayed-type hypersensitivity (DTH) negative$^{(-)}$; localized cutaneous leishmaniasis (LCL) due to *L.* (*L.*) *amazonensis* with two groups, DTH$^-$ and DTH positive$^{(+)}$, and LCL due to *L.* (*Viannia*) *braziliensis* with DTH$^+$. The average values of the CD4+ and CD8+ T-cell profiles were described previously (Silveira et al., 2004), and the average values of the LC densities were from the present study (Figure 1). For recognition of T-cells and LC, the monoclonal antibodies Anti-Human T cell CD45RO (clone OPD4), Anti-Human T cell CD8 Supressor/Cytotoxic (clone DK25) and Anti-Human CD1a (clone 010) (DAKO-Denmark) were used, respectively. The technical proceedings were as used by Silveira et al. (2004) and Xavier et al. (2005).

LCs residing in steady-state tissues are found in an immature state, functioning primarily by monitoring the environment for candidate danger signals and in capturing, processing and transducing them. When a certain threshold is reached, LCs become mature cells, acquiring the ability to migrate from the epidermis to regional lymph nodes, where they are thought to initiate adaptive immune responses and stimulate T-cells (Girolomoni et al., 2002; Kaplan et al., 2005). DCs provide a direct link between innate and adaptive immunity, as well as controlling T-cell differentiation and polarization (Guermonprez et al., 2002).

4. The role of the dendritic LC in ACL immunopathogenesis

According to the above considerations, the role of LCs in ACL immunopathogenesis was recently examined by comparing the epidermal density of LCs in the cutaneous lesions of LCL patients infected with *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* (Xavier et al., 2005). Surprisingly, the most interesting finding was that associated with the group of *L.* (*L.*) *amazonensis* LCL patients presenting negative DTH reactions, which showed a higher ($P < 0.05$) concentration of LCs than that of two other groups: *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis* LCL patients presenting positive DTH reactions. Thus, considering the functional role of epidermal LCs, known as crucial antigen-presenting cells (APCs), in stimulating an effective T-cell response (Moll, 1993, Moll et al., 1995), this finding was then interpreted as an attempt by non-reactive DTH *L.* (*L.*) *amazonensis* LCL patients to increase the concentration of LCs at the cutaneous lesions, leading to improvements in the modulation of T-cell response activation at the draining lymph nodes, as the DTH mechanism of the T-cell response of these patients appeared to be abrogated (DTH reaction was absent). However, a prior study revealed similar profiles for LC density in the skin lesions of LCL and ADCL patients infected by *L.* (*L.*) *mexicana*, a parasite closely related to *L.* (*L.*) *amazonensis*, indicating that even in an immune reactive form (LCL), the expression of LCs was similar to that found under immune suppression (ADCL) (Ritter et al., 1996). More recently, however, in a new comparison of LC density among the clinical—immunological forms of ACL, a progressive increase in LC density was shown from the reactive (DTH$^+$) LCL— central form caused by *L.* (*V.*) *braziliensis* to the non-reactive (DHT$^-$) extremity forms, BDCL and ADCL, caused by *L.* (*L.*) *amazonensis* (ADCL^{DTH-}>BDCL^{DTH-}>LCL^{DTH-}>LCL^{DTH+}) (Figure 1). These new findings seem to indicate a strong and species-specific negative correlation between the present LC density and those previously found in CD4+ and CD8+ T-cell profiles (Silveira et al., 2004) (Figure 2); i.e., along the clinical—immunological spectrum of ACL linked to *L.* (*L.*) *amazonensis*, it is possible that LCs may perform a pivotal role in the development of T-cell immune suppression, from the non-reactive (DTH$^-$) LCL patients toward the more immune-suppressed (Th2-type immune response activation) BDCL and ADCL cases.

5. The dendritic LC in the immunopathogenesis of experimental leishmaniasis

Following recent studies regarding the role of LCs in the immunopathogenesis of experimental leishmaniasis, it is currently believed that logical reasons were found that appear to confirm the observations associated with human *L.* (*L.*) *amazonensis* infection involving the induction of specific T-cell immune suppression (Lainson et al., 1986; Silveira et al., 1991, 1998). According to these studies, which have provided new insight into the functions of LCs in leishmaniasis, it was proposed that in BALB/c mice experimentally infected with *L.* (*L.*) *major*, the DCs capable of stimulating antigen-specific T-cell proliferation were Langerin-negative (LCs are Langerin-positive), suggesting that dermal DCs are crucial to the initiation of a specific T-cell response (Ritter et al., 2004) and not LCs, as was previously postulated (Moll, 1993; Moll et al., 1995). Another study also demonstrated that contact hypersensitivity was found to be exacerbated rather than abrogated in mice presenting an absence of LCs (Kaplan et al., 2005), suggesting that few LCs at the leishmanial focus of infection may help to develop a strong hypersensitivity reaction; by contrast, it is possible that the high LC density in non-reactive (DTH$^-$) *L.* (*L.*) *amazonensis* LCL patients, as well as in patients presenting BDCL and ADCL forms, may explain their progressive T-cell immune suppression. In addition, it has also been

demonstrated that LCs were able to process and present parasite antigens through the MHC class II receptor to CD4+ T-cells, which might then differentiate into regulatory T-cells, suggesting that LCs could be responsible for the suppression of inflammatory events against *L.* (*L.*) *major* infection in vivo (Ritter and Osterloh, 2007). In conclusion, these studies suggest that LCs may indeed represent a *Leishmania* sp. evasion mechanism in order to avoid a T-cell response and to induce immune suppression.

6. Concluding remarks

Taken together, these pieces of information seem to be important for a clearer understanding of the T-cell immune suppression found in human *L.* (*L.*) *amazonensis* infection, which may reflect in negative DTH and lymphocyte proliferation assays found in the majority of *L.* (*L.*) *amazonensis* LCL patients and in those BDCL and ADCL presenting more immune suppression. Thus, a progressive increase in LC density and a progressive decrease in CD4+ and CD8+ T-cell profiles exist, justifying a well-defined and species-specific negative correlation between LCs and CD4+/CD8+ T-cells in the spectrum of ACL associated with *L.* (*L.*) *amazonensis*. In addition, it is possible that some chemokines, such as MCP-1 and MIP-1[α], which have been shown to be the most effective mononuclear cell chemokines in LCL and ADCL caused by *L.* (*L.*) *mexicana* (Ritter et al., 1996), may also exert an attractant effect on the LCs in the clinical spectrum of human *L.* (*L.*) *amazonensis* infection. This might also explain the greater quantities of parasitized macrophages in the cellular infiltrate of LCL skin lesions by *L.* (*L.*) *amazonensis* than by *L.* (*V.*) *braziliensis*; a finding also characteristic of skin lesions in BDCL and ADCL patients (Silveira et al., 2004). Thus, recent data resulting from either ACL patients or from experimental leishmaniasis infection in mice, indicate that *L.* (*L.*) *amazonensis* may use LCs to prime CD4+ T-cells to function as regulatory T-cells (CD4+CD25+ Treg cells) and then to suppress the T-cell response; as shown by the DTH and lymphocyte proliferation assays (Ji et al., 2005). This mechanism may also be important for explaining the evolution of some early non-reactive (DTH$^-$) *L.* (*L.*) *amazonensis* LCL cases toward severe BDCL and ADCL, principally those cases that were mistakenly treated for a relatively long period before a definitive diagnosis of ACL (Silveira et al., 2005). In such cases, it is possible that *L.* (*L.*) *amazonensis* may use LCs as an evasive mechanism for conditioning a favorable CD4+ Th2-type immune response activation to the parasite. Moreover, corroborating this clinical observation, experimental evidence exists confirming that early stages of *L.* (*L.*) *amazonensis* infection in BALB/c mice may impair multiple immune functions, leading to an antigen-specific T-cell immune suppression (Ji et al., 2003). Lastly, using cultured human monocyte-derived DCs, a recent report shows that *L.* (*L.*) *amazonensis* may also impair the differentiation and function of DCs to suppress T-cell response (Favali et al., 2007).

Funding: This research was supported by the Instituto Evandro Chagas (Secretaria de Vigilância em Saúde, Ministério da Saúde, Brazil); Instituto de Medicina Tropical (Universidade Federal do Pará, Brazil); Wellcome Trust (London, UK); Laboratório de Investigação Médica (LIM)-50 [Hospital de Clínicas (HC)-Faculdade de Medicina (FM)-Universidade de São Paulo (USP), Brazil] and Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil (FAPESP: 06/56319-1, Brazil).

Conflicts of interest: None declared.

Ethical approval: Not required.

References

Barral, A., Costa, J.M.L., Bittencourt, A.L., Barral-Neto, M., Carvalho, E.M., 1995. Polar and subpolar diffuse cutaneous leishmaniasis in Brazil: clinical and immunopathologic aspects. Int. J. Dermatol. 34, 474—479.

Blackwell, J.M., 1999. Tumour necrosis factor alpha and mucocutaneous leishmaniasis. Parasitol. Today 15, 73—76.

Bonfim, G., Nascimento, C., Costa, J.M.L., Carvalho, E.M., Barral-Neto, M., Barral, A., 1996. Variation of cytokine patterns related to therapeutic response in diffuse cutaneous leishmaniasis. Exp. Parasitol. 84, 188—194.

Cáceres-Dittmar, G., Tapia, F.J., Sanchez, M.A., Yamamura, M., Uyemura, K., Modlin, R.L., Bloom, B.R., Convit, J., 1993. Determination of the cytokine profile in American cutaneous leishmaniasis using the polymerase chain reaction. Clin. Exp. Immunol. 91, 500—505.

Carvalho, E.M., Barral, A., Costa, J.M.L., Bittencourt, A., Marsden, P.D., 1994. Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis. Acta Trop. 56, 315—325.

Carvalho, E.M., Correia-Filho, D., Baccelar, O., Lessa, H., Rocha, H., 1995. Characterization of the immune response in subjects with self-healing cutaneous leishmaniasis. Am. J. Trop. Med. Hyg. 53, 273—277.

Castellucci, L., Menezes, E., Oliveira, J., Magalhães, A., Guimarães, L.H., Lessa, M., Ribeiro, S., Reale, J., Noronha, E.F., Wilson, M.E., Duggal, P., Beaty, T.H., Jerônimo, S., Jamieson, S.E., Bales, A., Blackwell, J.M., de Jesus, A.R., Carvalho, E.M., 2007. IL6-174 G/C promoter polymorphism influences susceptibility to mucosal but not localized cutaneous leishmaniasis in Brazil. J. Infect. Dis. 194, 519—527.

Castes, M., Trujilho, D., Calgano, M., Cabrera, M., Convit, J., 1993. Response Th1/Th2 in human American cutaneous leishmaniasis: its possible relevance for the design of a vaccine. Mem. Inst. Oswaldo Cruz 88, 42—43.

Convit, J., Ulrich, M., Fernandez, C.T., Tapia, F.J., Cáceres-Dittmar, G., Cástes, M., Rondon, A.J., 1993. The clinical and immunological spectrum of American cutaneous leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 87, 444—448.

Costa, J.M.L., Marsden, P.D., Llanos-Cuentas, E.A., Netto, E.M., Carvalho, E.M., Barral, A., Rosa, A.C., Cuba, C.C., Magalhães, A.V., Barreto, A.C., 1986. Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. Am. J. Trop. Med. Hyg. 89, 319—321.

Da-Cruz, A.M., De-Oliveira, M.P., De-Luca, P.M., Mendonça, S.C., Coutinho, S.G., 1996. Tumor necrosis fator-alpha in human American tegumentary leishmaniasis. Mem. Inst. Oswaldo Cruz 91, 225—229.

Favali, C., Tavares, N., Clarêncio, J., Barral, A., Barral-Neto, M., Brodskyn, C., 2007. *Leishmania amazonensis* infection impairs differentiation and function of human dendritic cells. J. Leukocyte Biol. 82, 1—6.

Girolomoni, G., Caux, C., Lebecque, S., Dezutter-Dambuyant, C., Ricciardi-Castagnoli, P., 2002. Langerhans cells: still a fundamental paradigm for studying the immunobiology of dendritic cells. Trends Immunol. 23, 6—8.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., Amigorena, S., 2002. Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol. 20, 621—667.

Ji, J., Sun, J., Soong, L., 2003. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. Infect. Immun. 71, 4278—4288.

Ji, J., Masterson, J., Sun, J., Soong, L., 2005. Regulatory T cells restrain pathogenic responses during *Leishmania amazonensis* infection. J. Immunol. 174, 7147—7153.

Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., Shlomchik, M.J., 2005. Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity 23, 611—620.

Katz, S.I., Tamaki, K., Sachs, D.H., 1979. Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature 282, 324—326.

Kissenpfennig, A., Malissen, B., 2006. Langerhans cells — revisiting the paradigm using genetically engineered mice. Trends Immunol. 27, 132—139.

Lainson, R., Shaw, J.J., 1987. Evolution, classification and geographical distribution. in: Peters, W., Killick-Kendrick, R. (Eds.), The Leishmaniases in Biology and Medicine, vol. 1. Academic Press, London, pp. 1—120.

Lainson, R., Shaw, J.J., 2005. Leishmaniasis in the New World, in: Collier, L., Balows, A., Sussman, M. (Eds), Topley & Wilson's Microbiology and Microbial Infections, vol. 5. Parasitology, tenth ed. Arnold, London, pp. 313—349.

Lainson, R., Shaw, J.J., Silveira, F.T., Braga, R.R., Ryan, L., Povoa, M.M., Ishikawa, E.A.I., 1986. A *Leishmania* e as leishmanioses, in: Instituto Evandro Chagas; 50 Anos de Contribuição às Ciências Biológicas e à Medicina Tropical. Fundação Serviços de Saúde Pública, Belém, Pará, Brazil, pp. 83—124.

Moll, H., 1993. Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis. Immunol. Today 23, 1595—1601.

Moll, H., Flohé, S., Rollinghoff, M., 1995. Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antingen-specific T-cell immune response. Eur. J. Immunol. 25, 693—699.

Palucka, A.K., Banchereau, J., 2006. Langerhans cells: daughters of monocytes. Nat. Immunol. 7, 223—224.

Petersen, E.A., Neva, F.A., Oster, C.N., Diaz, H.B., 1982. Specific inhibition of lymphocyte proliferation response by adherent suppressor cells in diffuse cutaneous leishmaniasis. N. Engl. J. Med. 306, 387—391.

Pirmez, C., Yamamura, M., Uyemura, K., Paes-Oliveira, M., Conceição-Silva, F., Modlin, R.L., 1993. Cytokine patterns in the pathogenegis of human leishmaniasis. J. Clin. Invest. 91, 1390—1395.

Ribeiro-de-Jesus, A., Almeida, R.P., Lessa, H., Baccelar, O., Carvalho, E.M., 1998. Cytokine profile and pathology in human leishmaniasis. Braz. J. Med. Biol. Res. 31, 143—148.

Ritter, U., Osterloh, A., 2007. A new view on cutaneous dendritic cell subsets in experimental leishmaniasis. Med. Microbiol. Immunol. 196, 51—59.

Ritter, U., Moll, H., Laskay, T., Bröcker, E.-B., Velazco, O., Becker, I., Gillitzer, R., 1996. Differential expression of chemokines in patients with localized and diffuse cutaneous American leishmaniasis. J. Infect. Dis. 173, 699—709.

Ritter, U., Meissner, A., Scheidig, C., Körner, H., 2004. CD8α- and Langerin-negative dendritic cells, but not Langerhans cells, act as principal antigen-presenting cells in leishmaniasis. Eur. J. Immunol. 34, 1542—1550.

Silveira, F.T., Lainson, R., Shaw, J.J., Garcez, L.M., de Souza, A.A.A., Braga, R.R., Ishikawa, E.A.I., 1990. Experimental skin leishmaniasis: II. The course of infection in *Cebus appella* monkey (Primates: Cebidae) by *Leishmania* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis*. Rev. Soc. Bras. Med. Trop. 23, 5—12.

Silveira, F.T., Lainson, R., Shaw, J.J., de Souza, A.A.A., Ishikawa, E.A., Braga, R.R., 1991. Cutaneous leishmaniasis due to *Leishmania* (*Leishmania*) *amazonensis* in Amazonian Brazil, and the significance of a negative Montenegro skin test in human infections. Trans. R. Soc. Trop. Med. Hyg. 85, 735—738.

Silveira, F.T., Lainson, R., de Brito, A.C., Oliveira, M.R.F., Paes, M.G., De Souza, A.A.A., Da Silva, B.M., 1997. Leishmaniose Tegumentar Americana, in: Leão, R.N.Q. (Ed), Doenças Infecciosas e Parasitárias: Enfoque Amazônico. Editora CEJUP, Belém, Pará, Brazil, pp. 619—630.

Silveira, F.T., Blackwell, J.M., Ishikawa, E.A., Braga, R.R., Shaw, J.J., Quinnell, R.J., Soong, L., Kima, P., McMahon-Prat, D., Black, G.F., Shaw, M.A., 1998. T-cell responses to crude and defined leishmanial antigens in patients from the lower Amazon region of Brazil infected with different species of *Leishmania* of the subgenera *Leishmania* and *Viannia*. Parasite Immunol. 20, 19—26.

Silveira, F.T., Lainson, R., Corbett, C.E.P., 2004. Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil. Mem. Inst. Oswaldo Cruz 99, 239—251.

Silveira, F.T., Lainson, R., Corbett, C.E.P., 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, M.L., Machado, P.R., Ko, A.I., Alves, F., Bittencourt, A., Almeida, R.P., Mobashery, N., Johnson Jr, W.D., Carvalho, E.M., 2002. Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. J. Infect. Dis. 186, 1829—1834.

Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., 2000. Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity 12, 71—81.

Xavier, M.B., Silveira, F.T., Demachki, S., Ferrreira, M.M.R., Nascimento, J.L., 2005. American tegumentary leishmaniasis: a quantitative analysis of Langerhans cells presents important differences between *L.* (*L.*) *amazonensis* and *Viannia* subgenus. Acta Trop. 95, 67—73.

ANEXO-10

Ex vivo and in vivo biological behavior of *Leishmania (Viannia) shawi*

Passero, L.F.D.; Sacomori, J.V.; Tomokane, T.Y.; Corbett, C.E.P.; Silveira, F.T.; **Laurenti, M.D.**

Parasitology Research 2009; 105: 1741-1747.

Parasitol Res (2009) 105:1741–1747
DOI 10.1007/s00436-009-1614-7

ORIGINAL PAPER

Ex vivo and in vivo biological behavior of *Leishmania* (*Viannia*) *shawi*

Luiz Felipe Domingues Passero · Juliano V. Sacomori · Thaíse Yumie Tomokane · Carlos Eduardo Pereira Corbett · Fernando Tobias da Silveira · Márcia Dalastra Laurenti

Received: 21 June 2009 / Accepted: 24 August 2009 / Published online: 16 September 2009

Abstract Since the first description of *Leishmania* (*Viannia*) *shawi*, few studies were performed with this parasite. In the present work, the in vivo and ex vivo behavior of *L.* (*Viannia*) *shawi* infection was studied using murine model. Peritoneal macrophages from BALB/c and C57BL/6 mice were infected with promastigotes in the stationary phase of growth; after 24 h, the infection index and nitric oxide (NO) levels in the supernatant of the cultures were analyzed. BALB/c and C57BL/6 mice were infected into the hind footpad, and at each 2 weeks, mice were sacrificed, and the histological changes of the skin inoculation site, parasitism, and humoral immune responses were evaluated during 8 weeks. Ex vivo experiments showed that macrophages of BALB/c presented higher infection index and lesser NO levels than macrophages of C57BL/6. In vivo experiments showed that BALB/c presented higher lesion size than C57BL/6 mice; similarly, the histopathological changes and the parasitism in skin were more exacerbate in BALB/c mice. In draining lymph nodes, the main change was increase of germinative centers, and parasites were detected from 6 weeks pi onwards in both mice strain. IgG was detected in BALB/c mice from 4 weeks, while in C57BL/6, from 6 weeks pi onwards. Taken together, these results indicate that BALB/c showed a classical behavior of susceptibility when compared to C57BL/6 mice.

L. F. D. Passero · T. Y. Tomokane · C. E. P. Corbett · M. D. Laurenti
Laboratório de Patologia de Moléstias Infecciosas (LIM-50), Depto. de Patologia, Universidade de São Paulo, São Paulo, SP, Brazil

J. V. Sacomori
Laboratório de Patologia de Moléstias Infecciosas (LIM-50), Departamento de Patologia, Universidade de São Paulo, Avenida Dr. Arnaldo, 455, 01246-903 São Paulo, Brazil

F. T. da Silveira
Núcleo de Medicina Tropical da Universidade Federal do Pará/Laboratório de Leishmanioses do Instituto Evandro Chagas, Belém, PA, Brazil

L. F. D. Passero (✉)
Department of Pathology, São Paulo University Medical School, Av. Dr. Arnaldo, 455, 01246-903 São Paulo, SP, Brazil
e-mail: felipepassero@usp.br

Introduction

Leishmaniasis is a disease caused by parasites of the genus *Leishmania*, which are capable to infect a broad range of mammalian species, including humans. Up to now, there are 27 species described in the World; among which, some few studied and others recently characterized, such as *Leishmania* (*Viannia*) *shawi* (Lainson et al. 1989), *Leishmania* (*Viannia*) *naiffii* (Lainson and Shaw 1989), *Leishmania* (*Viannia*) *colombiensis* (Kreutzer et al. 1991), *Leishmania* (*Viannia*) *lainsoni* (Silveira et al. 1987), and *Leishmania* (*Viannia*) *lindenbergi* (Silveira et al. 2002).

In the Amazon region of Brazil, *L.* (*Viannia*) *shawi* was described and characterized by Lainson et al. (1989), who isolated it from *Cepus apella* and *Chiropotes satanas* monkeys, *Choloepus didactylus* sloth, edentate *Bradypus tridactylus*, coatimundis *Nasua nasua*, and sandfly *Lutzomyia whitmany* in the Serra dos Carajás, Pará State, Brazil. In these animals, the parasite had different tissue tropisms: in the sloths, it was found in the spleen and liver, and in the other reservoirs, it was found limited to the skin; only in the hamster the parasite caused a self-limited infection in the skin with moderate densities of amastigote forms. This first report on *L.* (*Viannia*)

shawi showed that it is capable of infecting a wide range of animals and with tissue specificity regarding host species.

Two years afterwards, the same research group found this species related to the human American Cutaneous Leishmaniasis (ACL). In most cases, around 60%, the disease was characterized by single ulcerated skin lesion, although in some individuals, many lesions were recorded in the body (Shaw et al. 1991). More recently, however, the opinion is that *L.* (*Viannia*) *shawi* infection has increased in its importance as an agent of ACL in the Brazilian Amazon region, being surpassed only by *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *guyanensis* (Silveira, personal communication).

Despite these few studies, information regarding the interaction between *L.* (*Viannia*) *shawi* and the host is scarce. Therefore, the aims of this study was evaluate the infectivity of *L.* (*Viannia*) *shawi* in macrophages from BALB/c and C57BL/6 mice, as well as in vivo evaluating the lesion size, histopathological changes, parasite density, and humoral immune response to better understand the events that take place in the course of infection, characterizing the murine model for *L.* (*Viannia*) *shawi* infection.

Materials and methods

Parasites

L. (*Viannia*) *shawi* (MHOM/BR/96/M15789 strain) was isolated from a patient with cutaneous leishmaniasis in the Buriticupu County, Maranhão State, Brazil. It was classified by monoclonal antibodies and isoenzymes at the Evandro Chagas Institute, Belém, Pará State, Brazil. The parasites maintained in BALB/c mice footpad were isolated and expanded in RPMI-1640 medium, supplemented with 10% heat-inactivated serum, 0.25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mg/ml gentamicin, and 100 IU/ml penicillin and maintained at 25°C. On the sixth day of culture, the promastigote forms were washed three times with phosphate buffer (PBS) pH7.4, centrifuged at 1,200 g, 10 min, 4°C, for mice infection and production of soluble antigen.

Antigen production

Stationary phase promastigotes were harvested from 5 to 6 days of culture and washed three times in cold PBS, resuspended in sterile distillated water, subjected to frozen in liquid nitrogen, and thawed at room temperature three times, and the solution was centrifuged at 10,000 g for 30 min. The supernatant was collected, and protein concentration was determined using a commercial kit (Biorad, USA). Aliquots were stored at −80°C.

Experimental animals

Eight-week-old BALB/c and C57BL/6 male mice obtained from the Animal Facility of the São Paulo University, Medical School Brazil were maintained in our laboratory during the experiments according to the guidelines of the institutional rules regarding the welfare of experimental animals and with the approval of the Animal Ethics Committee of São Paulo University.

Ex vivo experiments: macrophage infection and NO quantification

Approximately 10^5 BALB/c and C57BL/6 mice peritoneal macrophages were placed in a 24-well plate in RPMI medium. *L.* (*Viannia*) *shawi* promastigotes in a stationary phase of culture growth were added to the macrophage monolayers at a ratio of 5 parasites to 1 macrophage, and the culture was incubated at 5% CO_2 at 35°C. After 24 h, the plate was centrifuged (1,000 g, 10 min, 4°C), and supernatants were collected and stored at −80°C for nitric oxide (NO) quantification, according to Green et al. 1990. The NO levels were estimated using standard curve constituted of nitrite. The slides were dried at room temperature, fixed in methanol, and stained by Giemsa. The infection index was determinate in both macrophages from BALB/c and C57BL/6 mice.

BALB/c and C57BL/6 mice infection

Stationary phase promastigote forms were washed three times in PBS, and the parasites concentration was adjusted to 2×10^7 promastigotes/ml. Fifty microliters was injected subcutaneously into the hind footpads of the mice (around 10^6 promastigotes/mouse), and only PBS was injected in control animals. The lesion size was measured weekly with a dial micrometer and expressed as the difference in size between the infected and the contralateral uninfected footpad. At 2, 4, 6, and 8 weeks post-infection (pi), one group of five infected mice of both mouse strains were bled, and the sera were collected and stored at −80°C. Biopsies of the skin inoculation site and lymph nodes were collected and fixed in buffered 5% formalin for histopathological studies and quantification of amastigote forms. Imprints of spleen and liver were performed to evaluate the viscera parasitism. The experiment was performed three times.

Evaluation of histopathological changes and quantitative morphometric analysis

The histological changes were evaluated through routine histological HE-staining of 5 μm sections under the light

microscope and were further graded as negative, light, moderate, and intense, according to Giunchetti et al. (2008). The parasitism in skin and lymph nodes of BALB/c and C57BL/6 mice was evaluated using quantitative morphometric analysis in sections labeled by immunohistochemistry to evidence the amastigote forms, according to Passero et al. (2008).

Evaluation of the humoral immune response

The humoral immune response was measured by enzyme-linked immunosorbent assay (ELISA). High-binding plates (Costar, USA) were coated with 1 μg/well soluble antigen from *L.* (*Viannia*) *shawi* promastigote, overnight at 4°C. Plates were blocked with 10% of non-fat milk in PBS for 2 h at 37°C to prevent nonspecific binding. Sera of all animal groups (1:50) were added for 1 h at 37°C. Goat anti-mouse IgG monoclonal antibody conjugated with alkaline phosphatase (Sigma, USA) in a dilution of 1:1,000 was added for 1 h, 37°C. The substrate p-nitrophenyl phosphate (1.0 mg/ml) was added for 30 min, and the absorbance was read in ELISA reader (Uniscience, USA).

Statistical analysis

The results were expressed as mean±standard deviation of three independent experiments, and the non-parametric test of Mann–Whitney was employed to compare infection index, NO levels, lesion size, parasite density, and humoral immune response between BALB/c and C57BL/6 mice. Differences were considered statistically significant when $p<0.05$. Statistical analysis were performed using Biostat 5.0 software, using $N=3$.

Results

Macrophage infection index and NO levels

Macrophages from BALB/c mice presented higher infection index than those from C57BL/6 with statistical significance ($p<0.05$). Moreover, macrophages from BALB/c mice showed tendency to present diminished levels of NO levels compared to macrophages from C57BL/6; however, no statistical significance was observed (Fig. 1). Uninfected macrophages showed no expressive NO levels (data not shown).

Lesion size

The infection caused by *L.* (*Viannia*) *shawi* induced an increase in the hind footpad swelling in BALB/c and C57BL/6 mice. The lesion size showed no significant differences at 1, 2, and 3 weeks pi between both mice, but from 4 weeks pi, the lesion size became significantly higher in BALB/c mice ($p<0.05$) compared with C57BL/6 mice (Fig. 2), and at 8 weeks pi, the footpad of BALB/c and C57BL/6 mice increased 2.1 and 1.6 times in relation to both healthy mice, respectively.

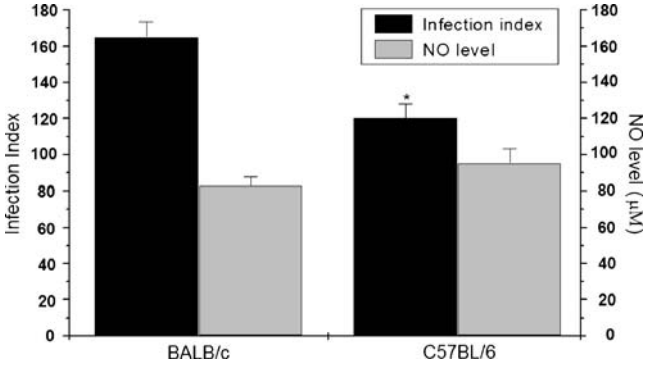

Fig. 1 Peritoneal macrophages from BALB/c and C57BL/6 mice were isolated and infected with a rate of five promastigote/one macrophages. After 24 h, the production of nitric oxide levels from both mice macrophages were quantified, and the infection index was calculated. *$p<0.05$ indicates statistical significance between infection index of macrophages from C57BL/6 with macrophage from BALB/c mice

Histopathological changes in the skin

Microscopically, a discrete inflammatory infiltrate in the dermis, consisting of moderated densities of polymorphonuclear, mononuclear cells, and light densities of amastigote forms inside the macrophages (Figs. 3a and 5a) was observed in BALB/c mice at 2 weeks pi. At 4 weeks pi, the inflammatory infiltrate enlarged, showing moderate densities of polymorphonuclear cells, high densities of infected

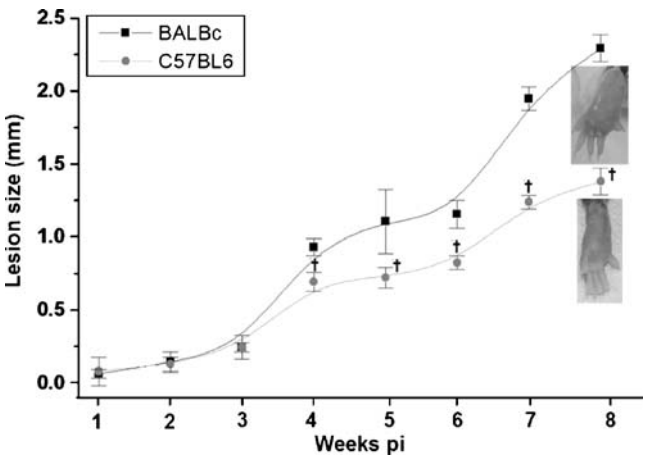

Fig. 2 Evolution of footpad swelling in BALB/c and C57BL/6 mice infected with 10^6 promastigotes/animal. *Cross*: $p<0.05$ indicates statistical significance between BALB/c and C57BL/6 infected mice

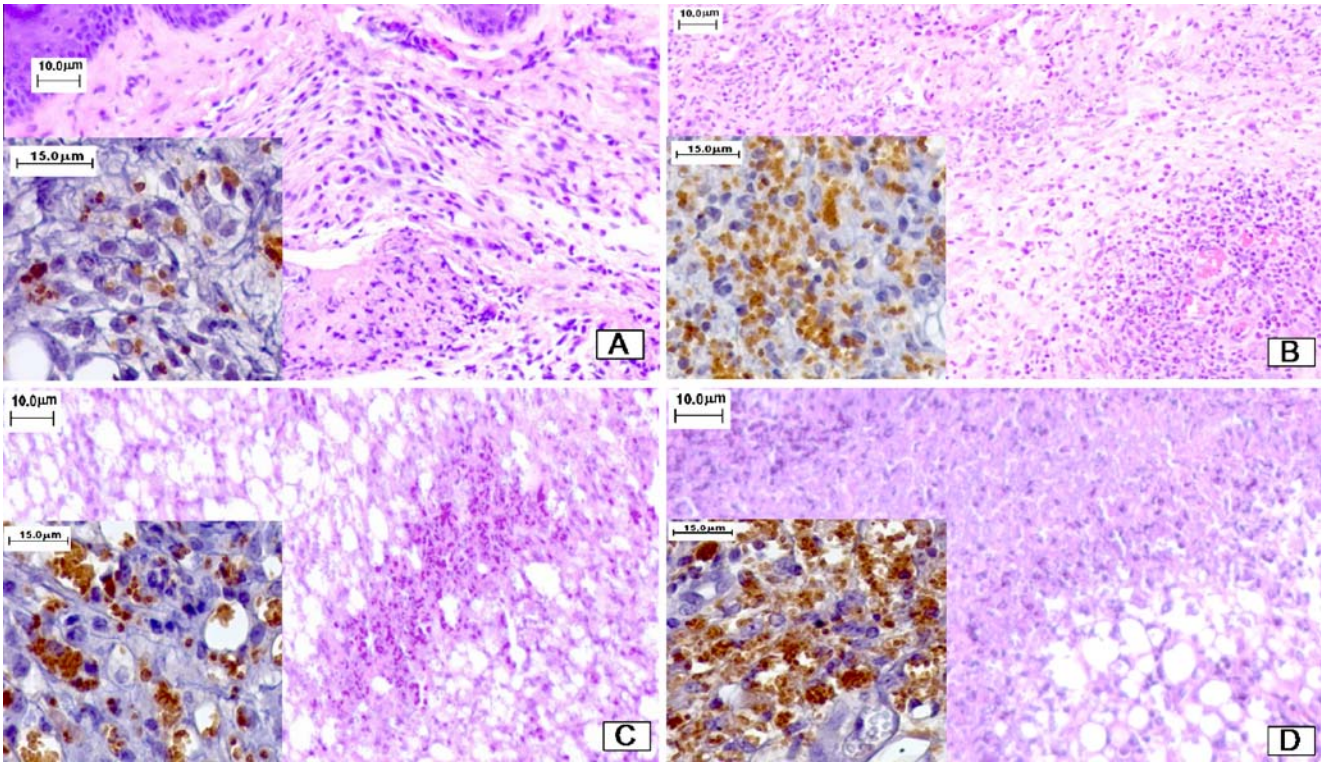

Fig. 3 Histopathology of the skin lesion produced in BALB/c mice by *Leishmania* (*Viannia*) *shawi*. **a** Two weeks pi (×200), (**b**) 4 weeks pi (×200), **c** 6 weeks pi (×200), and **d** 8 weeks pi (×200); details are immunohistochemistry reactions showing amastigote forms (×400)

mononuclear cell, and moderated density of amastigote forms (Figs. 3b and 5a). A highly inflammatory infiltrate diffusely distributed in the dermis with light densities of lymphocyte, plasma cells, and highly infected macrophages was observed at 6 weeks pi. Moreover, a moderate presence of polymorphonuclear cells and focal necrosis areas (Figs. 3c and 5a) was also verified. At 8 weeks pi, an intense and diffuse inflammatory infiltrate in the dermis, with light densities of polymorphonuclear and plasma cells, moderate densities of lymphocytes, and intense density of heavily infected macrophages, was observed. The necrosis was considered moderate (Figs. 3d and 5a). The number of amastigote forms in the parasite inoculation site of BALB/c mice increased significantly with the time of the infection ($p<0.05$).

Concerning to C57BL/6 mice, they also showed inflammatory changes in the skin, but in lesser degree. A light focal inflammatory infiltrate characterized by reduced densities of polymorphonuclear and mononuclear cells and light amounts of amastigote forms (Figs. 4a and 5a) were observed at 2 weeks pi. At 4 weeks pi, the inflammatory infiltrate was also light but spread through the dermis; it was characterized by the presence of a moderate density of polymorphonuclear and mononuclear cells, light density of lymphocytes, and amastigote forms (Figs. 4b, a). A moderate inflammatory infiltrate with a diffuse distribution, moderate presence of polymorphonuclear and mononuclear cells, light densities of lymphocyte, and moderate densities of amastigote forms (Figs. 4c, a) were observed in the dermis at 6 weeks pi. An intense and diffuse inflammatory infiltrate represented by intensely parasitized macrophages (Fig. 4a), moderate densities of lymphocytes, and few polymorphonuclear cells, was observed in the dermis at 8 weeks pi, additionally, a light area of necrosis was also found (Fig. 4d).

Comparatively, BALB/c mice showed higher amastigote densities than C57BL/6 mice ($p<0.05$). The densities of amastigote forms at 4, 6, and 8 weeks pi in C57BL/6 mice increased in relation to 2 weeks pi with statistical significance ($p<0.05$), reinforcing the tendency of disease progression in this mice.

The lymph nodes of both mice strains showed prominent germinative centers in lymphoid follicles, which were mostly pronounced in BALB/c mice. The amastigote forms were detected at 6 and 8 weeks pi, and their localization and densities were similar in both mouse strains (Fig. 5b). The spleen and liver of both mouse strains showed no histological changes, and parasites were not found.

Humoral immune response

BALB/c mice infected with *L.* (*Viannia*) *shawi* presented detectable anti-*Leishmania* IgG from 2 weeks pi onward compared to uninfected BALB/c mice ($p<0.05$), while in

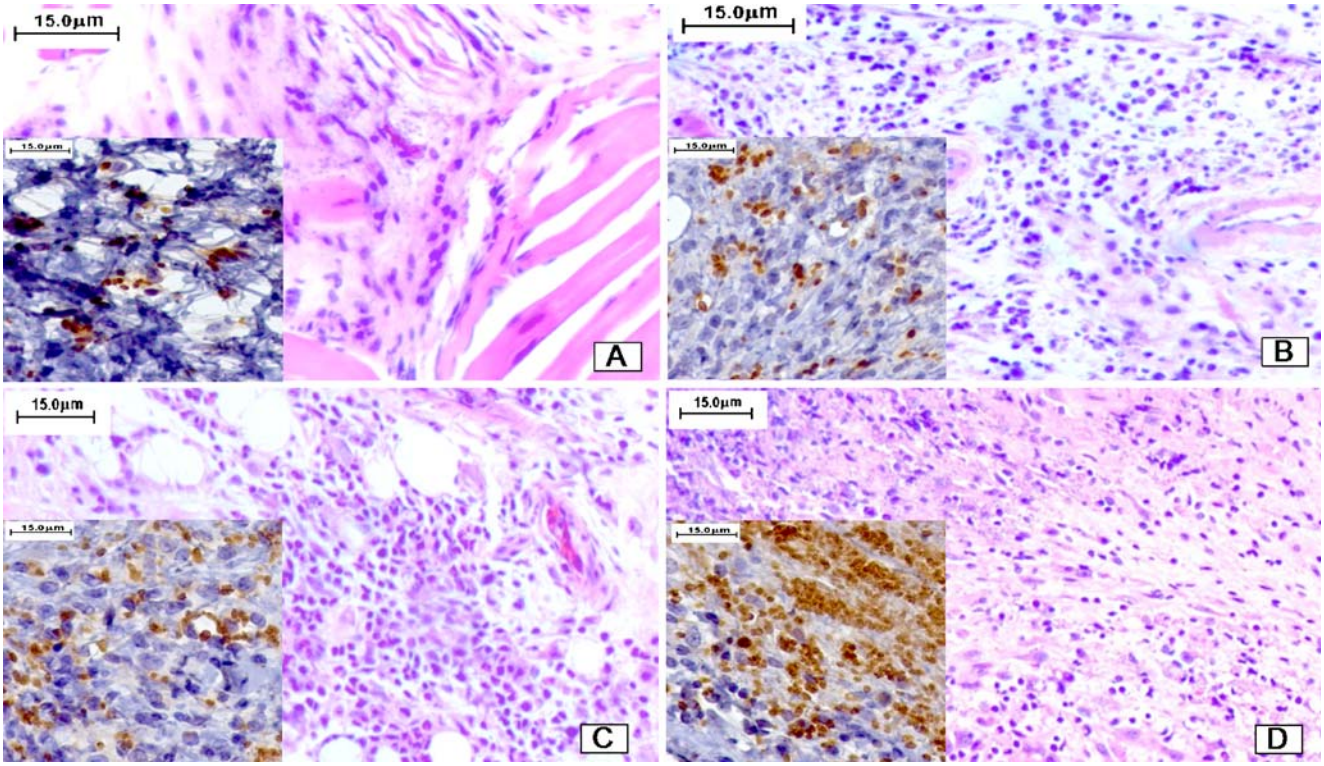

Fig. 4 Histopathology of the skin lesion produced in C57BL/6 mice by *Leishmania* (*Viannia*) *shawi*. **a** Two weeks pi (×200), **b** 4 weeks pi (×200), **c** 6 weeks pi (×200), and **d** 8 weeks pi (×200); details are immunohistochemistry reactions showing amastigote forms

C57BL/6 mice, it was detected only at 6 and 8 weeks pi compared to uninfected C57BL/6 mice ($p<0.05$). Moreover, the IgG levels were higher in BALB/c than C57BL/6 mice ($p<0.05$; Fig. 6).

Discussion

Studies on experimental models can help in the improvement of new approaches in drugs and vaccines studies. In the case of *L.* (*Viannia*) *shawi* parasites, information regarding their infectious evolution in ex vivo and in vivo is missing.

Studies in vitro or ex vivo have been useful in the understanding of some biological processes in the interaction of *Leishmania* sp. with host cells. The results showed that *L.* (*Viannia*) *shawi* infection induced higher infection index in macrophages of BALB/c than C57BL/6 mice, indicating that BALB/c mice presented high susceptibility to the parasite infection. On other hand, C57BL/6 mice were not capable of full elimination of intracellular amastigotes, demonstrating that they also present intermediate susceptibility to infection. In vitro studies demonstrated the essential role of NO in elimination of intracellular amastigotes (Liew et al. 1990a; Campos et al. 2008) and possibly, due to low levels of NO molecule in both macrophages from BALB/c and C57BL/6 mice, the infection could not be eliminated. However, other mechanisms associated to parasite elimination, such as hydrogen peroxide (Das et al. 2001), superoxide (Gantt et al. 2001), and hydroxyl radicals (Barr and Gedamu 2003) may be associated to lesser infection index observed in macrophages from C57BL/6 mice, but they were not sufficient for eliminate amastigotes within C57BL/6 macrophages.

Similarly, in vivo studies demonstrated that BALB/c suffered higher disease progression than C57BL/6 mice. In infected BALB/c mice, high lesion size was verified, associated to intense inflammatory reaction in dermis and high number of macrophages highly parasitized. C57BL/6 mice also presented increase in the footpad swelling with the presence of inflammatory cells and amastigote forms within macrophages, however, with low degree compared to BALB/c mice. These characteristics indicate that BALB/c and C57BL/6 mice were permissible to the infection caused by *L.* (*Viannia*) *shawi*; however, some immunological characteristic may be associated to lesser pathology and parasitism in C57BL/6 mice. In *Leishmania major*, infection is well defined that BALB/c presents a clear susceptible profile, while C57BL/6 mice present a resistant profile (Reiner and Locksley 1995). However, in New World leishmaniasis, this pattern cannot be the same, since *Leishmania mexicana* generated progressive disease and metastatic lesion in BALB/c mice, while C57BL/6 mice

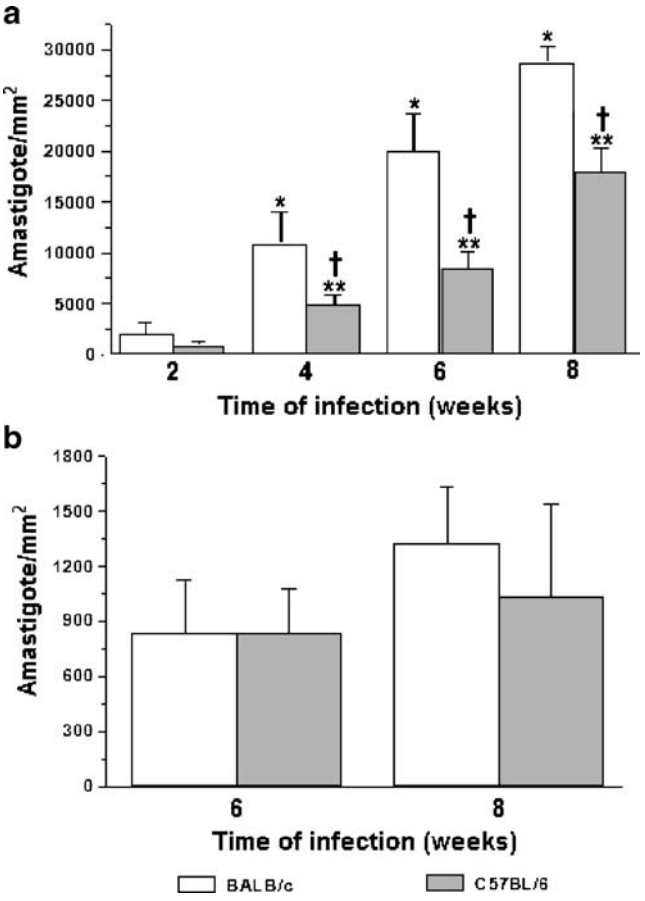

Fig. 5 Parasite density in skin (**a**) and lymph nodes (**b**) of BALB/c and C57BL/6 mice evaluated by image analysis system (AxioVison 5.0) in paraffin section stained by immunohistochemistry reaction. Each value represents the mean±standard deviation of amastigote/mm^2 of tissue of at least ten microscopy field. single and double *asterisks* $p<0.05$ indicates statistical significance comparing amastigote densities of BALB/c and C57BL/6 mice with those from of 2 weeks pi, respectively; cross: $p<0.05$ indicates statistical significance comparing amastigote densities of C57BL/6 with amastigote densities of BALB/c mice

developed measurable lesions in low levels, but they fail to heal for entire experimental time (Aguilar Torrentera et al. 2002). Studies conducted by Abreu-Silva et al. (2004) and Maioli et al. (2004) also demonstrated similar profile in both BALB/c and C57BL/6 infected with *Leishmania amazonensis*.

Moreover, in the dermis of both mice, the growing densities of amastigote forms inside macrophages indicate low capacity of effector cells, such as lymphocytes, to activate macrophages for kill amastigote forms (Liew et al. 1990b). Moreover, the presence of neutrophil and plasma cells observed in BALB/c mice skin may be associated to increasing susceptibility to infection, once these cells could be coupling to the progression of cutaneous lesions in experimental leishmaniasis (Wanasen et al. 2008; Tacchini-Cottier et al. 2000). Qualitative analysis showed light densities of lymphocytes in the dermis of C57BL/6 mice, at late stages of infection, indicating that lesser number of amastigote forms may be a direct consequence of lymphocytes presence into the site of infection. Necrosis areas were higher in BALB/c mice than in C57BL/6 mice and appear at early phase of infection. It could have a pathogenic role in leishmaniasis, since de Moura et al. (2005) found high lesion size associated with necrosis into ear dermis in susceptible mice infected with *Leishmania braziliensis*.

Into the draining lymph nodes close to skin infection site, the immune response against *Leishmania* sp. is developed. In *L. shawi* infection, the main histological finding in popliteal lymph nodes was increasing in the germinative center, which was higher in BALB/c than C57BL/6 mice (data not shown). Moreover, IgG antibodies were detected early in BALB/c mice; however, C57BL/6 mice also presented detectable IgG antibodies but in lesser levels and in later stages. Classically, high levels of total IgG antibodies have been associated to disease progression, indicating that both mice strain were in the direction of the disease establishment, but in BALB/c mice, high levels of IgG indicated a fast disease progression compared to C57BL/6 mice.

The characterization of biological behavior of new *Leishmania* species in experimental models can help in the understanding of basic events associated to pathological process. Moreover, experimental models of infection can be useful in the development of novel molecules with pharmacological interest as well as promising vaccine candidates. Studies with *L.* (*Viannia*) *shawi* demonstrated its potential to infect both BALB/c and C57BL/6 mice, where BALB/c mice presented classical signs of susceptibility, therefore, this mice strain could be an interesting target for further studies on host-parasite interplay for

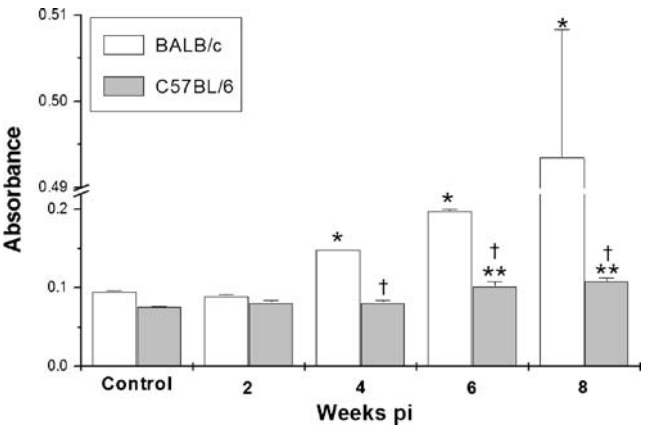

Fig. 6 Humoral response in BALB/c and C57BL/6 mice infected with *Leishmania* (*Viannia*) *shawi*. (**a**) anti-*Leishmania* IgG. single and double *asterisks* $p<0.05$ indicates statistical significance comparing IgG levels of infected BALB/c and C57BL/6, respectively, with mice at 2 weeks pi; cross: $p<0.05$ indicates statistical significance comparing IgG levels of infected BALB/c mice with C57BL/6 mice

understanding of immunopathogenesis of American Tegumentar Leishmaniasis, caused by this parasite species.

Acknowledgements This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and HCFMUSP-LIM50. It is part of the doctoral thesis of Luiz Felipe D. Passero under a FAPESP fellowship. The authors of this paper would like to thank Profa. Dra. Gabriela Santos-Gomes from Unidade de Leishmanioses, CMDT-LA, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal, for the critical reading of the manuscript.

References

Abreu-Silva AL, Calabrese KS, Cupolilo SM, Cardoso FO, Souza CS, Gonçalves da Costa SC (2004) Histopathological studies of visceralized *Leishmania* (*Leishmania*) *amazonensis* in mice experimentally infected. Vet Parasitol 121:179–187

Aguilar Torrentera F, Lambot MA, Laman JD, Van Meurs M, Kiss R, Noël JC, Carlier Y (2002) Parasitic load and histopathology of cutaneous lesions, lymph node, spleen, and liver from BALB/c and C57BL/6 mice infected with *Leishmania mexicana*. Am J Trop Med Hyg 66:273–279

Barr SD, Gedamu L (2003) Role of peroxidoxins in *Leishmania chagasi* survival. Evidence of an enzymatic defense against nitrosative stress. J Biol Chem 278:10816–10823

Campos MB, Gomes CM, de Souza AA, Lainson R, Corbett CE, Silveira FT (2008) In vitro infectivity of species of *Leishmania* (*Viannia*) responsible for American cutaneous leishmaniasis. Parasitol Res 103:771–776

Das M, Mukherjee SB, Shaha C (2001) Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. J Cell Sci 114:2461–2469

de Moura TR, Novais FO, Oliveira F, Clarêncio J, Noronha A, Barral A, Brodskyn C, de Oliveira CI (2005) Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. Infect Immun 73:5827–5834

Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, Britigan BE, Wilson ME (2001) Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. J Immunol 167:893–901

Giunchetti RC, Martins-Filho OA, Carneiro CM, Mayrink W, Marques MJ, Tafuri WL, Corrêa-Oliveira R, Reis AB (2008) Histopathology, parasite load and cell phenotypes of the popliteal lymph node in canine visceral leishmaniasis. Vet Immunol Immunopathol 121:23–33

Green SJ, Meltzer MS, Hibbs JB Jr, Nacy CA (1990) Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J Immunol 144:278–283

Kreutzer RD, Corredor A, Grimaldi G Jr, Grogl M, Rowton ED, Young DG, Morales A, McMahon-Pratt D, Guzman H, Tesh RB (1991) Characterization of *Leishmania colombiensis* sp. n (Kinetoplastida: Trypanosomatidae), a new parasite infecting humans, animals, and phlebotomine sand flies in Colombia and Panama. Am J Trop Med Hyg 44:662–675

Lainson R, Shaw JJ (1989) *Leishmania* (*Viannia*) *naiffi* sp. n. a parasite of the armadillo, *Dasypus novemcinctus* (L.) in Amazonian Brazil. Ann Parasitol Hum Comp 64:3–9

Lainson R, Braga RR, de Souza AAA, Pôvoa MM, Ishikawa EAY, Silveira FT (1989) *Leishmania* (*Viannia*) *shawi* sp. a parasite of monkeys, sloths and procyonids in Amazonian Brazil. Ann Parasitol Hum Comp 64:200–207

Liew FY, Li Y, Millott S (1990a) Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J Immunol 145:4306–4310

Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S (1990b) Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. J Immunol 144(12):4794–4797

Maioli TU, Takane E, Arantes RM, Fietto JL, Afonso LC (2004) Immune response induced by New World *Leishmania* species in C57BL/6 mice. Parasitol Res 94:207–212

Passero LFD, Laurenti MD, Tomokane TY, Corbett CEP, Toyama MH (2008) The effect of phospholipase A2 from *Crotalus durissus collilineatus* on *Leishmania* (*Leishmania*) *amazonensis* infection. Parasitol Res 102:1025–1033

Reiner SL, Locksley RM (1995) The regulation of immunity to *Leishmania major*. Annu Rev Immunol 13:151–177

Silveira FT, Shaw JJ, Braga RR, Ishikawa E (1987) Dermal leishmaniasis in the Amazon region of Brazil: *Leishmania* (*Viannia*) *lainsoni* sp.n. a new parasite from the State of Pará. Mem Inst Oswaldo Cruz 82:289–291

Silveira FT, Ishikawa EA, De Souza AA, Lainson R (2002) An outbreak of cutaneous leishmaniasis among soldiers in Belém, Pará State, Brazil, caused by *Leishmania* (*Viannia*) *lindenbergi* n. sp. A new leishmanial parasite of man in the Amazon region. Parasite 9:43–50

Shaw J, Ishikawa EAY, Lainson R, Braga RR, Silveira FT (1991) Cutaneous leishmaniasis of man due to *Leishmania* (*Viannia*) *shawi* Lainson, de Souza, Póvoa, Ishikawa & Silveira, in Pará state, Brazil. Ann Parasitol Hum Comp 66:243–246

Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, Milon G, Louis JA (2000) An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. J Immunol 165:2628–2636

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

ANEXO-11

Histopathology, humoral and cellular immune response in the murine model of *Leishmania (Viannia) shawi*

Passero, L.F.D.; Marques, C.; Vale-Gato, I.; Corbett, C.E.P.; **Laurenti, M.D.**, Santos-Gomes, G.

Parasitology International 2010; [Epub ahead of print]

Parasitology International xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*

Luiz Felipe Domingues Passero [a], Cláudia Marques [b], Inês Vale-Gato [b], Carlos Eduardo Pereira Corbett [a], Márcia Dalastra Laurenti [a], Gabriela Santos-Gomes [b,*]

[a] *Laboratorio Patologia de Moléstias Infecciosas, Faculdade de Medicina da Universidade de São Paulo, Brazil. Av. Dr. Arnaldo, 455, São Paulo, Brazil*
[b] *UEI de Leishmanioses, Centre for Malaria and Tropical Diseases Associated Laboratoire, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira, 96, 1349-008, Lisboa, Portugal*

ARTICLE INFO

Article history:
Received 1 August 2009
Received in revised form 28 December 2009
Accepted 29 December 2009
Available online xxxx

Keywords:
Leishmania (*Viannia*) *shawi*
Histopathology
IgG1
Cytokines
IL-10
TGF-β

ABSTRACT

Leishmania (*Viannia*) *shawi* was recently characterized and few studies concerning modifications in cellular and humoral immune responses in experimental leishmaniasis have been conducted. In this work, immunopathological changes induced by *L. shawi* in chronically infected BALB/c mice were investigated. Infected BALB/c mice developed increased lesion size associated with strong inflammatory infiltrate diffusely distributed in the dermis, with highly infected macrophages. The humoral immune response was predominantly directed toward the IgG1 isotype. The functional activity of $CD4^+$ and $CD8^+$ T cells showed significantly increased TNF-α mRNA levels associated with reduced IFN-γ expression by $CD4^+$ T cells and the double negative (dn) CD4CD8 cell subset. High IL-4 levels expressed by $CD8^+$ T cells and dnCD4CD8 and TGF-β by $CD4^+$ and $CD8^+$ T cells were detected, while IL-10 was highly expressed by all three cell subpopulations. Taken together, these results show an evident imbalance between TNF-α and IFN-γ that is unfavorable to amastigote replication control. Furthermore, *L. shawi* seems to regulate different cell populations to express deactivating cytokines to avoid its own destruction. This study indicates BALB/c mice as a potentially good experimental model for further studies on American cutaneous leishmaniosis caused by *L. shawi*.

1. Introduction

American tegumentary leishmaniasis (ATL) is a zoonotic disease caused by *L.* (*Leishmania*) *amazonensis, L.* (*Viannia*) *braziliensis, L.* (*V.*) *guyanensis, L.* (*V.*) *shawi, L.* (*V.*) *lainsoni, L.* (*V.*) *naiffi and L.* (*V.*) *linderbegi* parasites [1] that are transmitted through sandfly bites. In humans, ATL can develop into cutaneous (ACL) or mucosal (AML) lesions [2].

The abundant experimental studies of cutaneous leishmaniasis involving *L.* (*L.*) *major* permitted a more comprehensive picture of the clinical, pathological, physiological and immunological aspects of the disease [3]. The work conducted on experimental animal models has furthered our understanding of the immune responses associated with resistance and susceptibility to infection. Resistance is related to the predomination of $CD4^+$ Th1 and $CD8^+$ Tc1 cell populations, which are responsible for the activation of macrophages to kill intracellular parasites and/or to lyse the infected cells through a complex network of cytokines. In contrast, susceptibility is related to $CD4^+$ Th2 type cells and the production of interleukin (IL)-4, IL-5 and IL-13 [4]. In human cutaneous leishmaniasis, the Th1/Th2 paradigm has been used in prognosis to predict the development of resistance or progression of the disease. Ajdary et al. (2000) [5] observed high levels of IL-4 and low amounts of IFN-γ in active lesions. On the other hand, high levels of IFN-γ were detected in PBMC from patients with healing lesions. Similar results have been reported by Louzir et al. (1998) [6] in cutaneous lesions of patients infected with *L. major*. However, the pathological and immunological characteristics displayed by Old World species of *Leishmania* can be profoundly different from those elicited by New World parasites.

BALB/c mice have a recognized high susceptibility to *L. major*, while C57BL/6 is resistant to infection. In these mice strains, *L. braziliensis* induces diminished footpad swelling and cure of the lesions is achieved in a few weeks [7], whereas *L. amazonensis* is able to infect and cause severe and progressive pathology [9]. Furthermore, Rocha et al. (2007) [8] identified differences between the cytokine profile, signaling pathways and effector molecules in the clinical course of *L. major* and *L. braziliensis*, showing that the requirements for the control of cutaneous leishmaniasis differ significantly within *Leishmania* species.

Therefore, experimental studies with *Leishmania* species from the New World may elicit important insights into the immunopathological mechanisms of ATL.

* Corresponding author. Instituto de Higiene e Medicina Tropical, Rua da Junqueira, 96, 1349-008 Lisboa, Portugal. Tel.: +351 21 3652600; fax: +351 21 3622458; +351 21 3632105.
E-mail address: santosgomes@ihmt.unl.pt (G. Santos-Gomes).

1383-5769/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2009.12.010

L. shawi was characterized in the Amazon region in 1989 by Laison et al. (1989) [10]. In 1991 Shaw et al. [11] demonstrated the importance of this species as an agent of cutaneous leishmaniasis in the Amazon region and described the pattern of lesions generated ranging from single to multiple ulcers. However, in the Atlantic rainforest region of northeastern Brazil (Pernambuco state) five human isolates were recently identified, by multilocus enzyme electrophoresis, as *L. shawi* [12]. The above considerations suggest that *L. shawi* may have a non-negligible importance in the epidemiology of ATL, which could be confirmed in a near future. Therefore, the present work aims to study the histopathological alterations, parasite load, humoral and cellular immune responses in mice chronically infected with *L. shawi*.

2. Materials and methods

2.1. Experimental animals

Eight week-old male BALB/c mice were purchased from the Medical School of São Paulo University and maintained according to the guidelines of institutional rules regarding the welfare of experimental animals, with the approval of the Animal Ethics Committee of São Paulo University. Groups of 12 mice each were divided into control and infected mice.

2.2. Parasites

L. shawi (MHOM/BR/96/M15789) parasites were isolated from a patient with cutaneous leishmaniasis in Buriticupu County, Maranhão State, Brazil and identified by monoclonal antibodies and multilocus enzyme electrophoresis at the Evandro Chagas Institute (Belém, Pará State, Brazil). The parasites maintained in BALB/c mice footpad were isolated and grown in RPMI-1640 medium (Gibco Invitrogen, USA) supplemented with 10% heat-inactivated FCS, 0.25 mM HEPES, 10 mg/ml gentamicin and 100 IU/ml penicillin and incubated at 25 °C. On day 6 of culture, promastigote forms were centrifuged (1200 *g* for 10 min) with phosphate buffer saline solution (PBS, pH 7.4) and used for mice infection and production of leishmania soluble antigen.

2.3. Infection, lesion size and parasite load

Mice were intradermally injected in the hind footpads with 1×10^6 parasite/mouse. Control mice were injected with PBS. The lesion size was measured weekly with a dial micrometer and expressed as the difference in size between the infected and the contralateral uninfected footpad. At 5 weeks post infection, mice were bled by cardiac punction and biopsies of skin and popliteal lymph nodes were collected. Sera were collected and stored at −80 °C to study the humoral immune response. The biopsies were used for histopathological examination and estimation of parasite load by limiting dilution assay according to Rodrigues et al. [13]. Moreover, RNA isolated from $CD4^+$ and $CD8^+$ T cells and double negative (dn) CD4CD8 cells purified from popliteal lymph nodes of infected and control mice were used to assay the *ex vivo* cellular immune response. The experiment was repeated three times.

2.4. Evaluation of histopathological changes in skin and lymph nodes

Histological modifications were evaluated by routine histological hematoxylin and eosin (HE) staining of 5 μm sections of skin under a light microscope. Histopathological alterations were further graded as negative, light, moderate and intense, according to Giunchetti et al. [14]. Slides from skin and lymph nodes were labeled by immunohistochemistry to reveal amastigote forms inside macrophages, according to Passero et al. (2008) [15].

2.5. Humoral immune response

The humoral immune response was evaluated by enzyme-linked immunosorbent assay (ELISA). High-binding plates (Costar, USA) were coated with 1 μg/well of *L. shawi* soluble antigen overnight at 4 °C. Next the plates were blocked with 10% of non-fat milk in PBS (2 h at 35 °C) to prevent nonspecific binding. Mouse sera (1:100) were added and the plates were incubated for 1 h at 35 °C. Biotinylated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Biorad, USA) (1:1000) were used for 1 h at 35 °C. After washing, streptavidin conjugated with peroxidase was added to each well for 1 h at 35 °C. After 3 washes, orthophenylenediamine (OPD) (1.0 mg/ml) plus 0.2% hydrogen peroxide (20 volumes) were placed into the wells and the plates were incubated for 8 min. The reaction was stopped with 50 μl of 4 N sulfuric acid and the plates were read at 492 nm in an ELISA Reader (Unisciences, USA). Sera from healthy and *L. shawi* chronically infected mice were also used as negative and positive controls, respectively. PBS with 0.05% Tween 20 was used in all washing steps.

2.6. $CD4^+$ and $CD8^+$ T cell isolation

$CD4^+$ and $CD8^+$ T cells and dnCD4CD8 cells were purified from lymph nodes of infected and uninfected BALB/c mice using the $CD4^+$ and $CD8^+$ (L3T4) microbeads on a MidiMACS system (Miltenyi Biotec, Germany). The lymph nodes were homogenized in RPMI 1640 and cell numbers were counted and adjusted to 10^7cells/90 μl of elution buffer [PBS + 0.5% bovine serum albumin (BSA, Boehringer, Germany)] with 10 μl of anti-mouse CD4 and CD8 microbeads. After 20 min of incubation, cells were washed (300 g, 10 min, 4 °C), resuspended in 500 μl of elution buffer and applied to a LS column on a magnetic separator (MACS Separator, Miltenyi Biotec), followed by 3 washes (500 μl/each) with elution buffer. dnCD4CD8 cells unbound to CD4 or CD8 microbeads were also collected. $CD4^+$ and $CD8^+$ T cells were isolated by positive selection and dnCD4CD8 cells by negative selection. After the wash steps, the column was removed from the magnetic separator and $CD4^+$ (purity of 94.1%) or $CD8^+$ T cells (purity of 91.7%) were eluted. RNA was extracted from 1×10^7 $CD4^+$ T and dnCD4CD8 cells and from 2×10^6 $CD8^+$ T cells.

2.7. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. The extraction procedure included a DNase treatment using RNase Free-DNase Set Protocol (Qiagen) to prevent DNA contamination. Target RNA was reverse transcribed into cDNA using 200UM-MLVRT (Life technologies, Gibco BRL, USA), at 37 °C for 60 min in the presence of 3 mM 5× M-MLV RT buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 10 mM BSA, 1 mM dNTPs (Gibco BRL), 40 U rRNAsin ribonuclease inhibitor and 10× Oligo (dT)15 (Promega, USA). The samples were then heated for 10 min at 95 °C for RT inactivation and cooled to 4 °C.

Quantitative real-time PCR was performed in the ABI GeneAmp 5700-sequence detection system (Perkin-Elmer/Applied Biosystems, USA). Reaction conditions were powered on a Pentium III Dell Opti Plex GX110 linked directly to the sequence detector. PCR amplifications were performed by using SYBR® Green as a double-strand DNA-specific binding dye with continuous fluorescence monitorization. Amplification was performed in a total volume of 20 μl containing 2 μl of cDNA sample, 10 μl of 2× SYBR® Green I dye PCR Master Mix [(AmpliTaq Gold® DNA polymerase, dNTP mix with dUTP, passive reference I and optimized buffer components) (Perkin-Elmer/Applied Biosystems)] and primers for IFN-γ, IL-10, TNF-α, IL-4 and TGF-β as described by Rosa et al. (2005) [16] and Rodrigues et al. (2006) [17]. PCR amplification was performed in triplicate wells using the following conditions: 10 min at 95 °C for AmpliTaq Gold activation

Please cite this article as: Passero LFD, et al, Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*, Parasitol Int (2010), doi:10.1016/j.parint.2009.12.010

followed by a total of 40 cycles (thermal profile for each cycle: 15 s at 95 °C, 1 min at 60 °C). To quantify the cytokine expression, cDNA plasmid standards for each cytokine and HPRT [16] were used to construct a standard curve in each PCR run.

2.8. Statistical analysis

The results are expressed as mean ± standard deviation of three independent experiments and the nonparametric Mann–Whitney *U* test was used to compare lesion size between acute and chronic phases of infected BALB/c mice and levels of immunoglobulin and cytokines between infected and control mice. A 5% significance level ($p<0.05$) was adopted to determine statistical differences. Statistical analysis was performed with the SPSS 13.0 for Windows software (SPSS Inc. USA).

3. Results

3.1. Lesion size and parasite load

Lesion size revealed progressive growth according to the time of infection. Significant size increases were observed from week 3 post infection (pi) until the end of the observation period ($p_{\text{week 3pi}}=0.0381$, $p_{\text{week 4pi}}=0.0021$, $p_{\text{week 5pi}}=0.0014$), corresponding to the chronic phase of infection, compared to the first week of infection (acute phase of infection). At week 5 pi, lesion size achieved 1.1 mm of footpad swelling (Fig. 1A) and parasite load was 2.6×10^4 promastigote/g of skin (Fig. 1B). The parasite was not detected in lymph nodes of infected BALB/c mice.

3.2. Histopathological alterations in skin and lymph nodes and humoral immune response

After 5 weeks of infection, an intense inflammatory infiltrate diffusely distributed in the dermis associated with highly infected macrophages (Fig. 2A, B and D), light densities of lymphocytes and plasma cells, and moderate levels of polymorphonuclear cells (Fig. 2C) surrounding focal necrotic areas in skin biopsies was verified.

Lymph nodes of infected mice showed the activation of germinal centers from lymphoid follicles (data not showed). In tissue sections stained with HE or labeled by immunohistochemistry, amastigote forms were not detected and viable promastigotes were not observed in limiting dilution assay.

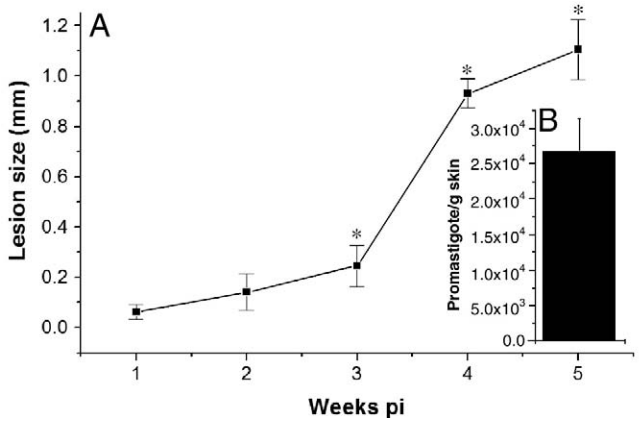

Fig. 1. BALB/c mice were infected in the right footpad and once a week for 5 consecutive weeks the size of the lesion was measured (A). At week 5 pi, BALB/c mice were sacrificed and fragments of skin were collected to assess parasite load by limiting dilution assay (B). The results are expressed as means ± standard deviation of three independent experiments. *($p<0.05$) indicates statistically significant differences compared to lesion size in the first week of infection.

At week 5 pi, infected mice presented a significant increase in anti-leishmania IgG1 antibodies ($p<0.0081$). The levels of IgG2a and IgG2b isotypes were similar to healthy mice (Fig. 3) and the IgG3 isotype was not detected in either group.

3.3. Profile of cytokine expression in cell subsets

$CD4^+$ T (Fig. 4A) and dnCD4CD8 (Fig. 4B) cells from infected mice expressed low levels of IFN-γ mRNA ($p_{CD4+}=0.0294$; $p_{\text{dnCD4CD8}}=0.0256$), however $CD8^+$ T cells presented similar IFN-γ levels in both groups. TNF-α expression was increased in $CD4^+$ ($p=0.0143$) and $CD8^+$ T cells ($p=0.0023$), but dnCD4CD8 cells showed few copies of TNF-α mRNA ($p=0.0345$). $CD4^+$ T cells from infected mice presented diminished IL-4 expression ($p=0.0212$), while $CD8^+$ ($p=0.0145$) and dnCD4CD8 cells ($p=0.0286$) revealed high levels compared to control mice.

$CD4^+$ (Fig. 5A) and $CD8^+$ T cells (Fig. 5B) and dnCD4CD8 cells (Fig. 5C) of infected mice showed high IL-10 expression ($p_{CD4+}=0.0056$; $p_{CD8+}=0.0401$; $p_{\text{dnCD4CD8}}=0.0001$); moreover, both $CD4^+$ ($p=0.0129$) and $CD8^+$ T cells ($p=0.0002$) showed high accumulation of TGF-β mRNA, whereas dnCD4CD8 cells revealed levels of TGF-β expression similar to control mice.

4. Discussion

Both Old and New World *Leishmania* species can cause tegumentary leishmaniasis, but the majority of the known mechanisms capable of generating susceptibility and resistance have been deciphered in experimental models of *L. major*. However, different strategies can be elicited by New World *Leishmania* species in the development of the lesion after a successful infection process. Until now, no studies have been conducted that investigated the histopathological alterations, anti-leishmanial antibodies and the balance of cytokine expression elicited in different cell subpopulations by murine *L. shawi* infection.

In human ATL, the healing process of cutaneous lesions caused by *L. braziliensis* is strongly related to high densities of T cells, activated macrophages and fibrosis [18]. In BALB/c mice a similar histopathological pattern is reproduced, despite the spontaneous cure of the lesions [19]. The present results revealed that in experimental infection by *L. shawi*, the increase of footpad swelling is a consequence of a strong skin inflammation, characterized by an infiltrate of mononuclear cells associated to a high density of infected macrophages and moderate densities of neutrophils surrounding the necrotic zone. In fact, the increased levels of viable parasites observed in skin after five weeks of infection indicates that BALB/c mice are unable to control the replication of *L. shawi* amastigotes, leading to the development of extensive inflammation in the absence of fibrosis. Previous studies reported that in human and experimental infections caused by *L. amazonensis* there was high density of heavily parasitized macrophages in the presence of lymphocytes, polymorphonuclear and plasma cells [20–23]. Studies conducted with *L. braziliensis* experimental infections also confirm the involvement of these cells in the pathogenesis of ATL [20,21,24,25]. Moreover de Moura and collaborators demonstrated that the expansion of neutrophil population is associated to a strong inflammatory process and necrosis [26]. These cells have also been associated to a negative prognostic in human infections, since high neutrophil densities are correlated with increased amastigote levels and the amount of specific drug required to cure the lesions [27]. In a similar way the presence of heavily parasitized macrophages associated to polymorphonuclear, plasma cells and necrosis in *L. shawi* infected BALB/c mice are a consequence of the pathogenicity of this parasite.

Th1 immune response is characterized by a set of proinflammatory cytokines with strong potential to eliminate intracellular pathogens. The main Th1 cytokine is IFN-γ, which is capable of activating

Please cite this article as: Passero LFD, et al, Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*, Parasitol Int (2010), doi:10.1016/j.parint.2009.12.010

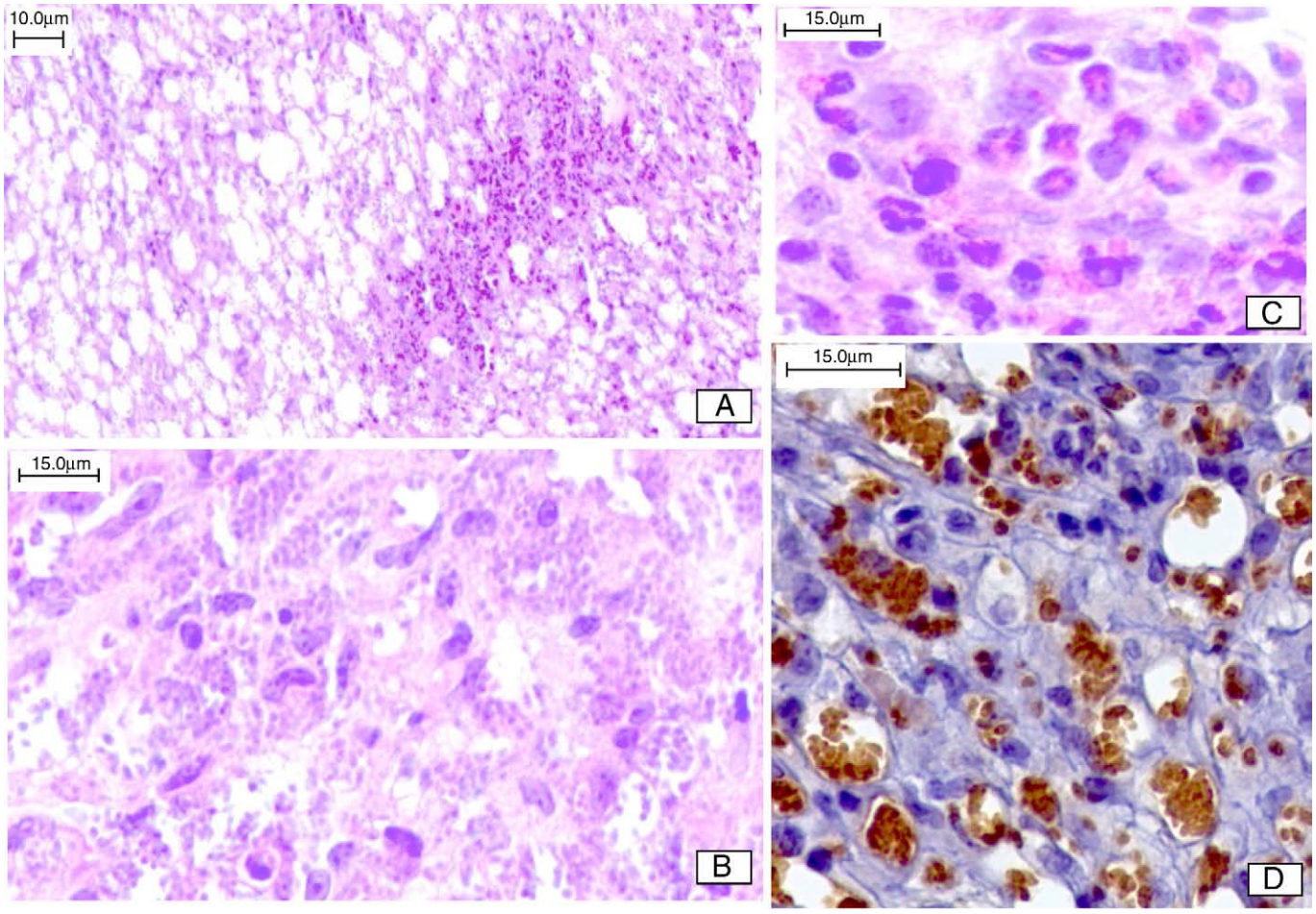

Fig. 2. In the skin, the inflammatory infiltrate mainly consisted of infected macrophages (A, B and D) and moderate densities of polymorphonuclear cells (C). Immunohistochemistry to reveal amastigote forms inside macrophages (D).

macrophages. Therefore, the decrease of IFN-γ by $CD4^+$ T cells in mice infected with *L. shawi* may be related to deactivation of effector cells, favoring parasite survival. IL-4 has a role in the development of a Th2 subpopulation and in the inhibition of Th1 cytokines. However, in infection by *L. shawi*, the expression of IL-4 by $CD4^+$ T cells was inhibited. Taken together, these results indicate that the parasite negatively regulates the expression of both Th1 and Th2 cytokines. This profile of low IL-4, also verified in human cutaneous leishmaniosis generated by *L. braziliensis* [28,29] and in murine cutaneous leishmaniosis caused by *L. amazonensis* [30], can be a common feature of certain strains of *Leishmania* sp. from the New World. Other studies disregard the effect of IL-4 in the generation of susceptibility to *L. amazonensis* in C3H mice and to *L. panamensis* and *L. mexicana* in BALB/c mice [31,32]. In fact the low levels of IL-4 and IFN-γ, observed in *L. shawi* infected mice, could be a direct consequence of the overexpression of deactivating cytokines, such as IL-10 and TGF-β. These cytokines are associated to the immunosuppressor activity of regulatory T cells and Th3 immune response, respectively, and to downregulation of class I and II molecules of major histocompatibility complex and co-stimulatory molecules [33]. These cytokines, which have a key role in the modulation of immunological homeostasis reducing extensive inflammatory responses [34,35] are subject to posttranscriptional regulation.

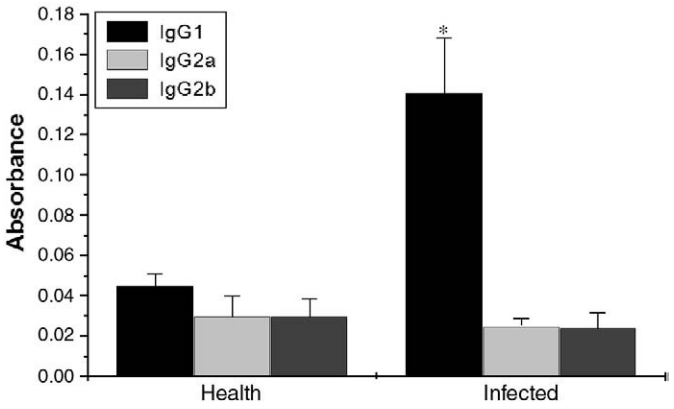

Fig. 3. Levels of anti-leishmania IgG antibodies at week 5 pi. The results are expressed as means ± standard deviation of three independent experiments. *($p<0.05$) indicates statistically significant differences compared to healthy mice.

TNF-α can synergize with IFN-γ to eliminate parasites through the activation of macrophage pathways leading to NO synthesis [36]. In the present study, the high expression of TNF-α by $CD4^+$ T cells was insufficient to control parasite replication. Thus the balance between TNF-α and IFN-γ seems to be vital for the activation of macrophage oxidative mechanisms leading to parasite load reduction [37].

$CD8^+$ T cells can be further characterized into a distinct effector type of cell based on their cytokine-secreting profiles after antigen encounter. Type 1 $CD8^+$ T cells (Tc1) predominantly secrete IFN-γ and TNF-α and kill either by perforin- or Fas-mediated mechanisms, while Tc2 releases mainly IL-4, IL-5 and IL-10 and can destroy target cell via the perforin pathway [38]. Although both cell subsets presented incontestable cytotoxic effects, in the present study $CD8^+$ Tc2 cells expressing IL-10 or TGF-β might be linked to the progression

Please cite this article as: Passero LFD, et al, Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*, Parasitol Int (2010), doi:10.1016/j.parint.2009.12.010

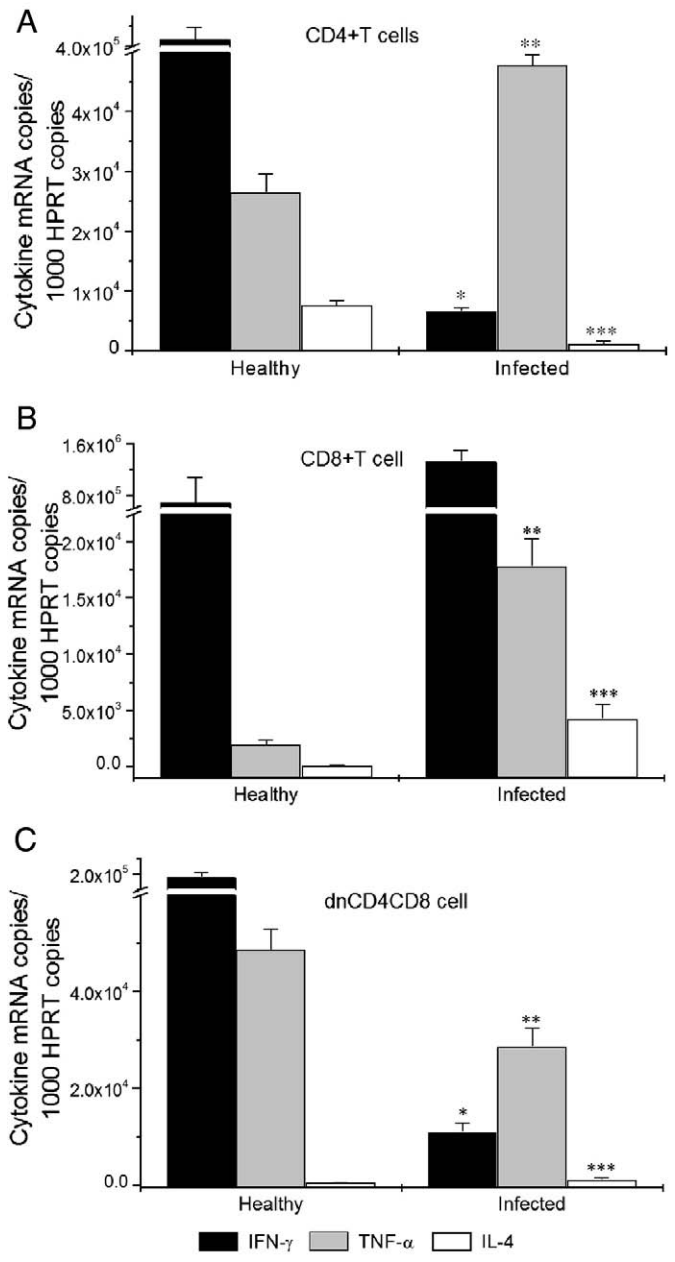

Fig. 4. Th1 and Th2 cytokines levels expressed by CD4+ (A) and CD8 T (B) cells and dnCD4CD8 cells (C) purified from popliteal lymph nodes of infected and healthy BALB/c mice. IFN-γ, TNF-α and IL-4 mRNA levels were quantified by real-time PCR. The results are expressed as number of cytokine mRNA copies per 1000 copies of HPRT. Values represent the means ± standard deviation of three independent experiments. *($p<0.05$) indicates statistically significant differences compared to healthy mice.

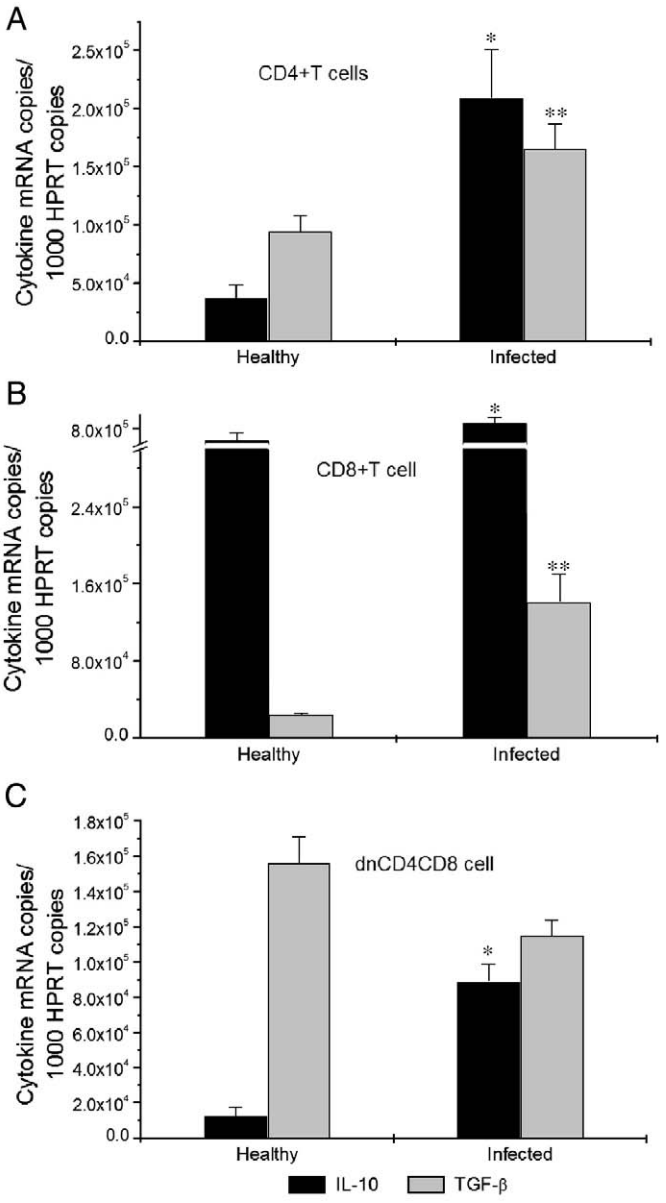

Fig. 5. IL-10 and TGF-β expressed by CD4+ (A) and CD8+ T cells (B) and dnCD4CD8 cells (B) purified from popliteal lymph nodes of infected and healthy BALB/c mice. Cytokine levels were determined by real-time PCR. The results are expressed as number of cytokine mRNA copies per 1000 copies of HPRT. Values represent the means ± standard deviation of three independent experiments. *($p<0.05$) indicates statistically significant differences compared to healthy mice.

of *L. shawi* as a possible additional source of Th2 or deactivating cytokines. The high expression of TNF-α elicited by $CD8^+$ T cells of infected mice may not be sufficient to counteract the probable deactivation of effector cells. Although some reports showed equivalence between IL-10 expression and its respective production [39,40] it was demonstrated that the availability of the respective protein is finely tuned by posttranscriptional mechanisms. Additionally, after being produced, TGF-β remains in its inactive form until is activated [41]. Therefore, a direct correlation between the expression and production of IL-10 or the functional activity of TGF-β is not always observed.

In this study, the dnCD4CD8 cell subset from infected mice, which includes neutrophils, eosinophils, dendritic, B and T cells [42], showed increased expression of IL-4 and IL-10. Thus, these cells are also involved in the host immune response following infection by *L. shawi* and may play an important role in disease progression by expressing significant levels of cytokines associate with immunosuppression of Th1 cells and downregulation of cytokines related with the control of infection. In a previous work, it was reported that $\alpha\beta^+$ dnCD4CD8 T cells contribute to the control of human cutaneous leishmaniosis caused by *L. braziliensis*, while $\gamma\delta^+$ dnCD4CD8 T cells appear to induce disease progression [43,44]. Therefore, in infection by *L. shawi*, the dnCD4CD8 cell subpopulation seems to function as a negative regulator of cell-mediated immunity.

All cell subpopulations isolated from infected BALB/c mice showed increased IL-10 expression. The importance of this cytokine in ATL was also verified in human populations exposed to *L. braziliensis*, since the appearance of active lesions was correlated with elevated levels of IL-10 [45]. Moreover, in experimental infection by *L. amazonensis* and *L. mexicana*, IL-10 seems to play a significant role in regulating the

Please cite this article as: Passero LFD, et al, Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*, Parasitol Int (2010), doi:10.1016/j.parint.2009.12.010

development of a protective Th1 immune response, as well as maintenance of the disease [46]. Taken together, the above considerations indicate IL-10 as an important factor in disease progression elicited by *L. shawi* and by New World species in general.

In the present study, the humoral immune response, characterized by high production of anti-leishmanial IgG1 antibodies, seems to be associated with disease progression. Moreover in human cases of *L. braziliensis* high antibody titers had been associated to the presence of amastigote and disease [47]. In mice, the production of specific class of antibodies has been linked to the induction of a polarized immune response. The IgG1 isotype has been associated with Th2 immune response and disease progression, while production of IgG2 has been correlated with Th1 immune response and control of infection [48]. In fact, previous studies demonstrated that the production of IgG1 is dependent on IL-4, which can be provided by different T cell populations [49,50]. However, in the present study, the results point to an apparent discrepancy between high levels of IgG1 and low Th2 immune response, which was also verified in *L. amazonensis* experimental infections [24]. Although the amount of IL-4 released by each cell subset was not evaluated, a significant increase in the expression of this cytokine by $CD8^+$ T and dnCD4CD8 cell populations was verified. Knowing that regulation of this cytokine occurs at transcription level [51], it is possible that the amount of IL-4 mRNA accumulated was correlated with the level of protein released by these two subpopulations, therefore regulating the differentiation of B cells to produce IgG1.

The outcome of *Leishmania* infection depends on a complex interaction between the parasite and the mammalian host, with the individual biological characteristics contributing to the development of disease manifestations or to an asymptomatic condition. *Leishmania* sp. from the New World presents high genetic variability and the induction of susceptibility or resistance is necessarily different among these species. To the best of our knowledge, this is the first report focusing on the study of immunopathogenesis caused by *L. shawi*. Infected BALB/c mice presented inefficient parasite control, lesion development, local inflammation and a strong humoral immune response. This parasite seems to influence different cell populations to preferentially express immunosuppressor or deactivating cytokines, thus avoiding its own destruction. The characterization of *L. shawi* infection in BALB/c mice elicits new insights for the establishment of an experimental animal model that represents natural human infection and can be used for future drug and vaccine studies.

Acknowledgments

Funding for this work was provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (07/56209-4), LIM50-HCFMUSP and by the Portuguese Foundation for Science and Technology (FCT) with co-participation of the European Union Fund (FEDER) through a research project (PTDC/CVT/70275/2006). This work is part of Luiz Felipe Domingues Passero' PhD thesis supported by Fapesp (07/50654-6).

References

[1] Shaw JJ. Taxonomy of the genus *Leishmania*: present and future trends and their implications. Mem Inst Oswaldo Cruz 1994;89:471–8.
[2] Sinha S, Fernández G, Kapila R, Lambert WC, Schwartz RA. Diffuse cutaneous leishmaniasis associated with the immune reconstitution inflammatory syndrome. Int J Dermatol 2008;47:1263–70.
[3] Louis JA, Gumy A, Voigt H, Launois P, Rocken M. Experimental cutaneous Leishmaniasis: a powerful model to study in vivo the mechanisms underlying genetic differences in Th subset differentiation. Eur J Dermatol 2002;12:316–8.
[4] Bourreau E, Gardon J, Pradinaud R, Pascalis H, Prévot-Linguet G, Kariminia A, et al. Th2 responses predominate during the early phases of infection in patients with localized cutaneous leishmaniasis and precede the development of Th1 responses. Infect Immun 2003;71:2244–56.
[5] Ajdary S, Alimohammadian MH, Eslami MB, Kemp K, Kharazmi A. Comparison of the immune profile of nonhealing cutaneous Leishmaniasis patients with those with active lesions and those who have recovered from infection. Infect Immun 2000;68:1760–4.
[6] Louzir H, Melby PC, Ben Salah A, Marrakchi H, Aoun K, Ben Ismail R, et al. Immunologic determinants of disease evolution in localized cutaneous leishmaniasis due to *Leishmania major*. J Infect Dis 1998;177:1687–95.
[7] Maioli TU, Takane E, Arantes RM, Fietto JL, Afonso LC. Immune response induced by New World Leishmania species in C57BL/6 mice. Parasitol Res 2004;94:207–12.
[8] Rocha FJ, Schleicher U, Mattner J, Alber G, Bogdan C. Cytokines, signaling pathways, and effector molecules required for the control of *Leishmania* (*Viannia*) *braziliensis* in mice. Infect Imun 2008;75:3823–32.
[9] Abreu-Silva AL, Calabrese KS, Cupolilo SM, Cardoso FO, Souza CS, Gonçalves da Costa SC. Histopathological studies of visceralized *Leishmania (Leishmania) amazonensis* in mice experimentally infected. Vet Parasitol 2004;121:179–87.
[10] Lainson R, Braga RR, De Souza AA, Pôvoa MM, Ishikawa EA, Silveira FT. *Leishmania (Viannia) shawi* sp. n., a parasite of monkeys, sloths and procyonids in Amazonian Brazil. Ann Parasitol Hum Comp 1989;64:200–7.
[11] Shaw JJ, Ishikawa EA, Lainson R, Braga RR, Silveira FT. Cutaneous leishmaniasis of man due to *Leishmania (Viannia) shawi* Lainson, de Souza, Póvoa, Ishikawa & Silveira, in Pará State, Brazil. Ann Parasitol Hum Comp 1991;66:243–6.
[12] Brito ME, Andrade MS, Mendonça MG, Silva CJ, Almeida EL, Lima BS, et al. Species diversity of *Leishmania (Viannia)* parasites circulating in an endemic area for cutaneous leishmaniasis located in the Atlantic rainforest region of northeastern Brazil. Trop Med Int Health 2009;14:1278–86.
[13] Rodrigues OR, Marques C, Soares-Clemente M, Ferronha MH, Santos-Gomes GM. Identification of regulatory T cells during experimental *Leishmania infantum* infection. Immunobiol 2009;214:101–11.
[14] Giunchetti RC, Martins-Filho OA, Carneiro CM, Mayrink W, Marques MJ, Tafuri WL, et al. Histopathology, parasite density and cell phenotypes of the popliteal lymph node in canine visceral leishmaniasis. Vet Immunol Immunopathol 2008;121:23–33.
[15] Passero LFD, Laurenti MD, Tomokane TY, Corbett CEP, Toyama MH. The effect of phospholipase A2 from Crotalus durissus collilineatus on *Leishmania (Leishmania) amazonensis* infection. Parasitol Res 2008;102:1025–33.
[16] Rosa R, Rodrigues OR, Marques C, Santos-Gomes GM. *Leishmania infantum*: soluble proteins released by the parasite exert differential effects on host immune response. Exp Parasitol 2005;109:106–14.
[17] Rodrigues OR, Moura RA, Gomes-Pereira S, Santos-Gomes GM. H-2 complex influences cytokine gene expression in *Leishmania infantum*-infected macrophages. Cell Immunol 2006;243:118–26.
[18] Morgado FN, Schubach A, Rosalino CM, Quintella LP, Santos G, Salgueiro M, et al. Is the *in situ* inflammatory reaction an important tool to understand the cellular immune response in American tegumentary leishmaniasis? Br J Dermatol 2008;158:50–8.
[19] Donnely KB, Lima HC, Titus RG. Histologic characterization of experimental cutaneous leishmaniasis in mice infected with *Leishmania braziliensis* in the presence or absence of sand fly vector salivary gland lysate. J Parasitol 1998;84:97–103.
[20] Wanasen N, Xin L, Soong L. Pathogenic role of B cells and antibodies in murine *Leishmania amazonensis* infection. Int J Parasitol 2008;38:417–29.
[21] Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, et al. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. J Immunol 2000;165:2628–36.
[22] Silveira FT, Lainson R, Corbett CE. Further observations on clinical, histopathological, and immunological features of borderline disseminated cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. Mem Inst Oswaldo Cruz 2005;100:525–34.
[23] Moraes MA, Silveira FT. Histopathology of the localized form of cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. Rev Inst Med Trop Sao Paulo 1994;36:459–63.
[24] McKinney LA, Hendricks LD. Experimental infection of *Mystromys albicaudatus* with *Leishmania braziliensis*: pathology. Am J Trop Med Hyg 1980;29:753–60.
[25] Lujan R, Dennis VA, Chapman Jr WL, Hanson WL. Blastogenic responses of peripheral blood leukocytes from owl monkeys experimentally infected with *Leishmania braziliensis panamensis*. Am J Trop Med Hyg 1986;35:1103–9.
[26] de Moura TR, Novais FO, Oliveira F, Clarêncio J, Noronha A, Barral A, et al. Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. Infect Immun 2005;73:5827–34.
[27] Gutierrez Y, Salinas GH, Palma G, Valderrama LB, Santrich CV, Saravia NG. Correlation between histopathology, immune response, clinical presentation, and evolution in *Leishmania braziliensis* infection. Am J Trop Med Hyg 1991;45:281–9.
[28] Pirmez C, Yamamura M, Uyemura K, Paes-Oliveira M, Conceição-Silva F, Modlin RL. Cytokine patterns in the pathogenesis of human leishmaniasis. J Clin Invest 1993;91:1390–5.
[29] de Souza-Neto SM, Carneiro CM, Vieira LQ, Afonso LC. *Leishmania braziliensis:* partial control of experimental infection by interleukin-12 p40 deficient mice. Mem Inst Oswaldo Cruz 2004;99:289–94.
[30] Laurenti MD, da Matta VL, Pernichelli T, Secundino NF, Pinto LC, Corbett CE, et al. Effects of salivary gland homogenate from wild-caught and laboratory-reared *Lutzomyia longipalpis* on the evolution and immunomodulation of *Leishmania (Leishmania) amazonensis* infection. Scand J Immunol 2009;70:389–95.
[31] Guevara-Mendoza O, Une C, Franceschi Carreira P, Orn A. Experimental infection of Balb/c mice with *Leishmania panamensis* and *Leishmania mexicana*: induction of early IFN-gamma but not IL–4 is associated with the development of cutaneous lesions. Scand J Immunol 1997;46:35–40.
[32] Jones DE, Buxbaum LU, Scott P. IL-4-independent inhibition of IL—12 responsiveness during *Leishmania amazonensis* infection. J Immunol 2000;165:364–72.

Please cite this article as: Passero LFD, et al, Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*, Parasitol Int (2010), doi:10.1016/j.parint.2009.12.010

[33] Kane MM, Mosser DM. The role of IL10 in promoting disease progression in leishmaniasis. J Immunol 2001;166:1141–7.
[34] Ghalib HW, Piuvezam MR, Skeiky YA, Siddig M, Hashim FA, el-Hassan AM, et al. Interleukin 10 production correlates with pathology in human *Leishmania donovani* infections. J Clin Invest 1993;92:324–9.
[35] Stenger S, Thüring H, Röllinghoff M, Bogdan C. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. J Exp Med 1994;180:783–93.
[36] Liew FY, Li Y, Millott S. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J Immunol 1990;145:4306–10.
[37] Alves CF, de Amorim IF, Moura EP, Ribeiro RR, Alves CF, Michalick MS, et al. Expression of IFN-gamma, TNF-alpha, IL-10 and TGF-beta in lymph nodes associates with parasite load and clinical form of disease in dogs naturally infected with *Leishmania (Leishmania) chagasi*. Vet Immunol Immunopathol 2009;128:349–58.
[38] Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol 2004;172:1380–90.
[39] Norsworthy NB, Sun J, Elnaiem D, Lanzaro G, Soong L. Sand fly saliva enhances *Leishmania amazonensis* infection by modulating interleukin-10 production. Infect Immun 2004;72:1240–7.
[40] Charmoy M, Megnekou R, Allenbach C, Zweifel C, Perez C, Monnat K, et al. *Leishmania major* induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. J Leukoc Biol 2007;82:288–99.
[41] Taylor AW. Review of the activation of TGF-beta in immunity. J Leukoc Biol 2009;85:29–33.
[42] Magerus-Chatinet A, Stolzenberg MC, Loffredo MS, Neven B, Schaffner C, Ducrot N, et al. FAS-L, IL-10, and double-negative CD4- CD8- TCR alpha/beta + T cells are reliable markers of autoimmune lymphoproliferative syndrome (ALPS) associated with FAS loss of function. Blood 2009;113:3027–30.
[43] Antonelli LR, Dutra WO, Oliveira RR, Torres KC, Guimarães LH, Bacellar O, et al. Disparate immunoregulatory potentials for double-negative (CD4- CD8-) alpha beta and gamma delta T cells from human patients with cutaneous leishmaniasis. Infect Immun 2006;74:6317–23.
[44] Gollob KJ, Antonelli LR, Faria DR, Keesen TS, Dutra WO. Immunoregulatory mechanisms and CD4-CD8- (double negative) T cell subpopulations in human cutaneous leishmaniasis: a balancing act between protection and pathology. Int Immunopharmacol 2008;8:1338–43.
[45] Salhi A, VJr Rodrigues, Santoro F, Dessein H, Romano A, Castellano LR, et al. Immunological and genetic evidence for a crucial role of IL-10 in cutaneous lesions in humans infected with *Leishmania braziliensis*. J Immunol 2008;180:6139–48.
[46] Padigel UM, Alexander J, Farrell JP. The role of interleukin-10 in susceptibility of BALB/c mice to infection with *Leishmania mexicana* and *Leishmania amazonensis*. J Immunol 2003;171:3705–10.
[47] Garcez LM, Silveira FT, el Harith A, Lainson R, Shaw JJ. Experimental cutaneous leishmaniasis. IV. The humoral response of *Cebus apella* (Primates: Cebidae) to infections of *Leishmania (Leishmania) amazonensis, L. (Viannia) lainsoni and L. (V.) braziliensis* using the direct agglutination test. Acta Trop 1997;68:65–76.
[48] Coffman RL, Lebman DA, Rothman P. Mechanism and regulation of immunoglobulin isotype switching. Adv Immunol 1993;54:229–70.
[49] Lebrun P, Lucas AH, McKenzie DT, Spiegelberg HL. Interleukin 4 acts on both high- and low-density murine B cell subpopulations to induce IgE and IgG1 synthesis in vitro. Int Arch Allergy Appl Immunol 1989;88:108–10.
[50] Fischer K, Collins H, Taniguchi M, Kaufmann SH, Schaible UE. IL-4 and T cells are required for the generation of IgG1 isotype antibodies against cardiolipin. J Immunol 2002;168:2689–94.
[51] Guo L, Hu-Li J, Paul WE. Probabilistic regulation of IL-4 production. J Clin Immunol 2005;25:573–81.

Please cite this article as: Passero LFD, et al, Histopathology, humoral and cellular immune response in the murine model of *Leishmania* (*Viannia*) *shawi*, Parasitol Int (2010), doi:10.1016/j.parint.2009.12.010

ANEXO-12

Revisão sobre a patogenia da leishmaniose tegumentar americana na Amazônia, com ênfaase a doença causada por *Leishmania (V.) braziliensis* e *Leishmania (L.) amazonensis*

Silveira, F.T.; Müller, S.R.; de Souza, A.A.A.; Lainson, R.; Gomes, C.M.C.; **Laurenti, M.D.;** Corbett, C.E.P.

Revista Paraense de Medicina 2008; 22(1): 9-20.

Fundação Santa Casa de Misericórdia do Pará

REVISTA PARAENSE DE MEDICINA

V. 22 (1) janeiro-março 2008

ARTIGO ORIGINAL

REVISÃO SOBRE A PATOGENIA DA LEISHMANIOSE TEGUMENTAR AMERICANA NA AMAZÔNIA, COM ÊNFASE À DOENÇA CAUSADA POR *Leishmania (V.) braziliensis* E *Leisshmania (L.) amazonensis*

REVISITING THE PATHOGENESIS OF THE AMERICAN TEGUMENTARY LEISHMANIASIS IN AMAZONIAN, WITH EMPHASIS TO THE DISEASE DUE TO *Leishmania (V.) braziliensis* AND *Leishmania (L.) amazonensis*

Fernando T. **SILVEIRA**[1,2]/+, Silvia R. **MÜLLER**[3], Adelson A.A. **de SOUZA**[1], Ralph **LAINSON**[1], Claudia M.C. **GOMES**[4], Marcia D. **LAURENTI**[4], Carlos E.P. **CORBETT**[4]

RESUMO

A patogenia da leishmaniose tegumentar americana (LTA) na Amazônia foi revisada à luz dos mais recentes aspectos associados ao espectro clínico, histopatológico e imunopatológico da doença causada por Leishmania (V.) braziliensis e Leishmania (L.) amazonensis. Esta revisão mostrou a existência de uma dicotomia entre as duas espécies de Leishmania e a resposta imune celular; enquanto a L. (V.) braziliensis mostra forte tendência em dirigir a infecção, a partir da forma central do espectro clínico-imunológico, a leishmaniose cutânea localizada (LCL), para o pólo imunológico hiperreativo, representado pela leishmaniose cutâneo-mucosa (LCM), com exacerbação da hipersensibilidade e perfil da resposta CD4 tipo-Th1, a L. (L.) amazonensis mostra o oposto, dirige a infecção para o pólo imunológico hiporreativo, representado pela leishmaniose cutânea anérgica difusa (LCAD), com forte inibição da hipersensibilidade e perfil da resposta CD4 tipo-Th2. Entre a forma central LCL e as formas polares LCM e LCAD a infecção passa por uma fase intermediária, a leishmaniose cutânea disseminada borderline (LCDB), com inibição parcial da hipersensibilidade e perfil da resposta CD4 Th1+Th2. Estes são, provavelmente, os principais mecanismos imunológicos que modulam a patogenia da LTA causada por L. (V.) braziliensis e L. (L.) amazonensis.

DESCRITORES: Leishmaniose tegumentar americana; patogenia; *Leishmania (Viannia) braziliensis*; *Leishmania (Leishmania) amazonensis*; Amazônia; Brasil.

CONSIDERAÇÕES GERAIS

A patogenia da leishmaniose tegumentar americana (LTA) na Amazônia brasileira representa, sem dúvida, um grande desafio no sentido do aprimoramento dessa protozoose, em razão da complexa interação entre as múltiplas espécies de *Leishmania* que atuam como agentes etiológicos da doença e a resposta imune do homem infectado. A título de esclarecimento, hoje são reconhecidas,

+Autor para correspondência: silveiraft@lim50.fm.usp.br

[1] Laboratório de leishmanioses, Instituto Evandro Chagas (SVS, MS), Belém, Pará, Brasil;
[2] Instituto de Medicina Tropical, Universidade Federal do Pará, Belém, Pará, Brasil;
[3] Serviço de Dermatologia, Faculdade de Medicina, Universidade Federal do Pará, Belém, Pará, Brasil;
[4] Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brasil.

na referida região, sete espécies de *Leishmania* que podem produzir sintomatologia da LTA, sendo seis do subgênero *Viannia* e uma do subgênero *Leishmania*[1,2]. Entretanto, considerando o rico espectro das manifestações de natureza clínica, histopatológica e imunopatológica que são encontradas na doença causada por *Leishmania (Viannia) braziliensis* e *L. (Leishmania) amazonensis*, parece não haver dúvida de que essas espécies são, realmente, as que apresentam o maior potencial patogênico para o homem; são responsáveis não só pela forma mais simples da doença, a leishmaniose cutânea localizada (LCL), mas também, por formas clínicas mais graves, de longa evolução, com lesões cutâneas e/ou mucosas bastante destrutivas e, de difícil manejo terapêutico. A título de exemplo, a *L. (V.) braziliensis* está fortemente associada à leishmaniose cutâneo-mucosa (LCM), e a *L. (L.) amazonensis* à leishmaniose cutânea anérgia difusa (LCAD); as duas formas clínicas de maior gravidade no espectro clínico-imunológico da LTA; a primeira, associada ao pólo imunológico hiperreativo, com forte hipersensibilidade celular e, a segunda, ao pólo imunológico hiporreativo, com fraca (ou ausente) hipersensibilidade celular. Além disso, as duas espécies podem, ainda, ser agentes causais da leishmaniose cutânea disseminada borderline (LCDB), uma forma intermediária, hiporreativa, entre as formas polares LCM e LCAD. As outras espécies que também atuam como agentes da LTA na região, tais como, *L. (V.) guyanensis*, *L. (V.) lainsoni*, *L. (V.) shawi*, *L. (V.) lindenbergi* e *L. (V.) naiffi*, em ordem decrescente da infecção no homem, são agentes somente da leishmaniose cutânea localizada (LCL), de perfil imune celular bem equilibrado, o que lhe confere alto grau (~100%) de resolução com tratamento à base do antimonial pentavalente (Sb^v), o antimoniato de meglumina. Por essa razão, foi considerado de interesse revisar neste artigo a patogenia da LTA na Amazônia brasileira, com ênfase ao espectro das manifestações clínicas, histopatológicas e imunopatológicas da doença causada por *L. (V.) braziliensis* e *L. (L.) amazonensis*, já que ambas perpassam todas as formas clínicas do espectro da LTA. Antes, porém, de adentrar nesses aspectos propriamente, serão focalizados, primeiramente, de forma sucinta, alguns aspectos da patogenia que precedem as manifestações clínicas, ou seja; primeiro, os eventos responsáveis pelo estabelecimento da infecção e, segundo, os que determinam a evolução da infecção no homem.

EVENTOS QUE PRECEDEM A LTA

A infecção humana, pelas espécies de *Leishmania* que causam a LTA, tem seu início logo após a inoculação das formas promastigotas do parasito na pele, o que acontece durante a hematofagia pelas espécies de flebotomíneos vetores. A partir desse momento, inicia-se um processo de escape do parasito frente às defesas do organismo, o qual, quando vencido pelo parasito, resultará na sua fagocitose pelas células do sistema fagocítico mononuclear (SFM), principalmente o macrófago. Em outras palavras, algumas das formas promastigotas metacíclicas infectantes, que conseguiram escapar da ação lítica do complemento e dos eosinófilos e neutrófilos, são fagocitadas por macrófagos, nos quais, transformam-se em formas amastigotas dentro dos vacúolos parasitóforos dessas células; nesse micro-ambiente passam a multiplicar-se por divisão binária. Em seguida, a evolução da infecção dependerá do perfil imunogenético do homem, fortemente associado à resposta imune celular, e da virulência da espécie de *Leishmania* infectante. Nesse sentido, a patogenia da doença será definida em conseqüência do tipo de interação entre a espécie de *Leishmania* e o perfil imunogenético do hospedeiro (*Leishmania*/macrófago), da qual resultarão as diferentes formas clínicas da LTA[3].

1. Eventos determinantes da infecção: Interação *Leishmania*-macrófago

Conforme mencionado, antes do estabelecimento da infecção pela *Leishmania* no macrófago, ocorre uma série de eventos que irão preceder a entrada da forma promastigota metacíclica infectante na célula hospedeira, os quais terão grande participação no sucesso ou não da infecção.

a) Ação do sistema do complemento

Após a inoculação das formas promastigotas na pele, a maioria é destruída pela

ação lítica do complemento[4,5]. Entretanto, algumas das formas promastigotas inoculadas, promastigotas metacíclicas[6,7], são resistentes à ação lítica do complemento[8,9]; apresentam na membrana plasmática moléculas de glicoconjugados (lipofosfoglicanos) e glicoproteinas, as quais, não só favorecem a fixação de componentes do complemento, C3b e iC3b[10], como também, representam uma barreira de proteção contra a lise pelo complemento[11].

b) Adesão do parasito à membrana do macrófago

Vencido o primeiro obstáculo, o próximo passo da forma promastigota metacíclica é sua adesão ao macrófago. Esta é uma etapa crítica para a infecção, sendo mediada por receptores da membrana do macrófago que se ligam às moléculas da membrana das formas promastigotas metacíclicas, chamadas ligantes do parasito[12,13,14]. Vários receptores presentes na membrana do macrófago já foram identificados; receptor para molécula de manose-fucose em *L. (L.) donovani*[15,16,17] e para manose-6-fosfato em *L. (L.) amazonensis*[18]. Receptores para lectina, glicoconjugados e lipofosfoglicanos (LPG), estão envolvidos na adesão de *L. (L.) major* e de *L. (L.) donovani*[19,20]. Receptor para a principal glicoproteina de superfície, a gp63, foi identificado em *L. (L.) mexicana*[21,22].

Além disso, foi demonstrado que promastigotas metacíclicas de *L. (L.) major* e *L. (L.) donovani* são capazes de ativar o complemento pela via alternada e fixar o componente C3 na sua superfície[23], facilitando a adesão e posterior fagocitose através dos receptores CR3 e CR1[24,25]; essa ligação promove, também, aumento da sobrevida do parasito no macrófago, pela inibição da produção do óxido nítrico[26,27].

c) Fagocitose e sobrevivência do parasito no macrófago

Depois da adesão, a fagocitose é um processo rápido, acompanhado pela ativação da respiração do macrófago, resultando na produção de peróxido e superóxido de hidrogênio, os quais são altamente tóxicos para o parasito[28]. Desse modo, após a fagocitose o parasito transforma-se em amastigota dentro do vacúolo parasitóforo; este funde-se com grânulos lisossomais para formar os fagolisossomos, onde as formas amastigotas deverão se multiplicar. Entretanto, nesse micro-ambiente o parasito vai enfrentar vários mecanismos microbicidas, tais como, produtos do metabolismo do oxigênio, baixo pH e proteínas catiônicas. Em resposta, o parasito lança mão dos receptores CR3 e CR1, a fim de inibir a ativação do sistema de oxigênio do macrófago[29,30,31,32]. Desse modo, considerando que a forma amastigota é capaz de superar os mecanismos microbicidas do macrófago, a sua sobrevivência dependerá então da capacidade de resistir, também, aos mecanismos da resposta imune celular.

2) Eventos determinantes da evolução da infecção: Interação *Leishmania*/célula de Langerhans/células T CD4/CD8

Além dos macrófagos da derme, outras células com capacidade fagocítica na pele possuem receptores para o componente C3 do complemento, como a célula de Langerhans (CL), que também pode ser infectada por *Leishmania*[33]. Além disso, a CL tem função crítica na LTA, já que é considerada a principal apresentadora de antígenos parasitários para os linfócitos T em repouso nos linfonodos regionais[34,35,36]. A CL origina-se na medula óssea e forma verdadeira rede nas camadas basal e supra-basal da epiderme, de onde migra para a derme a fim de capturar e fagocitar o parasito para, em seguida, transportar os antígenos da pele até os linfonodos regionais via linfáticos aferentes[37]. Após deixar a pele, é reposta a partir de precursores circulantes ou por divisão intra-epitelial. A sua superfície expressa várias moléculas com propriedades funcionais; MHC II, receptores Fc e C3b, CD1, ICAM-1, ICAM-3, LFA-3, CD4, receptores de IL-2 e atividade de ATPase de membrana[38,39]. Contem no seu citoplasma organelas denominadas grânulos de Birbeck, provavelmente relacionadas ao processo de endocitose. Sua capacidade de apresentar antígenos da pele foi confirmada em cultura celular; processamento, migração, maturação e estimulação das células T[40]. Desse modo, a CL desempenha um papel crucial na patogênese da LTA, já que representa o veículo que promoverá o primeiro contato do antígeno com a resposta imune,

resultando na estimulação diferencial das subpopulações dos linfócitos T CD4, Th1 e Th2, cujas citocinas irão regular positivamente (IL-2, INF-γe TNF-á) ou negativamente (IL-4, IL-5, IL-6, IL-10 e IL-13) a atividade do macrófago na eliminação do parasito. A ativação do macrófago, através da resposta imune CD4 Th1, resulta na produção do óxido nítrico ("NO"), que juntamente com o stress oxidativo representa importante mecanismo de eliminação do parasito[41,42,43,44].

Recentemente, porém, estudos realizados sobre o papel da CL na imunopatogênese da leishmaniose cutânea por *L. (L.) major* em camundongo isogênico BALB/c tem evidenciado que, diferente do pressuposto sobre a importância da CL na iniciação da resposta imune por célula T[34,35], são as células dendríticas da derme as verdadeiras responsáveis pela estimulação antígeno-específica dessas células[45]. Além disso, foi observado, também, que a hipersensibilidade do tipo tardia (delayed type hypersensitivity) mostrava-sevisivelmente exacerbada em camundongo geneticamente deficiente (knock-out) para CL[46], sugerindo que, quanto menor a presença da CL na infecção por *Leishmania*, mais forte seria a resposta de hipersensibilidade. Por último, foi evidenciado que a CL mostrou-se capaz de processar e apresentar antígeno parasitário através dos receptores classe II do MHC para células CD4, que diferenciaram depois em células T reguladoras ("Treg cells"), sugerindo que a CL poderia inibir os eventos inflamatórios na infecção pela *L. (L.) major*[47]. Em conclusão, esses estudos sugerem que a CL representaria, de fato, um mecanismo de evasão do parasito da resposta imune por célula T, induzindo um estado de supressão imunológica. Esta condição foi recentemente examinada no espectro clínico-imunológico da LTA, comparando-se a densidade da CL nas diferentes formas clínicas por *L. (V.) braziliensis* e *L. (L.) amazonensis*, sendo observado progressivo aumento na densidade da CL no sentido da forma LCL imune reativa (DTH$^+$) por *L. (V.) braziliensis* até as formas LCL, LCDB e LCAD imunes não reativas (DTH$^-$) por *L. (L.) amazonensis*. Esse achado, combinado com o perfil das células CD4 e CD8 no mesmo espectro, no qual essas células mostraram progressivo declínio no mesmo sentido, ou seja, da forma LCL imune reativa (DTH$^+$) por *L. (V.) braziliensis* em direção às formas LCL, LCDB e LCAD imunes não reativas (DTH$^-$) por *L. (L.) amazonensis*[3], indica a existência de forte correlação negativa, espécie-específica, entre a densidade da CL e das células CD4 e CD8 no espectro clínico da LTA. Em outras palavras, enquanto a densidade da CL aumenta ao longo do espectro clínico-imunológico da infecção por *L. (L.) amazonensis*, no sentido das formas imune-suprimidas LCDB e LCAD, a densidade das células CD4 e CD8 diminui no mesmo sentido, sugerindo que a CL pode estar modulando uma estimulação antígeno-específica CD4 tipo-Th2, o que explicaria a tendência da infecção por *L. (L.) amazonensis* cursar com supressão da resposta imune e, conseqüentemente, desenvolver as formas imune-suprimidas LCDB e LCAD[48].

EVENTOS INERENTES À LTA

Conforme referido antes, a patogenia da LTA é fortemente influenciada pelo perfil imunogenético do homem, ao qual, encontra-se igualmente associada a resposta imune mediada por célula T[49,50,51] e, ainda, pelo perfil de virulência da espécie de *Leishmania* infectante. Como resultado da interação entre as diferentes espécies de *Leishmania* e os mecanismos da resposta imune do homem ocorre um espectro de manifestações clínicas, histopatológicas e imunopatológicas, as quais serão comentadas adiante.

Espectro clínico

Com base nessas premissas, a infecção pode manter-se assintomática em indivíduos naturalmente resistentes, com resposta imune inata capaz de controlar a progressão da infecção ou, como acontece em indivíduos com susceptibilidade imunológica, resultar em um espectro de manifestações clínicas na pele e/ou mucosas naso-buco-faríngea, traduzidas pelas formas clínicas já conhecidas; LCL, LCDB, LCM ou LM e, LCAD.

Entretanto, essa classificação não é exclusivamente clínica, já que faz parte também de um contexto imunológico da infecção, entre as espécies de *Leishmania* e a resposta imune celular. Nesse sentido, no centro do espectro identifica-se a forma LCL, representada, na grande maioria dos casos (e" 95%), por lesões cutâneas ulceradas, tendo como agente principal a *L. (V.) braziliensis* (Tabela). Contudo, devem ser lembradas as outras

espécies do subgênero *Viannia* que podem causar a forma LCL, tais como, *L. (V.) guyanensis*, *L. (V.) lainsoni*, *L. (V.) shawi*, *L. (V.) lindenbergi* e *L. (V.) naifi*, além da *L. (L.) amazonensis*, único representante do subgênero *Leishmania*.

Saindo do centro do espectro, as infecções não controladas pela resposta imune celular podem evoluir para um dos pólos imunológicos do espectro: i) para o pólo de hiperreatividade, caracterizado por forte hipersensibilidade e representado pelas formas LCM ou LM, nas quais, a necrose do tecido mucoso é a principal manifestação do estado de hipersensibilidade dessas formas; ou ii) para o pólo de hiporreatividade, caracterizado por inibição parcial ou total da hipersensibilidade e representado pela forma LCAD, na qual, a grande disseminação das lesões nodulares na pele demonstra a fragilidade da resposta imune celular em controlar a infecção. Essa dicotomia da resposta imune, de caráter subgênero-específica, tem papel fundamental na patogenia da LTA; somente infecções por espécies do subgênero *Viannia*, como a *L. (V.) braziliensis*, evoluem para o pólo imunológico de hiperreatividade, enquanto somente infecções por espécies do subgênero *Leishmania*, como a *L. (L.) amazonensis*, evoluem para o pólo imunológico de hiporreatividade. Desse modo, fica mais fácil entender o caráter imunogênico dos principais agentes da LTA na Amazônia; enquanto a *L. (V.) braziliensis* mostra forte tendência de dirigir a infecção para o pólo da hipersensibilidade, marcado por forte resposta CD4 tipo-Th1, a *L. (L.) amazonensis* mostra o posto, dirigindo a infecção para o pólo de fraca hipersensibilidade, associado à resposta CD4 tipo-Th2[48].

Entre as formas polares LCM ou LM (DTH^{+++}) e LCAD (DTH^{-}) e a forma central LCL (DTH^{+}), a infecção pode passar, ainda, por uma fase intermediária de disseminação, a LCDB, durante a qual a resposta imune celular apresenta-se parcialmente inibida ($DTH^{\pm}$)[3]. Com respeito aos seus agentes, mais uma vez parecem importantes algumas diferenças entre as duas espécies; nos casos por *L. (V.) braziliensis*, a disseminação é relativamente rápida, dois a três meses, gerando o aparecimento de dezenas ou até centenas de lesões cutâneas pápulo-ulcerosas (ectmatóides), enquanto nos casos por *L. (L.) amazonensis*, a disseminação é lenta, resultando em número limitado (cerca de dez) de lesões cutâneas eritemto-infiltradas. O termo borderline foi usado para caracterizar a supressão parcial da resposta imune celular, observada, principalmente, nos casos de LCDB por *L. (L.) amazonensis*; a reação intradérmica de Montenegro (=DTH) e a proliferação de linfócitos são sempre negativas. Não obstante, existem evidências mostrando que a resposta imune celular não está completamente ausente, como nos casos da forma LCAD; tem sido observada pronta recuperação após terapia com antimoniato de meglumina, o dobro do que é utilizado para a forma LCL[52,53,54,55].

Espectro histopatológico

A reação histopatológica na LTA tem sido alvo de inúmeros trabalhos buscando um entendimento das lesões teciduais encontradas na doença. Como resultado, existe um consenso que considera a LTA doença de natureza inflamatória crônica, histiolinfoplasmocitária, acompanhada ou não de necrose dos tecidos e reação granulomatosa[56,57,58,59,60,61,62,63,64].

Contudo, existem evidências demonstrando que em determinadas formas clínicas e dependendo do agente envolvido, o infiltrado celular pode apresentar variações, sugerindo que a resposta histopatológica está sujeita, também, à influência do agente específico (Tabela). A título de exemplo, deve-se destacar um estudo com mais de 30 casos da forma LCL por *L. (L.) amazonensis* na Amazônia, no qual o principal achado foi a presença de grande quantidade de macrófagos vacuolizados, ricamente parasitados, junto ao infiltrado celular linfoplasmocitário. Além disso, fazia parte do quadro áreas incipientes de necrose cercadas por células epitelióides[60]. Por outro lado, nos casos da forma LCL por *L. (V.) braziliensis* o infiltrado dérmico apresenta-se diferente; nestes casos o que predomina é a reação linfoplasmocitária, enquanto macrófagos com parasitos são raros. Além dessa reação, são observadas, ainda, extensas áreas de necrose associadas ou não à granulomas epitelióides, relacionados aos fenômenos de hipersensibilidade celular fortemente induzidos pela *L. (V.) braziliensis*[58,59]. É interessante citar, também, um estudo experimental no primata Cebus apella (Primates: Cebidae), comparando as lesões

produzidas por *L. (V.) braziliensis* e *L. (L.) amazonensis;* até o segundo mês da infecção, o quadro foi parecido nos dois grupos de animais, mostrando infiltrado histiolinfoplasmocitário em ambos, porém, no grupo inoculado com *L. (V.) braziliensis* era notório reduzido número de macrófagos parasitados. Ao contrário, no grupo inoculado com *L. (L.) amazonensis* havia predomínio dos macrófagos vacuolizados, ricamente parasitados, sobre a reação linfoplasmocitária. Com a evolução do processo, até nove meses no grupo inoculado com *L. (V.) braziliensis,* houve a formação de extensas áreas de necrose no infiltrado, circundadas por granulomas epitelióides que, mais tarde, eram invadidas por tecido fibroso até a cicatrização final. No grupo inoculado com *L. (L.) amazonensis,* a evolução foi mais rápida, apenas quatro meses, mostrando reduzidas áreas de necrose no infiltrado e raros grupos de células epitelióides; a cura ocorreu também por cicatrização das lesões[64]. Nos casos da forma LCDB por *L. (V.) braziliensis,* predomina a reação exudativa, linfoplasmocitária, com poucos macrófagos parasitados, além de áreas de necrose associadas ou não ao granuloma epitelióide. Nos casos da forma LCDB por *L. (L.) amazonensis,* o predomínio é dos macrófagos vacuolizados, ricamente parasitados, com raros grupos de células epitelióides[54]. Nas formas LCM ou LM (hiperreativas), o quadro é caracterizado por infiltrado linfoplasmocitário denso e difuso, entremeado por áreas de necrose e reação granulomatosa epitelióide; em casos de longa evolução (mais de 5 anos) esta reação granulomatosa pode ser tuberculóide[58,59], refletindo forte estado de hipersensibilidade celular desta forma. Na forma LCAD (hiporreativa), o quadro é caracterizado por infiltrado macrofágico vacuolizado, ricamente parasitado e com escassa reação linfoplasmocitária. Como não há hipersensibilidade, o macrófago está inativo, não há necrose e nem reação granulomatosa; existe apenas intensa reação macrofágica, conhecida como nódulo macrofágico ou histiocitoma, ricamente parasitado[64]. Em casos de LCAD muito avançados, com lesões ósseas de extremidade, foi descrito, recentemente, um tipo de osteomielite causada por *L. (L.) amazonensis*[65,66].

Espectro imunopatológico

A resposta imune na LTA tem sido abordada, principalmente, com base no perfil dos linfócitos CD4 e CD8 e de algumas citocinas produzidas por essas células nas lesões dos pacientes, com especial interesse no IFN-γ e IL-4 (Tabela), face ao papel que parecem desempenhar na resposta imune adquirida, resistente (Th1) ou susceptível (Th2), respectivamente, contra a infecção[67,68]. Desse modo, tendo a forma LCL representando a maioria dos casos de LTA (e"95%), acredita-se que apresente perfil predominante da resposta Th1, resultando na produção de níveis significativos de IFN-γ nas lesões, com ativação do macrófago e eliminação do parasito[69,70]. Entretanto, tem sido demonstrado que a modulação da resposta imune celular pode ser influenciada pela espécie de *Leishmania* infectante; pacientes com LCL por *L. (V.)* braziliensis apresentaram reatividade para o teste intradérmico de Montenegro e de proliferação de linfócitos maior ($p< 0.05$) do que pacientes com LCL por *L. (L.)* amazonensis[71,72]; a maioria dos casos (>50%) de LCL por *L. (L.)* amazonensis não apresenta reatividade para esses testes, sugerindo que esta espécie apresenta mecanismos mais eficientes de escape da resposta imune celular do que a *L. (V.) braziliensis*. Além disso, foi demonstrado, ainda, através da análise semi-quantitativa por RT-PCR aumento significativo da expressão do RNA mensageiro para IFN-γ em lesão de pacientes com LCL por *L. (V.) braziliensis*, enquanto nenhuma expressão foi detectada do RNA mensageiro para IL-4. Por outro lado, nos pacientes com LCL por *L. (L.) amazonensis* foi demonstrado aumento significativo da expressão do RNA mensageiro para IL-4, embora não houvesse diminuição da expressão de RNA mensageiro para IFN-γ, sugerindo que a IL-4 na forma LCL por *L. (L.) amazonensis* poderia estar competindo com IFN-γ e contribuindo para inibir a resposta de hipersensibilidade[3].

Nas formas LCM ou LM, associadas à forte resposta de hipersensibilidade e proliferação de linfócitos[71,72], o perfil de linfócitos CD4 e CD8 no infiltrado das lesões tem mostrado predomínio de CD4[73,74,75,76], o que também foi encontrado nessas formas da LTA na Amazônia, reiterando a importância do linfócito CD4 na patogênese da

LCM/LM[3]. Desse modo, parece significativo que na forma LCM/LM tenha sido identificado um perfil de células CD4 maior que nas outras formas da LTA (LCL, LCDB e LCAD), sugerindo a possibilidade de níveis significativos de IFN-γ e TNF-á nessas formas, já que estas citocinas estão fortemente associadas à resposta de hipersensibilidade. Entretanto, um perfil misto de resposta Th1/Th2 foi diagnosticado em casos de LCM/LM na Venezuela[69] e no sudeste do Brasil[70], contrastando com os resultados na Amazônia, onde foi evidenciada resposta imune claramente Th1; altos níveis de RNA mensageiro para IFN-γ em lesões mucosas, em contraste com resultados negativos de RNA mensageiro para interleucina (IL)-4 nas mesmas amostras[3]. Assim, parece possível que a exacerbação da resposta imune celular na LCM/LM possa representar o resultado de um prolongado estímulo imunogênico pela *L. (V.) braziliensis*, uma vez que o início dos sintomas aparece cerca de cinco anos depois da lesão cutânea primária, propiciando um longo tempo de sensibilização[77]; esse estímulo prolongado culminaria com a produção de altos níveis de IFN-γ e TNF-á, consideradas importantes na gênese da lesão mucosa[78]. Contudo, foi evidenciado, recente, que um fator genético do hospedeiro, o polimorfismo do promotor IL-6-174 G/C, poderia também contribuir na patogênese da LCM/LM[79].

Na forma LCAD, a reação de hipersensibilidade celular e o teste de proliferação de linfócitos são sempre negativos, indicando um forte bloqueio da resposta imune celular, impossibilitando aos pacientes o controle da infecção[80,81]. Corroborando com estes aspectos, foi demonstrada, na Amazônia, a menor concentração de linfócitos CD4 e CD8 no infiltrado celular das lesões de pacientes com LCAD. Este encontro, junto com a constatação da menor expressão de RNA mensageiro para IFN-γ e da maior expressão de RNA mensageiro para IL-4 nas lesões dos pacientes[3], veio confirmar que a resposta imune celular é claramente Th2, o que explica as freqüentes recaídas nesses pacientes após tratamento com diferentes quimioterápicos[82].

Com respeito à forma LCDB, é importante salientar que durante a disseminação da infecção, uma fase crítica da doença, tanto a reação de hipersensibilidade como a proliferação de linfócitos são negativos, refletindo a existência de uma inibição dos mecanismos de imunidade celular nos pacientes. Além disso, tem sido observado que essa inibição parcial da resposta imune, sugerindo uma resposta CD4 mista (Th1+Th2), é mais pronunciada nos casos por *L. (L.) amazonensis* do que por *L. (V.) braziliensis*, embora a disseminação da infecção seja mais lenta nos casos por *L. (L.) amazonensis*, o que exige esquema de tratamento com antimonial pentavalente o dobro da dose total usada para *L. (V.) braziliensis*[54].

CONSIDERAÇÕES FINAIS

Com base nesses comentários, parece indiscutível a importância da espécie de *Leishmania* na patogenia da LTA na Amazônia, onde a doença pode significar o resultado de uma complexa interação da resposta imune do homem com sete espécies bem definidas do parasito, com potenciais patogênicos bastante distintos, como é o caso das duas espécies de maior importância médica, *L. (V.) braziliensis* e *L. (L.) amazonensis*. Neste sentido, foi demonstrado que ambas podem induzir amplo espectro de manifestações clínicas, histopatológicas e imunopatológicas das mais complexas, as quais, à medida que são melhor traduzidas, podem ter grande contribuição no sentido de promover novas perspectivas no manejo terapêutico da doença. Desse modo, apesar de não ser prioridade abordar o tratamento da doença nesta revisão, parece claro que um tratamento bem orientado da forma LCL, seguro da espécie de *Leishmania* envolvida, principalmente quando *L. (V.) braziliensis* ou *L. (L.) amazonensis*, poderá ser de extrema importância no sentido de prevenir complicações futuras da infecção, já que ambas são capazes de escapar dos mecanismos da resposta imune celular e desviar a infecção para os pólos imunológicos do espectro da doença; a *L. (V.) braziliensis* pode conduzir a infecção para o pólo imunológico hiperreativo, representado pela forma LCM ou LM, com forte exacerbação da hipersensibilidade, enquanto a *L. (L.) amazonensis* pode conduzir a infecção para o pólo imunológico hiporreativo, representado pela forma LCAD, com inibição parcial ou total da hipersensibilidade; conforme demonstrado, estes desvios da resposta imune celular, com exacerbação ou supressão dos

seus mecanismos de defesa (a hipersensibilidade, por exemplo), podem apresentar efeitos deletérios para o indivíduo com infecção por essas espécies de *Leishmania*. A este respeito, é importante mencionar que a constatação desse fundamento imunológico na patogenia da LTA foi possível pelo simples fato de que jamais foi observado, entre mais de mil pacientes com a forma LCL por *L. (V.) braziliensis* ou *L. (L.) amazonensis* e que receberam tratamento específico (antimoniato de meglumina) no serviço de leishmanioses do Instituto Evandro Chagas, em Belém, estado do Pará, Brasil, algum caso que tenha evoluído mais tarde, pós-tratamento, para uma das formas graves, LCM/LM ou LCAD, da LTA (Silveira, observação pessoal). Dessa forma, essa constatação não só representa forte evidência sobre o papel dos fundamentos imunológicos aqui considerados, como também, vem reforçar a importância do tratamento precoce da forma LCL na redução da prevalência das formas graves da LTA, assim como, sua morbidade e custos com tratamento.

SUMMARY

REVISITING THE PATHOGENESIS OF THE AMERICAN TEGUMENTARY LEISHMANIASIS IN AMAZONIAN, WITH EMPHASIS TO THE DISEASE DUE TO *Leishmania (V.) braziliensis* AND *Leishmania (L.) amazonensis*

Fernando T. **SILVEIRA**, Silvia R. **MÜLLER**, Adelson A.A. **de SOUZA**, Ralph **LAINSON**, Claudia M.C. **GOMES**, Marcia D. **LAURENTI** and Carlos E.P. **CORBETT**

The pathogenesis of American tegumentary leishmaniasis (ATL) was reviewed in the light of more recent features of clinical, histopathological and immunopathological spectrum of disease caused by Leishmania (V.) braziliensis and Leishmania (L.) amazonensis. This review has shown a dichotomy in the interaction between these two species of Leishmania with the human cellular immune response; while L. (V.) braziliensis shows a clear tendency to direct infection, from the localized cutaneous leishmaniasis (LCL) in the center of the clinical-immunological spectrum of disease, to the hyperactive immunologic pole represented by mucocutaneous leishmaniasis (MCL), which shows exacerbated hypersensitivity reaction and CD4 Th1-type immune response, L. (L.) amazonensis shows the opposite, directing infection to the hypoactive immunologic pole consisted by anergic diffuse cutaneous leishmaniasis (ADCL), associated with a marked inhibition of hypersensitivity reaction and CD4 Th2-type immune response. Between the central LCL and the two polar MCL and ADCL forms the infection may present an intermediary phase, borderline disseminated cutaneous leishmaniasis (BDCL), which shows partial inhibition of hypersensitivity reaction and a mixed CD4 Th1 plus Th2 immune response. These are probably the main immunological mechanisms regarding the immune response dichotomy that modulates the pathogenesis of ATL caused by these Leishmania parasites.

KEY WORDS: American tegumentary leishmaniasis; pathogenesis; *Leishmania (Viannia) braziliensis*; *Leishmania (Leishmania) amazonensis*; Amazon region; Brasil.

APOIO FINANCEIRO: Este trabalho recebeu apoio financeiro do Instituto Evandro Chagas (Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasil); Instituto de Medicina Tropical (Universidade Federal do Pará, Brasil); Wellcome Trust (London, UK); Laboratório de Investigação Médica (LIM)-50 (Hospital de Clínicas (HC)-Faculdade de Medicina (FM)-Universidade de São Paulo (USP), Brasil) e Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP: 06/56319-1, Brasil).

REFERÊNCIAS

1. LAINSON R, SHAW JJ. Leishmaniasis in the New World. *In L Collier, A Balows, M Sussman (eds), Topley & Wilson's Microbiology and Microbial Infections, 10th ed., Vol 5, Parasitology, Arnold, London* 2005; 313-349.

2. SILVEIRA FT, ISHIKAWA EAI, de SOUZA AAA, LAINSON R. An outbreak of cutaneous leishmaniasis among soldiers in Belém, Pará State, Brazil caused by *Leishmania (Viannia) lindenbergi* n. sp., a new leishmanial parasite of man in the Amazon region. *Parasite* 2002; 9: 43-50.
3. SILVEIRA FT, LAINSON R, CORBETT CEP. Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil. *Mem Inst Oswaldo Cruz* 2004; 99: 239-251.
4. MOSSER DM, EDELSON PJ. Activation of the alternative complement pathway by *Leishmania* promastigotes: parasite lysis and attachment to macrophages. *J Immunol* 1984; 132: 1501-1505.
5. MOSSER DM, BURK SK, COUTAVAS EE, WEDGEWOOD, JF, EDELSON PJ. *Leishmania* species: mecanisms of complement activation by five strains of promastigotes. *Exp Parasitol* 1986; 62: 394-404.
6. SACKS DL, PERKINS PV. Identification of an infective stage of *Leishmania* promastigotes. *Science* 1984; 223: 1417.
7. SACKS DL, PERKINS PV. Development of infective stage *Leishmania* promastigotes within phlebotomine sandflies. *Am J Trop Med Hyg* 1985; 34: 456.
8. SACKS DL, da SILVA RP. The generation of infective stage *Leishmania major* promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *J Immunol* 1987; 139: 3099.
9. PIMENTA PF, da SILVA R, SACKS DL, da SILVA P. Cell surface nano nanatomy of *Leishmania major* as reveled by fractureflip. A suface meshwork of 44 nm fusiform filaments identifies infective developmental stage promastigotes. *Eur J Cell Biology* 1989; 48: 180-190.
10. PUENTES SM, SACKS DL, JOINER KA. Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *J Exp Medical* 1988; 167: 887-902.
11. SACKS DL, BROCLIN NT, TURCO SJ. Developmental modification of the lipophosphoglycan from *Leishmania major* promastigotes during metacyclogenesis. *Mol Biochem Parasitol* 1990; 42: 225-234.
12. CHANG KP. *Leishmania donovani* macrophage binding mediated by surface glycoproteins/antigens. *Mol Biochem Parasitol* 1981; 4: 67-76.
13. KLEMPNER MS, CEDRN M, WYLER DJ. Attachment of plasma membrane vesicles of human macrophages to *Leishmania tropica* promastigotes. *J Infec Dis* 1983; 148: 377-384.
14. CHANNON JY, ROBERTS MB, BLACKWELL JM. A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages. *Immunology* 1984; 53: 345-355.
15. BLACKWELL JM, EZEKWITZ AB, ROBERTS MB, CHANNON JY, SIM RB, GORDON S. Macrophage complement and lectin-like receptores bind *Leishmania* in the absence of serum. *J Exp Med* 1985; 162: 224-231.
16. WILSON ME, PEARSON RD. Evidence that *Leishmania donovani* utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. *J Immunol* 1986; 136: 4681.
17. WILSON ME, PEARSON RD. Roles of CR3 and mannose receptors in the attachment and ingestion of *Leishmania donovani* by human mononuclear phagocytes. *Inf Immunol* 1988; 56: 363-369.
18. SARAIVA EMB, ANDRADE AFB, de SOUZA W. Involvment of the macrophage mannose-6-phosphate receptor in the recognotion of *Leishmania mexicana amazonensis*. *Parasitol Research* 1987; 73: 411-416.
19. HANDMAN E, GODING JW. The *Leishmania* receptor for macrophage is a lipid containing glycoconjugate. *Eur Mol Biol Org J* 1985; 4: 329-336.
20. PALATNIK CB, BOROJEVIC R, PREVIATO JO, MENDONÇA-PREVIATO L. Inhibition of *Leishmania donovani* promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate ligands. *Inf and Immunity* 1989; 57: 754-763.
21. RUSSEL DG, WILHELM H. The involvment of the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophages. *J Immunol* 1986; 136: 2613-2620.
22. CHANG CS, CHANG KP. Monoclonal antibody affinity purification of a *Leishmania* membrane glycoprotein and its inhibition of *Leishmania*-macrophage binding. *Proc Nat Acad Sci USA* 1986; 83: 100-104.
23. FRANKE ED, MAC-GREVY PB, KATZ SP, SACKS DL. Growth cycle dependent generation of complement resistant *Leishmania* promasigotes. *J Immunol* 1985; 134: 2713-2718.
24. MOSSER DM, EDELSON PJ. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. *J Immunol* 1985; 135: 2786-2789.
25. da SILVA RP, HALL BF, JOINER KA, SACKS DL. CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. *J Immunol* 1989; 43: 617-622.
26. MOSSER DM, EDELSON PJ. The third component of complement (C3) is responsible for the intracellular survival of *Leishmania major*. *Nature* 1987; 327: 329-331.

27. WRIGHT SD, SILVERSTEIN SC. Receptors for C3b and C3bi promote phagocytosis but not release of toxic oxygen from human phagocytes. *J Exp Med* 1983; 159: 2016-2022.
28. MURRAY HW. Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J Exp Med* 1981; 153: 1302-1315.
29. MURRAY HW. Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *J Immunol* 1982; 129: 351-357.
30. PEARSON RD, HARCUS JL, SUMES PH, ROMITO R, DONOVITZ GR. Failure of the phagocytic oxidative response to protect human monocyte-derived macrophage from infection by *Leishmania donovani*. Journal of Immunology 1982; 129: 282-287.
31. TURCO SJ. The lipophosphoglycan of *Leishmania*. *Parasitol Today* 1988; 4: 255-257.
32. REALEY AT, KUNHS DB, BASFORD RE, GLEW RH, KAPLAN SS. Leishmanial phosphatase blocks neutrophil O_2 producion. *J Biol Chem* 1984; 259: 173-175.
33. BLANK C, FUCHS H, RAPPERSBERGER K, RÖLLINGHOFF M, MOLL H. Parasitism of epidermal Langerhans Cells in experimental cutaneous leishmaniasis with *Leishmania major*. *J Inf Dis* 1993; 167: 418-425.
34. MOLL H. Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis. *Immunol Today* 1993; 23: 1595-1601.
35. MOLL H, FLOHÉ S, RÖLLINGHOFF M. Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antingen-specific T-cell immune response. *Eur J Immunol* 1995; 25: 693-699.
36. AXELROD O, KLAUS S, FRANKENBURG S. Antigen presentation by epidermal Langerhans cells in experimental cutaneous leishmaniasis. *Parasite Immunol* 1994; 16: 593-597.
37. KAPSENBERG ML, TEUNISSEN MBM, BOS JD. Langerhans cells : a unique subpopulation of antigen-presenting dendritic cells. In: Skin Immune System (SIS). *J.D. Bos (Ed.) Boca Ratón, CRC Press* 1990, 109-124.
38. BOS JD, KAPSENBERG ML. The skin immune system. Its cellular constituents and their interactions. *Immunol Today* 1986; 7: 235-240.
39. TEUNISSEN MBM, WORMEESTER J, KRIEG SR, PETERS PJ, VOGELS IMC, KAPSENBERG ML, BOS J.D. Human epidermal Langerhans cells under profound morphologic and phenotypical changes during *in vitro* culture. *J Invest Dermatol* 1990, 94, 166-173.
40. SCHMIT D. La présentation antigenique au niveau de la peau. *Ann Dermatol Vener* 1990; 117: 405-413.
41. LIEW FY, MILLOT S, PARKINSON C, PALMER RMJ, MONCADA S. Macrophage killing of *Leishmania* parasite *"in vivo"* is mediated by nitric oxide from L-arginine. *J Immunol* 1990; 144: 4794-4797.
42. LIEW FY, LI Y, MOSS D, PARKINSON C, ROGERS MV, MONCADA S. Resistance to Leishmania major infection correlates with the induction of nitrix oxide synthase in murine macrophages. *Eur J Immunol* 1991; 21: 3009-3014.
43. MAUEL J, RANSIJN A, BUCHMULLER-ROULER Y. Killing of *Leishmania* parasites in activated murine macrophages is based on an L-arginina-dependent process that produces nitrogen derivatives. *J Leuk Biol* 1991; 49: 73-82.
44. STENGER S, THURING H, RÖLLINGOFF M, BOGDAN C. Tissue expression in indicible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *J Exp Med* 1994; 180: 783-789.
45. RITTER U, MEISSINER A, SCHEIDIG C, KÖRNER H. CD8á- and Langerin-negative dendritic cells, but not Langerhans cells, act as principal antigen-presenting cells in leishmaniasis. *Eur J Immunol* 2004; 34: 1542-1550.
46. KAPLAN DH, JENISON MC, SAELAND S, SHOLOMCHIK WD, SHOLOMCHIK MJ. Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 2005; 23: 611-620.
47. RITTER U, OSTERLOH A. A new view on cutaneous dendritic cell subsets in experimental leishmaniasis. *Med Microbiol and Immunol* 2007, 196, 51-59.
48. SILVEIRA FT, LAINSON R, GOMES CMC, LAURENTI MD, CORBETT CEP. Reviewing the role of dendritic Langerhans cell in the immunopathogenesis of American cutaneous leishmaniasis. *Trans Roy Soc Trop Med Hyg* 2008, (no prelo).
49. BLACKWELL JM. A murine model of genetically controlled host responses to *Leishmania*. In: Ecology and genetics of host parasite interactions (eds. Rollinson, D., Anderson, R.M.). *New York: Academic Press* 1985; 147-57.
50. CARVALHO EM, JOHSON WD, BARRETO E, MARSDEN PD, COSTA JML, REED S, ROCHA H. Cell mediated immunity in American cutaneous and mucosal leishmaniasis. *J Immunol* 1985; 135: 4144-4148.
51. CÁSTES M, AGNELLI A, VERDE O, RONDON AJ. Characterization of the cellular immune response in American cutaneous leishmaniasis. *Clin Immunol and Inunopathol* 1983; 27: 176-186.

52. CARVALHO EM, BARRAL A, COSTA JML, BITTENCOURT A, MARSDEN PD. Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis. *Acta Tropica* 1994; 56: 315-325.
53. COSTA JML, MARSDEN PD, LLANOS-CUENTS EA, NETTO EM, CARVALHO EM, B ARRAL A, ROSA AC, CUBA CC, MAGALHÃES AV, BARRETO AC. Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. *J Trop Med and Hyg* 1986; 89: 319-321.
54. SILVEIRA FT, LAINSON R, CORBETT CEP. Further observations on clinical, histopathological and immunological features of borderline disseminated cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. *Mem Inst Oswaldo Cruz* 2005; 100: 525-34.
55. TURETZ ML, MACHADO PR, KO AI, ALVES F, BITTENCOURT A, ALMEIDA RP, MOBASHERY N, JOHSON WD Jr, CARVALHO EM. Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. *J Inf Dis* 2002; 186: 1829-1834.
56. ESPERANZA AC. Histopathological diagnosis of cutaneous and mucocutaneous leishmaniasis in Bolívia. *Mem Inst Oswaldo Cruz* 1983; 78: 13-20.
57. DIMIER-DAVID L, RAVISSE P, BUSTILLOS R, ROLLANO F, MALLEA F, DAVID C, LVÈVRE L, DEDET JP. Histopathology of mucocutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis*. *Ann de Dermatol e Venereol* 1994; 121: 387-392.
58. MAGALHÃES AV, MORAES MAP, RAICK NA, LLANOS-CUENTS EA, COSTA JML, CUBA CC, MARSDEN PD. Histopatologia da leishmaniose tegumentar por *Leishmania braziliensis braziliensis*. *Rev Inst Med Trop São Paulo* 1986[a]; 28: 253-262.
59 .MAGALHÃES AV, MORAES MAP, RAICK NA, LLANOS-CUENTAS EA, COSTA JML, CUBA CC, MARSDEN PD. Histopatologia da Leishmaniose Tegumentar por *Leishmania braziliensis braziliensis*: 3. Reação celular nos tecidos. *Rev Inst Med Trop São Paulo* 1986[b]; 28: 300-311.
60. MORAES MAP, SILVEIRA FT. Histopatologia da forma localizada de leishmaniose cutânea por *Leishmania (Leishmania) amazonensis*. *Rev Inst MedTrop São Paulo* 1994; 36: 459-463.
61. RIDLEY DS. A histological classification of cutaneous leishmaniasis and its geographical expression. *Trans Roy Soc Trop Med Hyg* 1980; 74: 515-521.
62. RIDLEY DS, MAGALHÃES AV, MARSDEN PM. Histological analysis and the pathogenesis of mucocutaneous leishmaniasis. Journal of Pathology 1989; 159: 293-299.
63. SANGUEZA OP, SANGUEZA JM, STILLER MJ, SANGUEZA P. Mucocutaneous leishmaniasis: a clinicopathology classification. *J Ame Acad Dermatol* 1993; 28: 927-932,
64. SILVIRA FT, MORAES MAP, LAINSON R, SHAW JJ. Experimental cutaneous leishmaniasis. III – Histopathological aspects of the developmental behavior of cutaneous lesions induced in the *Cebus apella* monkey (Primates: Cebidae) by *Leishmania (Viannia) lainsoni, L. (V.) braziliensis e L. (Leishmania) amazonensis*. *Rev Inst Med Trop São Paulo* 1990; 32: 387-394.
65. COSTA AA, SALDANHA AC, LEITE BM, RAMOS B, JUNIOR IA, NORONHA AL, BARRAL A, CORBETT CEP, COSTA JML. Imaging exams of bone lesions in patients with diffuse cutaneous leishmaniasis (DCL). *Acta Tropica* 2005; 96: 9-15.
66. COSTA AA, ABREU AL, GOMES CM, SALDANHA AC, BARRAL A, COSTA JML, CORBETT CEP. Experimental modelof chronic osteomyelitis caused by *Leishmania (L.) amazonensis*. *Actra Tropica* 2006; 98: 125-129.
67. BARRAL-NETTO, M, BRODSKYN, C, CARVALHO EM, BARRAL A. Human-leishmaniasis@cytokines.bahia.br. *Braz J Med Biol Res* 1998; 31: 149-155.
68. RIBEIRO-DE-JESUS, A, ALMEIDA RP, LESSA H, BACELLAR O, CARVALHO EM. Cytokine profile and pathology in human leishmaniasis. *Braz J Med Biol Res* 1998; 31: 143-148.
69. CÁCERES-DITTIMAR G, TAPIA FJ, SANCHEZ MA, YAMAMURA M, UYEMURA K, MODLIN RL, BLOOM BR, CONVIT J. Determination of the cytokine profiles in American cutaneous leishmaniasis using the polymerase chain reaction. *Clin Exp Immunol* 1993; 91: 500-505.
70. PIRMEZ C, YAMAMURA M, UYEMURA K, PAES-OLIVEIRA M, CONCEIÇÃO-SILVA F, MODLIN RL. Cytokine patterns in the pathogenegis of human leishmaniasis. *J Clin Invest* 1993; 91: 1390-1395.
71. SILVEIRA FT, LAINSON R, SHAW JJ, de SOUZA AAA, ISHIKAWA EA, BRAGA RR. Cutaneous leishmaniasis due to *Leishmania (Leishmania) amazonensis* in Amazonian Brazil, and the significance of a Montenegro skin-test in human infections. *Trans Roy Soc Trop Med Hyg* 1991; 85: 735-738.
72. SILVEIRA FT, BLACKWELL JM, ISHIKAWA EAI, BRAGA RR, SHAW JJ, QUINNELL RJ, SOONG L, KIMA P, MCMAHON-PRAT D, BLACK GF, SHAW MA. T cell responses to crude and defined leishmanial antigens in patients from the lower Amazon region of Brazil infected with different species of *Leishmania* of the subgenera *Leishmania* and *Viannia*. *Parasite Immunol* 1998; 20: 19-26.
73. BARRAL A, JESUS AR, ALMEIDA RP, CARVALHO EM, BARRAL-NETTO M, COSTA JML, BARADÓ R, ROCHA H, JOHNSON WD. Evaluation of T-cell subsets in the lesion infiltrates of human cutaneous and mucocutaneous leishmaniasis. *Parasite Immunol* 1987; 9: 487-497.

74- PIRMEZ C, COOPER C, PAES-OLIVEIRA M, SHUBACH A, TORIGIAN VK, MODLIN RL. Immunologic responsiveness in American cutaneous leishmaniasis lesions. *J Immunol* 1990; 145: 3100-3104.
75. MARTINEZ-AARENDS A, TAPIA FJ, CÁCERES-DITTMAR G, MOSCA W, VALECIL L, CONVIT J. Immunocytochemical characterization of immune cells in lesions of American cutaneous leishmaniasis using novel T cell markers. *Acta Tropica* 1991; 49: 271-280.
76. ESTERRE P, DEDET JP, FRENAY C, CHEVALLIER M, GRIMAUD JA. Cell populations in the lesion of human cutaneous leishmaniasis: a light microscopical, immunocytochemical and ultrastructural study. *Virch Arch Anat-Pathol Anat-Histopathol* 1992; 421: 239-247.
77. SILVEIRA FT, DUARTE ERL, de FARIAS ECF, IKEDA CS, LOPES AP, CHAGAS EJP, TEIXEIRA LM, ISHIKAWA EA. Leishmaniose mucosa na Amazônia brasileira: Avaliação, retrospectiva, dos aspectos clínicos e epidemiológicos da doença, com ênfase ao estado do Pará. *Rev Soc Bras Med Trop* 1999; 32 (Supl. I): 9.
78. BLACKWELL JM. Tumor necrosis factor alpha and mucocutaneous leishmaniasis. *Parasitol Today* 1999; 15: 73-75.
79. *CASTELLUCCI L, MENEZES E, OLIVEIRA J, MAGALHÃES A, GUIMARÃES LH, LESSA M, RIBEIRO S, REALE J, NORONHA EF, WILSON ME, DUGGAL P, BEATY TH, JERÔNIMO S, JAMIESON SE, BALES A, BLACKWELL JM, de JESUS AR, CARVALHO EM. IL6-174 G/C promoter polymorphism influences susceptibility to mucosal but not localized cutaneous leishmaniasis in Brazil.* J Inf Dis *2007; 194: 519-527.*
80. BARRAL A, COSTA JML, BITTENCOURT A, BARRAL-NETTO M, CARVALHO EM. Polar and subpolar diffuse cutaneous leishmaniasis in Brazil: clinical and immunopathologic aspects. *Int J Dermatol* 1995; 34: 474-479.
81. PETERSEN EA, NEVA FA, OSTER CN, DIAZ HB. Specific inhibition of lymphocyte proliferation response by adherent suppressor cells in diffuse cutaneous leishmaniasis. *New Engl J Med* 1982; 306: 387-391.
82. BONFIM G, NASCIMENTO C, COSTA JML, CARVALHO EM, BARRAL-NETTO M, BARRAL A. Variation of cytokine patterns related to therapeutic response in diffuse cutaneous leishmaniasis. *Exp Parasitol* 1996; 84: 188-194.

Tabela 1. Patogenia da LTA determinada por *L. (V.) braziliensis* e *L. (L.) amazonensis* na Amazônia, Brasil.

	Espectro clínico, histopatológico e imunopatológico				
Formas clínicas	**LCAD**	**LCDB**	**LCL**	**LCDB**	**LCM**
Parasito	*L. (L.) a.*	*L. (L.) a.*	*L. (L.) a. / L.(V.) b.*	*L. (V.) b.*	*L. (V.) b.*
Carga parasitária	++++	+++	+++/++	+	-
Tipo de lesão	nódulo	placa infiltrada	úlcera/úlcera	pápula ulcerada	necrótica
Distribuição	difusa	disseminada	única/múltipla	disseminada	naso-bucal
Macrófago	++++	+++	++/+	+	-
Plasmócito	+	++	++/+++	+++	+++
Linfócito	+	++	++/+++	+++	++++
Necrose	-	-	+/++	++	++++
Granuloma	-	epitelióide	epitelióide/epitelióide	epitelióide	tuberculóide
DTH	-	-	± / ++	±	++++
Linfoproliferação	-	-	± / ++	±	++++
IFN-γ	+	+	++/+++	++	++++
IL-4	++++	++	+/-	+	-
Células T CD4	Th2	Th1≥Th2	Th1/Th1	Th1>Th2	Th1
Tratamento (Sbv)	±	++	++++/++++	+++	++

LCAD = leishmaniose cutânea anérgica difusa; LCDB = leishmaniose cutânea disseminada borderline; LCL = leishmaniose cutânea localizada; LCM = leishmaniose cutâneo-mucosa; *L. (L.) a. = Leishmania (Leishmania) amazonensis* ; *L. (V.) b. = Leishmania (Viannia) braziliensis* ; DTH = hipersensibilidade do tipo retardada; IFN-g = interferon gamma; IL-4 = interleucina 4; Th1 = linfócito T auxiliador 1; Th2 = linfócito T auxiliador 2; Tratamento (Sbv) = terapêutica com antimônio pentavalente

ANEXO-13

A cross-section study on the clinical and immunological spectrum of human *Leishmania (L.) infantum chagasi* infection in the Brazilian Amazon region.

Crescente, J.A.B.; Silveira, F.T.; Lainson, R.; Gomes, C.M.C.; **Laurenti, M.D.;** Corbett, C.E.P.

Transaction of the Royal Society of Tropical Medicine and Hygiene 2009; 103: 1250-1256.

Transactions of the Royal Society of Tropical Medicine and Hygiene (2009) **103**, 1250—1256

available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/trstmh

A cross-sectional study on the clinical and immunological spectrum of human *Leishmania (L.) infantum chagasi* infection in the Brazilian Amazon region

José Ângelo B. Crescente[a], Fernando T. Silveira[a,b], Ralph Lainson[b], Claudia M.C. Gomes[c], Márcia D. Laurenti[c], Carlos E.P. Corbett[c,*]

[a] *Tropical Medicine Institute, Pará Federal University, Belém, PA, Brazil*
[b] *Parasitology Department, Evandro Chagas Institute (Surveillance Secretary of Health, Ministry of Health), Belém, PA, Brazil*
[c] *Pathology Department, Faculty of Medicine, São Paulo University, Avenida Dr. Arnaldo, 455 — Sala 1215, CEP 01246-903, São Paulo, SP, Brazil*

Received 23 August 2007; received in revised form 17 June 2009; accepted 17 June 2009
Available online 16 July 2009

KEYWORDS
Leishmania infantum chagasi;
Infection;
Clinical examination;
Immunologic tests;
Cross-sectional study;
Brazil

Summary The objectives of this study were to identify individuals with symptomatic and/or asymptomatic infection due to *Leishmania (L.) infantum chagasi*; to study the two types of infection, both clinically and immunologically, and to determine the prevalence rate of infection at the beginning of the study. This was a cross-sectional study with a cohort of 946 individuals, of both genders, from the age of 1 year, living in the municipality of Barcarena, PA, Brazil, an area endemic for American visceral leishmaniasis (AVL). The leishmanin skin test (LST) and the indirect fluorescent test (IFAT), were used for the diagnosis of infection. One hundred and twenty cases of infection were diagnosed, with a prevalence rate of 12.6%; eight cases showed high seroreactivity (1280—10 240, IgG) in IFAT and no LST reaction; four of these cases were typical AVL and four had subclinical oligosymptomatic infection. Using two immunological methods with a clinical examination of the infected individuals enabled the identification of five clinical-immunological profiles which may promote a better understanding of the interaction between *L. (L.) i. chagasi* and the human immune response: asymptomatic infection (AI) 73.4%; subclinical resistant infection (SRI) 15%; subclinical oligosymptomatic infection (SOI) 3%; symptomatic infection (AVL) 3% and indeterminate initial infection (III) 5%.

* Corresponding author. Tel.: +55 11 3081 7799.
E-mail address: ccorbett@usp.br (C.E.P. Corbett).

0035-9203/$ — see front matter
doi:10.1016/j.trstmh.2009.06.010

1. Introduction

The clinical manifestations of visceralizing *Leishmania* spp. are determined by the interactions of the parasite with the human immune system. To evaluate the human immune response to this disease, two types of assay have mainly been used: the IFAT or ELISA for evaluating the humoral response and the leishmanin skin test (LST) for evaluating the cellular, delayed-type hypersensitivity (DTH) response. However, in most situations these assays have been used separately, which has not given a view of the whole immune response to infection.

To illustrate this situation, some studies carried out in Africa, especially in Sudan[1,2] and Ethiopia[3–5], reported the epidemiological, clinical and immunological features of human infection caused by *Leishmania (L.) donovani* using either LST to measure DTH and IFAT or ELISA to measure the antibody response. In some Asian countries, such as India[6] and Nepal[7], which have a high prevalence of visceral leishmaniasis (VL) caused by *L. (L.) donovani*, similar immunological approaches were used to understand the features of human disease. In European Mediterranean countries, including Italy[8], Spain[9] and Greece[10], the clinical and immune responses to human infection with *Leishmania (L.) i. infantum* were investigated using similar approaches. In South America, where Brazil has the highest incidence of human VL due to *Leishmania (L.) infantum chagasi*, few studies have reported the epidemiological, clinical and immunological profiles of infection based on the same immunodiagnostic models.[11–16]

The clarification of the clinical manifestations that occur in the spectrum of human infection with *L. (L.) i. chagasi* infection has been the aim of some studies in Brazil.[14,17–20] In most of these studies, however, the diagnosis of infection was only based on the antibody response of the infected individuals allowing categorisation of the disease into three clinical forms: asymptomatic infection (AI), subclinical oligosymptomatic infection (SOI) and symptomatic infection [SI = American visceral leishmaniasis (AVL)]. It is quite possible, however, that this type of diagnostic approach may have neglected some clinical or immunological findings associated with the DTH response of infected individuals living in the endemic area.

For these reasons and because of the scarce information on the interaction between the human *L. (L.) i. chagasi* infection and the immune response in the Brazilian Amazon region,[21] we conducted a cross-sectional study of the prevalence rates and the clinical and immunological spectrum of infection by combining the two types of immune response assays: IFAT for the humoral response and LST for the DTH response aiming to improve our understanding on the clinical and immunological spectrum of the human *L. (L.) i. chagasi* infection in the Brazilian Amazon region.

2. Materials and methods

2.1. Study area

This study was carried out during October–November 2003 in Santana do Cafezal village, which is situated on the banks of the river Cafezal, 7 km from the administrative centre of Barcarena municipality (01°30′S; 48°37′W). It is considered to be within the metropolitan region of Belém, PA, in the north of Brazil. The climate is typically equatorial, with an average temperature of 27 °C and high humidity. The annual rainfall is in the region of 2500 mm or more, with the period from January to June forming the principal rainy season. Following extensive destruction of the primary forest, the area now consists mainly of plantations, with occasional patches of developing secondary forest. Approximately 70% of the inhabitants occupy wooden houses in non-flood land, which is surrounded by secondary forest, while the rest lives in the várzea, an area of low vegetation which is flooded twice daily by waters of the Cafezal River.

2.2. Study design and population

The study was designed to identify individuals of all ages, with symptomatic or asymptomatic infection with *L. (L.) i. chagasi*; to characterize the infection both clinically and immunologically and to determine the prevalence rate of infection. Specifically, we looked for a better understanding of the transmission dynamics as well as the clinical and immunological features of the different patterns of the disease within the spectrum of human *L. (L.) i. chagasi* infection.

The population enrolled in the study consisted of a cohort of 946 individuals (almost 90% of the total population), being 568 males and 378 females aged 1–89 years old with a median of 20 years, suggesting a relatively young population. When the study began, the number of inhabitants in the area was estimated to be 1064.[22] In order to obtain a clearer idea of the transmission dynamics of infection, the total population was divided into three age groups: 1–10, 11–20 and ≥21 years. The groups consisted of 260, 218 and 468 individuals, respectively. This stratification considered that people aged ≥21 years have the same ability to develop a specific immune response against infection. For the infection diagnosis, both IFAT and LST were performed in all individuals previously selected for the prevalence study. In addition, all individuals presenting any type of immune reaction were clinically examined in order to identify any signs and/or symptoms that could be associated with the classical features of AVL.

2.3. Criteria for identification of human infection

The definition of a case of human infection with *L. (L.) i. chagasi* was the presence of reactivity in either or both immunological tests. IFAT demonstrates humoral immunity associated with the CD4+ Th2 immune response (antibody response = susceptibility) and LST demonstrates cellular immunity associated with the CD4+ Th1 immune response (hypersensitivity = resistance).[23] With the objective of expressing the specificity of IFAT and LST, a scale of semi-quantitative results was used with scores varying from + to ++++. For IFAT, serological titres of 80–160 and 320–640 (IgG) received + and ++ and those of 1280–2560 and 5120–10 240 were given +++ and ++++, respectively; for LST, exacerbated intradermal reactions (≥16 mm) were regarded as ++++, strongly positive (13–15 mm) as +++,

moderately positive (9—12 mm) as ++ and weakly positive (5—8 mm) as +. Thus, it was assumed that serological reactions of 80 (IgG) titre and intradermal reactions forming papules or indurations of $\geq$5 mm in diameter were regarded as the positive cut-off in IFAT and LST, respectively.[24–27]

2.4. Immunological test procedures

The procedure used for LST was previously described in other studies on American cutaneous leishmaniasis.[26,27] The antigen used in Santana do Cafezal, a village situated in an area where cutaneous and visceral leishmaniasis are potentially concomitant, should have a high specificity for the visceral disease. Thus, to promote high specificity in LST, cultured promastigote forms from the stationary phase in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) of a regional strain of *L. (L.) i. chagasi* (MCAO/BR/2003/M22697/Barcarena, PA, Brazil) was used. They were fixed in merthiolate solution (1/10 000), with a final concentration of approximately 10×10^6 parasites/ml. As control for the *Leishmania* antigen, 0.1 ml of the merthiolate solution (1/10 000) was administered intradermally in the opposite forearm of each individual.

IFAT was performed as proposed by Lima et al.[24,25] who demonstrated that amastigote antigens of *L. (L.) i. chagasi* had a higher specificity and sensitivity than those of promastigotes of the same species and *L. (L.) major*-like (Bio-Manguinhos, RJ, Brazil) and than amastigote antigens of *L. (L.) amazonensis*. Briefly, amastigote antigens were impregnated in the IFAT slides by imprint of small fragments of spleen and liver from hamster infected with the parasite. Crude *L. (L.) i. chagasi* amastigote antigen has the best specificity and sensitivity for the serological diagnosis of human infection with *L. (L.) i. chagasi* using IFAT. For diagnosis of canine VL this method has also proved to be more specific than IFAT and ELISA (Bio-Manguinhos), which are available through the Brazilian AVL control program.[28]

2.5. Data analysis

The data were analyzed by the Bio-Estat 4.0 program.[29] The χ^2 and binomial tests were used for the significance of differences between the clinical-immunological profiles of infection with a confidence interval of 95%.

3. Results

3.1. Prevalence rates of human *Leishmania (L.) infantum chagasi* infection

An infection prevalence rate of 11.2% (106/946) was obtained by LST and of 3.4% (32/946) by IFAT ($P < 0.001$). Among the 106 LST-reactive individuals, 18 (17%) were also IFAT-reactive, and of the 32 IFAT-reactive, the same 18 (56.2%) were also LST-reactive. This combination permitted the identification of an actual prevalence rate of 12.6% (120/946) for the community, using both tests.

3.2. Frequency of human *Leishmania (L.) infantum chagasi* infection according to sex, age and LST and IFAT specificity

The distribution of the 120 cases of infection showed no difference between males (55.8%) and females (44.2%). The distribution of infection according to age also showed no difference between the younger age groups, with 19.2% of infected cases aged 1—10 years and 25.8% of cases aged 11—20 years. However, when the rates of these groups were compared with that of the older age group both were significantly smaller ($P < 0.001$), with more than half (55%) of infected individuals being $\geq$21 years old.

Regarding LST and IFAT response, among the 106 LST-reactive cases, 41.5% presented exacerbated reactivity (++++), 14.1% were strongly positive (+++), 19.8% moderately positive (++) and 24.6% weakly positive (+). Thus over half of the cases (55.6%) had marked (+++/++++) immunological resistance (hypersensitivity) to infection. Among the 32 IFAT-reactive cases, 21.8% presented low serological reactivity (+), 53.1% were moderately positive (++), 18.8% strongly positive (+++) and 6.3% highly positive (++++). Thus 25.1% of cases presented a significant humoral response of strongly (+++) or highly positive (++++), showing immunological susceptibility to infection.

3.3. Clinical-immunological evaluation of human *Leishmania (L.) infantum chagasi* infection

Of the 120 cases of infection, most (73.4%) were clinically asymptomatic and exhibited an immune response (LST+/++++ and IFAT−) consistent with a clinical-immunological profile of resistant AI. A few (6.6%) were represented by an immune response (LST− and IFAT+++/++++) that could be associated with either of two susceptible clinical-immunological profiles: (SI = AVL) or SOI. The difference was mainly clinical; the profile of SI was applied to four (3.3%) cases of active AVL (two children and two adults), resulting in an AVL prevalence rate of 0.42%, while SOI was used in another four (3.3%) cases (one child and three adults). These cases presented various manifestations, associated or not and of uncertain duration, which were not characteristic of acute AVL, such as: fever; asthenia; pallor; adenopathy and slight hepatomegaly, but without splenomegaly. There were also some haematological alterations such as mild-to-moderate anaemia and leukopenia. A further 15% of cases were asymptomatic with an immune response reaction in both tests (LST+/++ and IFAT+/++), compatible with an intermediate clinical-immunological profile showing a reasonable degree of resistance (LST+/++). This profile was categorized as subclinical resistant infection (SRI). Finally, 5% of cases were asymptomatic and presented a humoral immune response but with low serological titres (LST− and IFAT+/++). These patients were regarded as a very early infected group with the possibility of their disease evolving to either the resistant profiles SRI and AI or to the susceptible ones SOI and SI. This was considered an indeterminate initial infection (III) (Figure 1).

The frequency of the AI profile (73.4%) was higher ($P < 0.001$) than the other profiles, SRI (15%), III (5%), SI (3%)

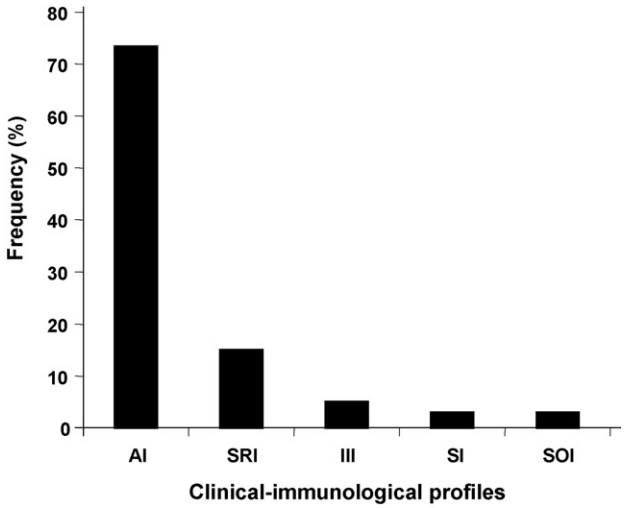

Figure 1 Frequency distribution of the clinical-immunological profiles of the human *Leishmania (L.) infantum chagasi* infection, in Santana do Cafezal village, Barcarena municipality, PA, Brazil. AI: asymptomatic infection; SRI: subclinical resistant infection; III: indeterminate initial infection; SI: symptomatic infection (= American visceral leishmaniasis); SOI: subclinical oligosymptomatic infection.

and SOI (3%) and that of the SRI profile was higher ($P < 0.001$) than those of the last three, III, SI and SOI, among which there were no differences in frequency rates. Based on this diagnostic approach, it was possible to distribute the 120 cases of infection into five clinical-immunological profiles, which represent a new proposal for the clinical and immunological spectrum of human *L. (L.) i. chagasi* infection in the New World (Table 1).

4. Discussion

This is one of the few epidemiological studies carried out in Brazil to evaluate the transmission dynamics of human *L. (L.) i. chagasi* infection in individuals aged 1 year and over, using two immunological methods, LST and IFAT. Even though some studies have previously reported on the transmission dynamics of infection,[11–13,15] they were limited to children up to 15 years old, which neglected the importance of older individuals in the epidemiology of infection.

With respect to the study population, Santana do Cafezal village consists of a community established for over a century, whose population selected for this study spanned at least four generations. Such long-term exposure to *L. (L.) i. chagasi* transmission may well favour the development of some degree of immune protection in the population. Although the general population has low purchasing power, food is provided principally by fishing and subsistence agriculture, providing a satisfactory feeding profile. Even though some recent evidence exists indicating that susceptibility to VL could be strongly controlled by a genetic mechanism,[30–32] it is possible that environment and/or nutrition could contribute to the promotion of an improved immune response against human *L. (L.) i. chagasi* infection, given that the prevalence (0.42%) of active AVL was low in this study.

With respect to the transmission dynamics of infection, this received two types of treatment in this study. The first analysed the infection prevalence by LST and IFAT and enabled a specific and comparative evaluation of the two types of immune response, cellular and humoral.[23] The second considered the combined analysis of LST and IFAT results, which allowed the determination of the actual rate of infection, in an attempt to evaluate the true situation of infection transmission in the study area. In the first analysis the infection prevalence determined by LST (11.2%) was higher ($P < 0.001$) than that determined by IFAT (3.4%). This demonstrated that among naturally infected individuals, a higher number presented immunological resistance (hypersensitivity) to infection, which may help to explain the low rate of prevalence (0.42%) of AVL in the present study. Moreover, considering that both tests were performed with the same antigen strain of *L. (L.) i. chagasi*, it is highly unlikely that the difference found in the prevalence rates determined by LST and IFAT could be attributed to the variability of the antigen specificity used in these tests. It should be highlighted that these findings can be influenced by some methodological procedures and/or epidemiological factors. In the Jacobina municipality, Bahia State, north-eastern region of Brazil, a soluble extract of *L. (L.) i. chagasi* antigen was used to study the infection prevalence by LST in children up to 15 years old.[11] By contrast with the present study, these authors found an infection rate of 34.1%. Considering that the immune response to infection is not limited to only one type, i.e. either a T-cell response or a humoral response, it is clear that these results underestimated the true rate of infection in that area. In another study in the municipality of Raposa, Maranhão State, also in the north-

Table 1 Clinical and immunological spectrum of human *Leishmania (L.) infantum chagasi* infection in the Amazon region, Brazil

Susceptible immunological pole		Resistant immunological pole
Symptomatic infection (AVL) LST− IFAT+++/++++	Indeterminate initial infection (III) LST− IFAT+/++	Asymptomatic infection (AI) LST+/++++ IFAT−
Subclinical oligosymptomatic infection (SOI) LST− IFAT+++/++++		Subclinical resistant infection (SRI) LST+/++ IFAT+/++

IFAT++++: 5120—10 240 (IgG); IFAT+++: 1280—2560 (IgG); IFAT++: 320—640 (IgG); IFAT+: 80—160 (IgG); IFAT−: negative reaction; LST: leishmanin skin test; LST++++: exacerbated reaction (≥16 mm); LST+++: strong reaction (13—15 mm); LST++: moderate reaction (9—12 mm); LST+: weak reaction (5—8 mm); LST−: negative reaction.

eastern region of Brazil, two different procedures were used: only children up to 5 years old were selected, representing a greater limitation compared to other studies and two different antigens were used for immune diagnosis; a soluble extract of *L. (L.) amazonensis* for LST and a soluble extract of *L. (L.) i. chagasi* for ELISA.[12,13] The results revealed infection prevalence rates of 18.6% by LST and 13.5% by ELISA, higher that those found in the present study (11.2% by LST and 3.4% by IFAT), suggesting a higher concentration of susceptible individuals (children up to 5 years old).

In another study also carried out in Maranhão State, municipality of São José de Ribamar, the prevalence rate of infection in children up to 15 years old was estimated using three antigen types: a soluble extract of *L. (L.) i. chagasi* for LST, and a crude antigen of the same parasite and a recombinant protein (rK39) for ELISA.[15] The data revealed the highest infection rates reported, 61.7% by LST and 19.7% (crude antigen) and 19.4% (rK39) by ELISA. These differences must be examined with caution, considering the procedures used and the epidemiological circumstances of each study area.

In Italy, where the disease is caused by *L. (L.) i. infantum*, a promastigote-antigen suspension of homologous parasite was used to demonstrate a 16.6% prevalence rate of infection in individuals of different ages by LST.[8] This most closely resembles the infection rate found in the present study and may reflect similar transmission dynamics of *L. (L.) i. infantum* and *L. (L.) i. chagasi*. In Africa, especially in Sudan, where the disease is caused by *L. (L.) d. donovani*, leishmaniasis represents a severe public health problem, being responsible for more than 100 000 deaths in the 1980s.[2] The epidemiological situation of human infection remains variable, as recent reports show prevalence rates ranging from 33% to 56% in two localities, Mushrau Koka and Um—Salala, respectively, using a promastigote-antigen suspension of the same parasite, demonstrating a higher infection transmission level than found in the Brazilian Amazon region.[1]

The second analysis was based on a combination of LST and IFAT data, which determined that the actual prevalence rate of infection in the study area is 12.6% (120/946). It should be highlighted that this appears to be an unpublished approach in the investigation of human *L. (L.) i. chagasi* infection epidemiology. Gender was not found to be a significant variable in the distribution of human *L. (L.) i. chagasi* infection. With respect to age, the younger age groups had similar infection rates, which were lower than that of the older group, demonstrating that infection progressively accumulates with age, similar to the pattern found in human *L. (L.) i. infantum* infection in Sicily, Italy.[8]

Regarding LST and IFAT specificity in the context of infection prevalence, the results showed that among the 106 LST-reactive cases, 56.6% presented a significant immunological resistance to infection (hypersensitivity +++/++++). This could be explained by a reasonable sample of infected individuals that have naturally received repeated antigenic stimulus through the infected phlebotomine sand fly vector (*Lutzomyia longipalpis*) over a prolonged period. In this context, it is possible that these 'natural infective doses' may represent a major strategy for consideration in a future vaccine program against infection. By contrast, it was observed that among the 32 IFAT-reactive cases, only 25% showed a susceptibility profile to AVL, with high serological titres ranging from 1280 (+++) to 10 240 (++++) (IgG). The great majority (75%) exhibited low serological reactivity 80 (+) to 640 (++) (IgG), indicating that only few cases expressing a humoral immune response (CD4 type 2 immune response) presented some degree of predisposition for developing AVL, which was confirmed in 12.5% of IFAT-reactive individuals and only in 3.3% of all 120 cases of infection. Moreover, considering the infection prevalence (12.6%), a ratio between infection and disease of 1:30 was estimated, while in the Jacobina municipality, Bahia State, this ranged from 1:6.5 to 1:18.5 with a prevalence rate of 34.1% by LST.[11] Again, it should be stated that in these locations the prevalence rates were estimated in children up to a maximum of 15 years of age.

In this study we propose a new, broad clinical-immunological spectrum for human *L. (L.) i. chagasi* infection in the New World using simple, inexpensive and reproducible methods. We propose five different clinical-immunological profiles of infection, adding two additional profiles, SRI and III, to the three already recognized by others: AI, SI (= AVL) and SOI[14,17–19], Pearson et al.[20] This proposed spectrum has the advantage of promoting a better view of the clinical and immunological factors in play in the interaction between *L. (L.) i. chagasi* and the human immune response, as well as providing a suitable tool for control programs of AVL. This spectrum may also reflect the genetic polymorphism behind the immunological mechanisms responsible for resistance to human VL infection.[30–33]

This experience also showed that the great majority (73.4%) of infected individuals living in the AVL-endemic area have an immune response profile of resistance to infection (LST+/++++ and IFAT−), confirming the significance of the hypersensitivity mechanism (T-cell immune response) in controlling the infection. This finding has also been demonstrated by others.[34] As a result, all these individuals were clinically asymptomatic (AI). The new profile SRI, which also showed a significant degree of resistance (LST+/++) to infection, was represented by a reasonable proportion (15%) of infected individuals. The SRI and AI profiles together were at least 88.4% of all infections in the endemic area, suggesting that most infected individuals present an immune resistance profile to the infection.

Another significant finding among infected individuals was the identification of the III profile (LST− and IFAT+/++), which consisted of individuals with the earliest stage of infection and with an apparent tendency for a predominantly humoral response, but with no definition of the ultimate immune response profile. This was considered as an indeterminate initial infection, which could evolve either to the resistant profiles SRI and AI or to the susceptible profiles SOI and SI, and may be useful in AVL control programs for monitoring recently infected individuals in the endemic area.

The finding that age may alter the features of infection was another conclusion of this work. A number of reports that documented a high number of subclinical oligosymptomatic cases, more than acute AVL cases, only included children up to 15 years old[17] or 5 years old.[18] In the present work, no difference in the prevalence rates between the SI (3.3%) and SOI profiles (3.3%) was identified, probably

because individuals of all ages were evaluated, suggesting an excess concentration of susceptible individuals in the prior reports. Indeed, the influence of age on the outcome of infection was detected between the age of these two profiles, an average age of 33.6 years was observed among individuals with the SOI profile, which was significantly higher ($P < 0.001$) than the average of 10.7 years old in the SI profile, indicating that older individuals seem to develop a better T-cell immune response to infection and present fewer conditions for the SI profile.

The feasibility of this approach for evaluating the clinical and immunological manifestations of human *L. (L.) i. chagasi* infection was confirmed in a recent cross-sectional study carried out in another locality (municipality of Cametá) in the northeast region of Pará State,[35] with a higher incidence of AVL than the municipality of Barcarena.[36] The prevalence rate of infection was 18.4% and the frequency rates of the clinical-immunological profiles were as follows: AI: 47.5%, SRI: 22.3%, SI: 0.5%, SOI: 3.9% and III: 25.7%. This suggests that an area with a higher level of transmission, where higher frequency rates of the SRI and III profiles occur, will contain a higher number of more recent cases of infection.

Authors' contributions: JABC, FTS and CEPC designed the study protocol; JABC, FTS, RL, CMCG, MDL and CEPC contributed to the collection and analysis of the data and preparation of the article. All authors read and approved the final manuscript. FTS and CEPC are guarantors of the paper.

Funding: This research was supported by the Instituto Evandro Chagas (Secretaria de Vigilância em Saúde, Ministério da Saúde, Brazil); Instituto de Medicina Tropical (Universidade Federal do Pará, Brazil); Wellcome Trust (London); Laboratório de Investigação Médica (LIM)-50 (Hospital de Clínicas (HC) Faculdade de Medicina (FM)-Universidade de São Paulo (USP), Brazil) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP: 06/56319-1, Brazil).

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Ethics Committee in Human Research of the Instituto Evandro Chagas (SVS), Brazil, with the protocol number CEP/IEC 16/2003 and CAPPesq/FMUSP 0255/07.

References

1. Khalil EA, Zijlstra EE, Kager PA, El Hassan AM. Epidemiology and clinical manifestations of *Leishmania donovani* infection in two villages in an endemic area in eastern Sudan. *Trop Med Int Health* 2002;**7**:35—44.
2. Zijlstra EE, El-Hassan AM, Ismael A, Ghalib HW. Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. *Am J Trop Med Hyg* 1994;**51**:826—36.
3. Ali A, Ashford RW. Visceral leishmaniasis in Ethiopia. I. Cross-sectional leishmanin skin test in an endemic locality. *Ann Trop Med Parasitol* 1993;**87**:157—61.
4. Ali A, Ashford RW. Visceral leishmaniasis in Ethiopia. III. The magnitude and annual incidence of infection, as measured by serology in an endemic area. *Ann Trop Med Parasitol* 1994;**88**:43—7.
5. Ali A, Ashford RW. Visceral leishmaniasis in Ethiopia. IV. Prevalence, incidence and relation of infection to disease in an endemic area. *Ann Trop Med Parasitol* 1994;**88**:289—93.
6. Sundar S. Indian kala-azar - better tools needed for diagnosis and treatment. *J Postgrad Med* 2003;**49**:29—30.
7. Schenkel K, Rijal S, Koirala S, Vanlerberghe V, Van der Stuyft P, Gramiccia M, et al. Visceral leishmaniasis in southeastern Nepal: a cross-sectional survey on *Leishmania donovani* infection and its risk factors. *Trop Med Int Health* 2006;**11**:1792—9.
8. Pampiglione S, Manson-Bahr PEC, La Placa M, Borgatti MA, Musumeci S. Studies in Mediterranean leishmaniasis: 3. The leishmanin skin test in kala-azar. *Trans R Soc Trop Med Hyg* 1975;**69**:60—8.
9. Garrote JI, Gutierrez MP, Izquierdo RL, Duenas MA, Zarzosa P, Canavate C, et al. Seroepidemiologic study of *Leishmania infantum* infection in Castilla-Leon, Spain. *Am J Trop Med Hyg* 2004;**71**:403—6.
10. Papadopoulou C, Kostoula A, Dimitriou D, Panagiou A, Bobojianni C, Antoniades G. Human and canine leishmaniasis in asymptomatic and symptomatic population in Northwestern Greece. *J Infect* 2005;**50**:53—60.
11. Badaró R, Jones TC, Lorenço R, Cerf BJ, Sampaio D, Carvalho EM, et al. A prospective study of visceral leishmaniasis in an endemic area of Brazil. *J Infect Dis* 1986;**154**:639—49.
12. Caldas AJM, Silva DRC, Pereira CCR, Nunes PMS, Silva BP, Silva AAM, et al. Infecção por *Leishmania (Leishmania) chagasi* em crianças de uma área endêmica de leishmaniose visceral americana na ilha de São Luis-MA, Brasil. *Rev Soc Bras Med Trop* 2001;**34**:445—51.
13. Caldas AJM, Costa JML, Silva AAM, Vinhas V, Barral A. Risk factors associated with infection by *Leishmania chagasi* in north-east Brazil. *Trans R Soc Trop Med Hyg* 2002;**96**:21—8.
14. Jeronimo SMB, Teixeira MV, de Queiroz Sousa A, Thielking P, Pearson RD, Evans TG. Natural history of *Leishmania (Leishmania) chagasi* infection in Northeastern Brazil: long-term follow-up. *Clin Infect Dis* 2000;**30**:608—9.
15. Nascimento MDSB, Souza EC, da Silva LM, da Cunha Leal P, de Lima Cantanhede K, de Barros Bezerra GF, et al. Prevalência de infecção por *Leishmania chagasi* utilizando os métodos de ELISA (rK39 e CRUDE) e intradermorreação de Montenegro em área endêmica do Maranhão, Brasil. *Cad Saúde Pública* 2005;**21**:1801—7.
16. Reed SG, Badaró R, Masur H, Carvalho EM, Lorenço R, Lisboa A, et al. Selection of a skin test antigen for American visceral leishmaniasis. *Am J Trop Med Hyg* 1986;**35**:79—85.
17. Badaró R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, et al. New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis* 1986;**154**:1003—12.
18. Gama MEA, Costa JML, Gomes CMC, Corbett CEP. Subclinical form of the American visceral leishmaniasis. *Mem Inst Oswaldo Cruz* 2004;**99**:889—93.
19. Holaday BJ, Pompeu MM, Evans T, Braga DN, Texeira MJ, Sousa AQ, et al. Correlates of *Leishmania*-specific immunity in the clinical spectrum of infection with *Leishmania chagasi*. *J Infect Dis* 1993;**167**:411—7.
20. Pearson RD, Souza AQ. Clinical spectrum of leishmaniasis. *Clin Infect Dis* 1996;**22**:1—13.
21. Silveira FT, Shaw JJ, Bichara CNC, Costa JML. Leishmaniose visceral americana. In: Leão RNG, editor. *Doenças infecciosas e parasitárias. Enfoque Amazônico*. Belém, PA: CEJUP; 1997. p. 631—44.
22. Instituto Brasileiro de Geografia e Estatística. *Contagem Nacional de Populações*. Rio de Janeiro, RJ: Superintendência de Estudos Geográficos e Sócio-econômicos; 2004.
23. Awasthi A, Mathur RK, Saha B. Immune response to *Leishmania* infection. *Indian J Med Res* 2004;**119**:238—58.

24. Lima LVR, de Souza AAA, Jennings YL, Campos MB, Corrêa Z, de Jesus R, et al. Comparação da reatividade entre antígenos de *Leishmania (L.) amazonensis* e *L. (L.) chagasi* no sorodiagnóstico (RIFI) da leishmaniose visceral humana no estado do Pará. *Rev Soc Bras Med Trop* 2003;**36**(Suppl I):312.
25. Lima LVR, de Souza AAA, Jennings YL, Corrêa Z, de Jesus R, Everdosa D, et al. Comparison of the reactivity between antigens of *Leishmania (L.) chagasi*, L. (L.) amazonensis e Leishmania sp. (Bio-Manguinhos) in the sero-diagnosis of visceral leishmaniasis by the indirect fluorescent antibody test (IFAT). *Rev Inst Med Trop São Paulo* 2003;**45**(Suppl 13):147.
26. Silveira FT, Lainson R, Shaw JJ, de Souza AA, Ishikawa EIA, Braga RR. Cutaneous leishmaniasis due to *Leishmania (Leishmania) amazonensis* in Amazonian Brazil, and the significance of a negative Montenegro skin-test in human infections. *Trans R Soc Trop Med Hyg* 1991;**85**:735—8.
27. Silveira FT, Blackwell JM, Ishikawa EA, Braga RR, Shaw JJ, Quinnell RJ, et al. T cell responses to crude and defined leishmanial antigens in patients from the lower Amazon region of Brazil infected with different species of *Leishmania* of the subgenera *Leishmania* and *Viannia*. *Parasite Immunol* 1998;**20**:19—26.
28. De Jesus RCS, Corrêa ZC, Everdosa DR, Martins AP, Eliseu LS, Campos MC, et al. Comparação das técnicas de RIFI (ag. IEC x ag. Bio-Manguinhos) e ELISA no sorodiagnóstico da leishmaniose visceral canina (LVC), Estado do Pará, Brasil. *Rev Soc Bras Med Trop* 2003;**36**:323.
29. Ayres M, Ayres Jr M, Ayres D, dos Santos AS. *Bioestat 4. 0: Aplicações estatísticas nas áreas das Ciências Biológicas e Médicas*. Belém, PA: Sociedade Civil Mamirauá-Brasília CNPq; 2004.
30. Blackwell JM, Mohamed HS, Ibrahim ME. Genetics and visceral leishmaniasis in the Sudan: seeking a link. *Trends Parasitol* 2004;**6**:268—74.
31. Jamieson SE, Miller EM, Peacock CS, Fakiola M, Wilson ME, Bales-Holst A, et al. Genome-wide scan for visceral leishmaniasis susceptibility genes in Brazil. *Genes Immun* 2007;**8**:84—90.
32. Peacock CS, Sanjeevi CB, Shaw MA, Collins A, Campbell RD, March R, et al. Genetic analysis of multicase families of visceral leishmaniasis in northeastern Brazil: no major role for class II or class III regions of HLA. *Genes Immun* 2002;**3**: 350—8.
33. Peacock CS, Collins A, Shaw MA, Silveira F, Costa J, Coste CH, et al. Genetic epidemiology of visceral leishmaniasis in northeastern Brazil. *Genet Epidemiol* 2001;**20**: 383—96.
34. Costa SR, D'Oliveira Jr A, Bacellar O, Carvalho EM. T cell response of asymptomatic *Leishmania chagasi* infected subjects to recombinant *Leishmania* antigens. *Mem Inst Oswaldo Cruz* 1999;**94**:367—70.
35. Rosas-Filho MS, Britto AB, Barbosa AP, Oliveira VM, da Cruz GL, Ribeiro LMF, et al. Caracterização das manifestações clínicas dos perfis clínico-imunológicos da infecção humana por *Leishmania (Leishmania) chagasi* em área endêmica de leishmaniose visceral no Pará, Brasil. *Rev Soc Bras Med Trop* 2007;**40**: 115.
36. Secretaria Executiva de Saúde do Estado do Pará (SESPA). *Leishmanioses visceral e tegumentar americana*. Belém, PA: Boletim Epidemiológico, Departamento de Controle de Endemias; 2004. p. 6—8.

ANEXO-14

A longitudinal study on the transmission dynamic of human *Leishmania (L.) infantum chagasi* infection in Amazonian Brazil, with special reference to its prevalence and incidence

Silveira, F.T.; Lainson, R.; Pereira, E.A.; de Souza, A.A.A.; Campos, M.B.; Chagas, E.J.; Gomes, C.M.C.; **Laurenti, M.D.;** Corbett, C.E.P.

Parasitology Research 2009; 104: 559-567

Parasitol Res (2009) 104:559–567
DOI 10.1007/s00436-008-1230-y

ORIGINAL PAPER

A longitudinal study on the transmission dynamics of human *Leishmania* (*Leishmania*) *infantum chagasi* infection in Amazonian Brazil, with special reference to its prevalence and incidence

Fernando T. Silveira · Ralph Lainson · Elza A. Pereira · Adelson A. A. de Souza · Marliane B. Campos · Eugênia J. Chagas · Claudia M. C. Gomes · Márcia D. Laurenti · Carlos E. P. Corbett

Received: 3 July 2008 / Accepted: 29 September 2008 / Published online: 21 October 2008

Abstract This was a longitudinal study carried out during a period over 2 years with a cohort of 946 individuals of both sexes, aged 1 year and older, from an endemic area of American visceral leishmaniasis (AVL) in Pará State, Brazil. The object was to analyze the transmission dynamics of human *Leishmania* (*Leishmania*) *infantum chagasi* infection based principally on the prevalence and incidence. For diagnosis of the infection, the indirect fluorescent antibody test (IFAT) and leishmanin skin test (LST) were performed with amastigote and promastigote antigens of the parasite, respectively. The prevalence by LST (11.2%) was higher ($p<0.0001$) than that (3.4%) by IFAT, and the combined prevalence by both tests was 12.6%. The incidences by LST were also higher ($p<0.05$) than those by IFAT at 6 (4.7%×0.6%), 12 (4.7%×2.7%), and 24 months (2.9%×0.3%). Moreover, there were no differences ($p>0.05$) between the combined incidences by both tests on the same point surveys, 5.2%, 6.3%, and 3.6%. During the study, 12 infected persons showed high IFAT IgG titers with no LST reactions: five children and two adults developed AVL (2,560–10,120), and two children and three adults developed subclinical oligosymptomatic infection (1,280–2,560). The combined tests diagnosed a total of 231 cases of infection leading to an accumulated prevalence of 24.4%.

F. T. Silveira (✉) · R. Lainson · A. A. A. de Souza · M. B. Campos
Parasitology Department, Evandro Chagas Institute (Surveillance Secretary of Health, Ministry of Health),
Av. Almirante Barroso, 492,
66090-000 Belém, Pará State, Brazil
e-mail: silveiraft@lim50.fm.usp.br

F. T. Silveira
Tropical Medicine Institute, Federal University of Pará,
Belém, Pará State, Brazil

E. A. Pereira
Control Department of Endemic Diseases,
Executive Secretary of Public Health,
Belém, Pará State, Brazil

E. J. Chagas
Public Health Department,
Health Secretary of Barcarena Municipality,
Barcarena, Pará State, Brazil

C. M. C. Gomes · M. D. Laurenti · C. E. P. Corbett
Pathology Department, Medical School of São Paulo University,
São Paulo, São Paulo State, Brazil

Introduction

American visceral leishmaniasis (AVL) is an anthropozoonosis, first recorded in Amazonian Brazil by Penna (1934) who, during an extensive study of postmortem necropsy tissues from individuals believed to have died from yellow fever in different parts of Brazil, diagnosed three cases of AVL in the State of Pará. Interestingly, all were from a forested area in that State, in the municipalities of Abaetetuba and Mojú where, in the following 2 years, other relevant information was obtained regarding the ecology and epidemiology of the disease. In 1937, Cunha and Chagas gave the name *Leishmania chagasi* to the causative agent of AVL, which was recently referred to as *Leishmania* (*Leishmania*) *infantum chagasi* Lainson and

Rangel 2005, and in 1938, Chagas et al. recorded eight more cases of AVL in the municipality of Abaetetuba and the presence of infected dogs. They found that the most common man-biting insect in and around the houses of infected individuals was the phlebotomine sandfly *Lutzomyia longipalpis* and suggested that it was the most likely parasite vector.

Unfortunately, following the premature death of Evandro Chagas in 1940, further observations regarding AVL in Amazonian Brazil were limited to a few clinical and epidemiological features regarding isolated cases in Pará State (Alencar 1962; Costa 1966). During some 46 years following Penna's first report of the disease in this part of Brazil, a mere 32 cases were recorded, and this resulted in Amazonian AVL being regarded as an occasional and sporadic disease. A progressive change in the epidemiology, however, was clearly taking place. Thus, in only 2 years (1984–1985), a total of 135 cases were recorded on the outskirts of the town of Santarém, west of Pará State (Lainson et al. 1984), and SESPA (2004) recorded a mean of approximately 235 cases per year in this State in a recent 5 years period.

The reason for this continuing increase in AVL during recent years is not completely understood. The principal factor involved, however, is increasing deforestation, which results in the invasion of the peridomestic habitat of developing human communities by the sandfly vector *L. longipalpis*. Infestation of animal housing may be intense, and in the presence of a large population of dogs, which are highly susceptible to infection with *L.* (*L.*) *infantum chagasi*, these animals became a major source of human infection (Lainson 1988; Lainson and Rangel 2005). Migration of nonimmunized individuals from other regions, improved diagnostic methods, and the increasing awareness of clinicians regarding the importance of including AVL in the differential diagnosis of febrile patients will continue to contribute the AVL increase (Lainson 1989).

Although the occurrence of AVL has increased drastically in recent years, there has been little field work in this region indicating the prevalence and/or incidence in terms of the symptomatic (acute AVL and subclinical forms) and/or asymptomatic infections. Such a study is, without doubt, of major importance for understanding more fully the dynamics involved in the transmission of *L.* (*L.*) *infantum chagasi* to man. In fact, the only available information regarding the interaction of the human immune response to infection with this parasite has come from the study of patients with AVL, an immune-suppressed condition which represents only the top of the iceberg in this interaction (Lainson and Shaw 2005; Silveira et al. 1997).

During the period 2000–2004, the mean annual incidence of AVL in the municipality of Barcarena, Pará State, north Brazil was 0.36:1,000 inhabitants (SESPA 2004): 67% were male patients, as well as 56% were under the age of 15 years old. In a locality known as Santana do Cafezal village, an annual average of 2.8 cases were recorded, indicating this area as a very suitable place in which to conduct a more elaborate study. This communication will comment the results of a longitudinal, prospective study of 946 individuals in Santana do Cafezal village over a period of 2 years (October/2003 to December/2005), using both the leishmanin skin test (LST) and the indirect fluorescent antibody test (IFAT) for trying to better understand the transmission dynamics of infection.

Materials and methods

Study area

This study was carried out in the Santana do Cafezal village, which is situated on the banks of the river Cafezal, only 7 km from the administrative center of Barcarena municipality (01° 30′ S: 48°37′ W), which is considered to be within the metropolitan region of Belém, Pará State, north of Brazil (Fig. 1). The climate is typically equatorial, with an average

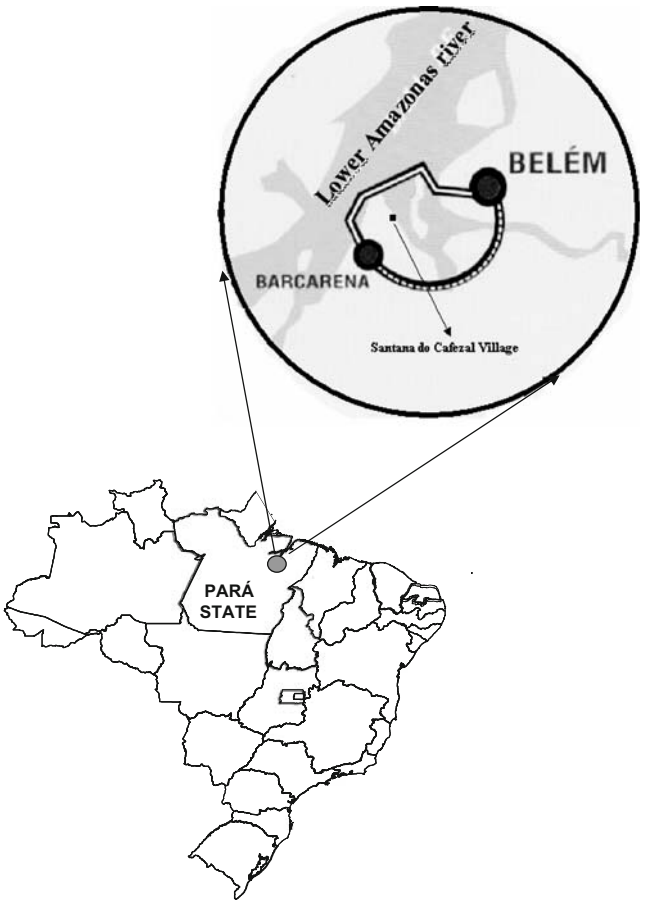

Fig. 1 Geographic localization of Santana do Cafezal village, Barcarena municipality, Pará State, in Amazonian Brazil

temperature of 27°C and high humidity. The annual rainfall is in the region of 2.500 mm or more, with the period from January to June forming the principal rainy season. Following extensive destruction of the primary forest, the area now consists mainly of plantations, with occasional patches of developing secondary forest. Approximately 70% of the inhabitants occupy wooden houses in non-flood land, which is surrounded by secondary forest, while the rest live in the várzea, an area of low vegetation which is flooded twice daily by waters of the river Cafezal.

Study design and population

The population enrolled in this study consisted of a cohort of 946 individuals (almost 90% of total population), being 568 males and 378 females aged between 1 (minimum) and 89 (maximum) years old, with a median age of 20 years old, suggesting to be a relatively younger population. When the study began, the number of inhabitants in the area was estimated to be 1,064 (Instituto Brasileiro de Geografia e Estatística 2004). Moreover, once the present study was performed with the objective of obtaining a clear idea regarding the transmission dynamics of infection, it was necessary to follow the design and the planning of a prospective analysis on the prevalence and incidence of infection during a follow-up period over 2 years. Thus, the indirect fluorescent antibody test and the leishmanin skin test were chronologically used at the same time point of the prevalence and the incidence surveys, which allowed to measure these infection rates by each one of these tests, as well as these real infection rates using the combined results of both tests. In this way, the IFAT and LST were used for all individuals previously selected for the prevalence, and for the following three incidences, at 6, 12, and 24 months, these tests were only performed in those individuals that had been negative for both in the prevalence and in the prior incidence survey. Because of different reasons, however, such as holidays or travel, for example, there was a loss of almost 10% (94 individuals) of original sample during the 2 years of the follow-up period. In addition, the total population was also divided into three age groups, 1–10, 11–20, and ≥21 years, which consisted of 260, 218, and 468 individuals, respectively, with the subject of analyzing the age distribution of infection.

Clinical evaluation of infected individuals

It was also regarded that all individuals presenting any immune reaction either by LST and/or by IFAT would be clinically examined (a complete physical examination) in order to identify any signs and/or symptoms that could be recognized as the classical feature of AVL, as well as that of the subclinical oligosymptomatic infection; only those cases presenting typical feature of AVL received conventional antimony therapy as recommended by the Brazilian program of AVL control (Brasil 2003). The subclinical oligosymptomatic cases were, in principle, only followed up to confirm their spontaneously clinical resolution, as it was observed by a prospective study carried out in the neighboring State of Maranhão, in the northeastern region of Brazil (Gama et al. 2004).

Criteria for identification of human infection

The definition of a human case of infection with *L.* (*L.*) *infantum chagasi* was based on two immunological parameters: the IFAT and LST. Nevertheless, as the IFAT evidences the humoral response (susceptibility) and the LST the T-cell response (resistance; Awasthi et al. 2004), the definition of human infection with *L.* (*L.*) *infantum chagasi* was assumed to be the presence of reactivity to either one or both tests, aiming to ensure as much as possible the real diagnosis of all infected individuals in the endemic area. Moreover, with the objective of expressing the specificity of IFAT and LST, a scale of semi-quantitative results was used with scores varying from + to ++++, as follows: for the IFAT, serological titers of 80–160 and 320–640 (IgG) received + and ++, and those of 1,280–2,560 and 5,120–10,240 were given +++ and ++++, respectively; for the LST, exacerbated (very strong) reactions (≥16 mm) were regarded as ++++, strong (13–15 mm) as +++, moderate (9–12 mm) as ++, and weak (5–8 mm) as +. Thus, it was assumed that serological reactions with 80 (IgG) titer and intradermal reactions forming papules or indurations of ≥5 mm in diameter were regarded as the positive cut-off for the IFAT and LST, respectively (Lima et al. 2003; Silveira et al. 1991, 1998).

Immunological test procedures

The proceedings for LST were previously described in other studies on the American cutaneous leishmaniasis (Silveira et al. 1991, 1998). The antigen used in Santana do Cafezal, a village situated in an area where cutaneous and visceral leishmaniasis are potentially concomitant, although the ecology of study area is not well promising for the cutaneous disease, should have a high specificity for the visceral disease. Thus, to promote high specificity in LST, cultured promastigotes forms from the stationary phase (RPMI 1640 media) of a regional strain of *L.* (*L.*) *infantum chagasi* (MCAO/BR/2003/M22697/Barcarena, Pará State) were used. They were fixed in a merthiolate solution (1:10,000), with a final concentration of approximately 10×10^6 parasites per milliliter. As control for the *Leishmania* antigen, 0.1 mL of the merthiolate solution (1:10,000) was intradermally used in the opposite forearm

of each individual. In addition, as the Instituto Evandro Chagas (IEC) is an official laboratory linked to the Health Ministry of Brazil, all reagents prepared for human research are previously evaluated by a Quality Control Program before the use for humans.

IFAT was performed as proposed by Lima et al. (2003), who showed that amastigote antigen of *L.* (*L.*) *infantum chagasi* had a higher specificity and sensitivity than those with promastigote antigen of the same species and of *Leishmania* (*Leishmania*) *major*-like (Bio-Manguinhos, Rio de Janeiro, Brazil), as well as with amastigote-antigen of *Leishmania* (*Leishmania*) *amazonensis*. Briefly, amastigote antigens were impregnated in the IFAT slides by printing of small fragments of spleen and liver, in the case of *L. infantum chagasi*, and skin, in the case of *L. amazonensis*, from "hamsters" (*Mesocricetus auratus*) infected with these parasites. It has been shown that the crude amastigote antigen of *L.* (*L.*) *infantum chagasi* has the best specificity and sensitivity for IFAT diagnosis of human *L.* (*L.*) *infantum chagasi* infection. For diagnosis of canine visceral leishmaniasis, this procedure has also been used with higher specificity than IFAT and enzyme-linked immunosorbent assay (ELISA; Bio-Manguinhos, Rio de Janeiro, Brazil; de Jesus et al. 2003).

Data analysis

The data obtained were analyzed by the Bio-Estat 4.0 program (Ayres et al. 2004) and the χ^2 and binomial tests were used for the significance of differences between the LST and IFAT surveys of infection with a confidence interval of 95% (p value<0.05).

Results

All results regarding the prevalence, incidence, and the accumulated prevalence of infection as well as its distribution according to the age and the specificity of LST and IFAT may be seen in Tables 1, 2, 3, and 4. However, presented below is a brief description of these results.

Prevalence of human *L.* (*L.*) *infantum chagasi* infection in the Santana do Cafezal village

The infection prevalence of 11.2% (106 of 946) by LST was higher (p<0.0001) than that of 3.4% (32 of 946) by IFAT. However, among the 106 LST and 32 IFAT reactors, there were 18 individuals reacting to both tests together, giving 17% between those LST and 56.2% those IFAT reactors. Thus, these combined results (88 by LST, 14 by IFAT, and 18 by both) gave a real prevalence of 12.6% (120 of 946) for the community (Table 1).

Table 1 Prevalence, incidence, and accumulated prevalence by LST and IFAT of human *L.* (*L.*) *infantum chagasi* infection in the Santana do Cafezal village, Barcarena municipality, Pará State, Brazil (October/2003–December/2005)

Surveys	Immunological procedure (%/*n*)		
	LST	IFAT	LST/IFAT
Prevalence (n=946)	11.2/106	3.4/32	12.6/120
Incidence 1 (n=798)	4.7/38	0.6/5	5.2/42
Incidence 2 (n=724)	4.7/34	2.7/20	6.3/46
Incidence 3 (n=644)	2.9/19	0.8/5	3.6/23
Incidence 1st year (n=798)	9.0/72	3.1/25	11.5/88
Incidence 2nd year (n=644)	2.9/19	0.8/5	3.6/23
Accumulated prevalence (n=946)	197 (20.8)	62 (6.5)	231 (24.4)

LST leishmanin skin test, *IFAT* indirect fluorescent antibody test

Frequency according to sex, age, and LST and IFAT specificity of the human *L.* (*L.*) *infantum chagasi* infection prevalence in the Santana do Cafezal village

The distribution of 120 cases of infection showed no difference (p>0.05) between men (55.8%) and women (44.2%). The age distribution also showed no difference (p>0.05) between the younger age groups, with 19.2% of cases aged 1–10 and 25.8% aged 11–20 years old. However, when these rates were compared with that of the older age group, both were smaller (p<0.05); more than half (55%) of infected cases were ≥21 years old (Table 2).

Regarding the LST and IFAT specificity, among the 106 LST reactors, 41.5% showed exacerbated reactivity, 14.1% strong, 19.8% moderate, and 24.6% weak. Thus, over half of the cases (55.6%) had marked (+++/++++) immunological resistance to infection (Table 3). Among the 32 IFAT reactors, 21.8% showed low reactivity, 53.1% moderate, 18.8% strong, and 6.3% high reactivity (Table 4). Thus, 25.1% of cases had significant immunological susceptibility to infection. Of these, four (3.3%) were typical AVL cases (two children and two adults) with a prevalence of 0.42%, whereas another four (3.3%) cases (one adolescent and three adults) exhibited a subclinical oligosymptomatic infection.

Incidence of human *L.* (*L.*) *infantum chagasi* infection in the Santana do Cafezal village

The first incidence by LST, 4.7% (38 of 798), was higher (p<0.05) than that by IFAT, 0.6% (five of 798); only one case reacted to both tests representing 2.6% (one of 38) of LST and 20% (one of five) of IFAT reactors. Thus, a real incidence of 5.2% was found in the first period of 6 months (42 new cases to 798 non-infected individuals). The second

Table 2 Age distribution of human *L.* (*L.*) *infantum chagasi* infection at the prevalence, incidence, and accumulated prevalence surveys in the Santana do Cafezal village, Barcarena municipality, Pará State, Brazil (October/2003-December/2005)

Age groups[a]	Epidemiology surveys		
	Prevalence (%)	Incidence (%)	Accumulated prevalence (%)
1–10	19.2	29.7	25.6
11–20	25.8	25.3	26.4
≥21	55	45	48

[a] years old

incidence by LST, 4.7% (34 of 724), was higher ($p<0.05$) again than that by IFAT, 2.7% (20 of 724); eight cases reacted to both tests consisting 23.5% (eight of 34) by LST and 40% (eight of 20) by IFAT. This association revealed a real incidence of 6.3% in the second period of 6 months (46 new cases to 724 non-infected individuals). The third incidence by LST, 2.9% (19 of 644), was also higher ($p<0.05$) than that by IFAT, 0.8% (five of 644); only one case reacted by both tests representing 1.6% (one of 19) by LST and 20% (one of five) by IFAT; this combination given a real incidence of 3.6% following a period of 12 months in the second year of study (23 new cases to 644 non-infected individuals; Table 1).

These three incidence surveys at 6 (5.2%), 12 (6.3%), and 24 (3.6%) months revealed 111 new cases of infection, giving a real incidence of 11.5% for the first year, which was higher ($p<0.0001$) than that of 3.6% found at the end of the second year study.

Frequency according to sex, age, and LST and IFAT specificity of the human *L.* (*L.*) *infantum chagasi* infection incidence in the Santana do Cafezal village

According to sex, the distribution of 111 new cases of infection showed no difference ($p>0.05$) between men (47.7%) and women (52.3%). Moreover, the distribution according to age also revealed no difference ($p>0.05$) between the younger age groups (1–10 × 11–20 years old), 29.7% and 25.3%, respectively, although both rates were lower ($p<0.05$) than that (45%) of the older age group (≥21 years old). However, in contrast to those rates found at the prevalence, the incidence of the younger age groups together were more than half (55%) of all cases recorded in the three incidence surveys, indicating that, among the new cases, most occurred in the two first decades of life, with almost 30% of cases in the first one (Table 2).

Table 3 Distribution of leishmanin skin test specificity of human *L.* (*L.*) *infantum chagasi* infection at the prevalence, incidence, and accumulated prevalence surveys in the Santana do Cafezal village, Barcarena municipality, Pará State, Brazil (October/2003-December/2005)

Immunological procedure LST intervals (mm)	Epidemiology surveys		
	Prevalence (%)	Incidence (%)	Accumulated prevalence (%)
5–8	24.6	27.5	25.9
9–12	19.8	24.2	21.8
13–15	14.1	12.0	13.2
≥16	41.5	36.3	39.1

Table 4 Distribution of indirect fluorescent antibody test specificity of human *L.* (*L.*) *infantum chagasi* infection at the prevalence, incidence, and accumulated prevalence surveys in the Santana do Cafezal village, Barcarena municipality, Pará State, Brazil (October/2003-December/2005).

Immunological procedure IFAT intervals (IgG)	Epidemiology surveys		
	Prevalence (%)	Incidence (%)	Accumulated prevalence (%)
80–160	21.8	40.0	30.6
320–640	53.1	50.0	51.6
1,280–2,560	18.8	6.7	13.0
5,120–10,240	6.3	3.3	4.8

Regarding the LST and IFAT specificity, it was revealed that, among the 91 LST reactors, 36.3% exhibited exacerbated reactivity, 12% strong, 24.2% moderate, and 27.5% weak reactivity, indicating that, among the 111 new cases, there was no difference ($p>0.05$) favoring exacerbated and strong LST reactors over moderate and weak LST reactors as occurred at the prevalence (Table 3). In contrast, among the 30 IFAT reactors, 40% showed low reactivity, 50% moderate, 6.7% strong, and 3.3% high reactivity, confirming that only three (10%) of new cases had humoral response compatible with AVL (Table 4). Of these, one case showed clinical feature suggestive of subclinical oligosymptomatic infection (febrile, asthenia, and adenopathy), while another two exhibited typical feature of AVL, who were diagnosed at the second incidence survey. Moreover, following 2 months, the first incidence, a 3-year-old girl who had showed low IFAT reactivity (80 IgG) with negative LST, developed to typical feature of AVL; these three AVL cases comprised an incidence of 0.37:1.000 inhabitants for the first year. During the second year, no recorded AVL cases occurred.

Accumulated prevalence of human *L.* (*L.*) *infantum chagasi* infection in the Santana do Cafezal village

Following these four surveys, there were diagnosed a total of 231 cases of infection (120 at the prevalence and 111 at

the three incidence surveys), giving an accumulated prevalence of 24.4%. Moreover, the accumulated prevalence by LST, 20.8% (197 of 946), was higher ($p<0.05$) than that by IFAT, 6.5% (62 of 946; Table 1).

Frequency according to sex, age, and LST and IFAT specificity of the human *L.* (*L.*) *infantum chagasi* infection accumulated prevalence in the Santana do Cafezal village

According to sex, the distribution of 231 cases of infection showed no difference ($p>0.05$) between men (53.2%) and women (46.8%), as well as in relation to all men, 21.6% (123 of 568), and women, 28.5% (108 of 378), in the sample examined. According to age, no difference ($p>0.05$) was found between the younger age groups (1–10 × 11–20 years old), 25.6% and 26.4%, respectively, although both rates were lower ($p<0.05$) than that (48%) of the older age group (≥21 years old), suggesting that a regular distribution of infection exists in the two first decades of life, with a progressive accumulation from the age 21 years old (Table 2).

Regarding the distribution of infection according to LST and IFAT specificity, it was found that, among the 197 LST reactors, 39.1% had exacerbate reactivity, 13.2% strong, 21.8% moderate, and 25.9% weak reactivity, indicating that more than half (52.3%) of infected individuals had significant immunological resistance against infection (Table 3). However, among the 62 IFAT reactors, 30.6% showed low reactivity, 51.6% moderate, 13% strong, and 4.8% high reactivity (Table 4), confirming that most IFAT reactors (82.2%) had low susceptibility to infection; among 51 cases with low IFAT reactivity, only one (1.9%) developed to active AVL.

Discussion

This represents the first longitudinal study carried out in Brazil which followed up a cohort of individuals with different ages regarding the transmission dynamics of human *L.* (*L.*) *infantum chagasi* infection by using two immunodiagnostic methods, LST and IFAT, simultaneously. As a result, this approach allowed to better estimate the real prevalence and incidence of infection in the endemic area, once the prior works regarding these epidemiology rates have used either a T-cell immune response assay (e.g., LST) or a humoral immune response assay (e.g., IFAT or ELISA, principally), which has raised some difficulties concerning a complete view of human immune response against the infection (Badaró et al. 1986; Caldas et al. 2001, 2002; Nascimento et al. 2005). In addition, these works have also been limited to the epidemiology of infection in children up to 15 years old, which underestimated the significance of older individuals in the epidemiology of infection. In the present study, however, it should be highlighted that the study population consisted of a stable community with a history of more than a century and composed of individuals within at least four generations. The inhabitants have thus had a long history of exposure to the transmission of *L.* (*L.*) *infantum chagasi* infection which, quite likely, might have stimulated the development of some degree of immunoprotection. Moreover, although a population of generally poor people, their diet consists largely of fish and basic agricultural products of fairly good nutritional value. For this reason, although there is some evidence suggesting that susceptibility to visceral leishmaniasis is strongly controlled by genetic mechanism (Peacock et al. 2002; Blackwell et al. 2004; Jamieson et al. 2007), it is equally possible that environmental and nutritional factors may also be influencing immune response.

With regards to a comparison between the two immunodiagnostic methods, it was found that LST infection rates were always higher ($p<0.05$) than those of IFAT, either in the prevalence (11.2% × 3.4%) or in the three incidences, at 6 (4.7% × 0.6%), 12 (4.7% × 2.7%), and 24 months (2.9% × 0.3%) of study, which indicated that among all infected individuals (231), the great majority (85%) had a consistent immunological character of resistance against the infection (Jeronimo et al. 2007). This finding might also explain the low prevalence (0.42%) and incidence (0.37 cases of 1,000 inhabitants) of acute AVL found in this study.

In any comparison, however, between the T-cell and humoral responses of human *L.* (*L.*) *infantum chagasi* infection, it must be regarded that results may be strongly influenced by different methodological proceedings. There are some prospective studies carried out in the northeastern region of Brazil, in Bahia, and Maranhão states, principally, which have used different antigens (soluble extract, crude antigen, and a recombinant protein (rK39) of *L.* (*L.*) *infantum chagasi* and a soluble extract of *L.* (*L.*) *amazonensis*) for determining either the prevalence or the incidence of infection by either LST or enzyme-linked immunosorbent assay. Thus, as the diagnosis of infection is not limited to a single type of immune response, it is evident that both prevalence and incidence have underestimated the true epidemiology of infection in that region (Badaró et al. 1986; Caldas et al. 2001, 2002; Nascimento et al. 2005).

On the other hand, in an old focus of visceral leishmaniasis in Sicily, Italy, where the disease is due to *Leishmania* (*Leishmania*) *infantum infantum*, a promastigote antigen of this parasite was used for LST diagnosis of infection in differently age individuals, which showed a prevalence of 16.6% (Pampiglione et al. 1975). This most closely resembles the result of the present study (11.2% by LST alone) and suggests similar transmission dynamics for

the two subspecies *L.* (*L.*) *infantum chagasi* and *L.* (*L.*) *infantum infantum*.

The diagnosis of infection based on both LST and IFAT assays, simultaneously, allowed to find a prevalence of 12.6%, which showed to be a new finding for Brazil. Regarding distribution of cases by sex, the result of 55.8% men and 44.2% women showed no significant difference. It was also of interest that the youngest age groups of 1–10 and 11–20 years old showed similar infection rates of 19.2% and 25.8%, respectively, being both smaller than that (55%) of the older age group (≥21 years old). The prevalence progressively increased with age, a fact also characteristic of human infection with *L.* (*L.*) *infantum infantum* in Sicily, Italy, which is only indicating that older people have had more time of exposure to infection than younger people (Pampiglione et al. 1975).

The specificity of the two tests showed that, among 106 LST reactors at the prevalence, 56.6% had an important degree of hypersensitivity, suggesting that a considerable number of individuals might have received repeated bites of infected sand flies over a long period. This should be considered in any future vaccination control program. On the other hand, it was noted that, among 32 IFAT reactors, only 25% had shown a susceptibility profile to *L.* (*L.*) *infantum chagasi* infection through their high IFAT titers ranging from 1,280 to 10,240 (IgG). Thus, the great majority (75%) had shown low IFAT reactivity from 80 to 640 (IgG), indicating that only few cases with humoral response had some degree of predisposition for developing AVL, which was confirmed in only 12.5% (four cases) of IFAT reactors.

With regards to the incidence, it should be emphasized that the differences found in the three surveys at 6 (5.2%), 12 (6.3%), and 24 (3.6%) months were not of any statistical significance ($p>0.05$), which suggested that the infection transmission during the 24 months may have stabilized and was without oscillation. Nevertheless, if the incidence had been evaluated in two periods of 12 months, the first would be 11.5% and the second 3.6%, thus showing that in the first year there was a higher rate of transmission ($p<0.05$) than that occurred in the second year. This may, in part, have an explanation in the results of control measures involving the elimination of IFAT-positive dogs in the study area. This not only decreased the canine infection rates from 43.8% in the first year to 29% in the second (Pereira et al. 2006), as well as may have also decreased human infection rates from 11.5% in the first year to 3.6% in the second. Thus, although it is not the subject to discuss the merits of culling seropositive dogs, advocated by the Brazilian Ministry of Health (Brasil 2003), these figures seem to support this type of control measure.

The distribution by sex among the 111 new cases of infection was 47.7% men and 52.3% women, with no significant difference ($p>0.05$). When these cases were analyzed by age group, it was noted that the youngest age groups of 1–10 and 11–20 years old had shown no significant difference ($p>0.05$) in their infection rates of 29.7% and 25.3%, respectively, but that both rates were lower than that 45% of the older age group of ≥21 years old. When combined, however, the infection rates of the two young groups totaled 55% of the incidence, indicating that, contrary to the prevalence profile, these cases were more frequent in the young groups and occurred principally in the children of 1–10 years (almost 30%). This finding correlates well with previous observations in the Brazilian Amazon region which have shown that transmission of *L.* (*L.*) *infantum chagasi* to man is principally intra-domiciliary or peridomestic, where children are particularly vulnerable (Lainson and Rangel 2003, 2005; Lainson and Shaw 2005; Silveira et al. 1997).

With regards to the LST in determining the incidence, there was no significant difference between 48.3% with exacerbated or strong positive reactions and 51.7% with moderate or weak reactions. This contrasted with the predominance of very strong reactions (56.6%) found in the prevalence survey, which suggests that the LST reactivity might be influenced by the exposure time of persons to the successive inoculation of the parasite by repeated bites of infected sandflies and indicates that, in any vaccination program, it is advisable to give repeated doses at regular intervals in order to maintain a good level of immunoprotection.

Among the 30 IFAT reactors, 90% presented moderate to weak positive results (80–640 IgG) and a low susceptibility to infection, although 2 months following the first incidence survey, one of these cases with a negative LST and a positive IFAT (80 IgG) reactions had evolved to typical AVL. Among the remaining 10% with high IFAT reactions (≥1,280 IgG), two cases were typical acute AVL, and another had a subclinical oligosymptomatic infection; all seven AVL cases diagnosed in this study (four at the prevalence and three during the incidence surveys) have received antimony therapy in accordance with the Brazilian AVL control program (Brasil 2003), while the subclinical oligosymptomatic cases have only been clinically followed up for confirming their self-healing. These findings have also confirmed that IFAT using *L.* (*L.*) *infantum chagasi* amastigote antigen is very efficient for determining human susceptible infection with this parasite (Lima et al. 2003).

In spite of a loss of 94 individuals from the original sample (946 individuals) of this study, it was possible to show that the accumulated prevalence of 20.8% by LST was higher than that of 6.5% by IFAT, indicating a LST/IFAT ratio of 3.2:1. In other words, there were three times more resistant individuals than susceptible in the endemic area. Moreover, when all LST and IFAT diagnosed cases

were considered together, there was found an accumulated prevalence of 24.4% for the 2 years study.

In conclusion, the above commented results have raised speculations regarding the possibility that either the control measures or certain environmental or ecological factors may have influenced the incidence of human *L.* (*L.*) *infantum chagasi* infection transmission in the endemic area of this study, leading this infection rate from 11.5% in the first year to 3.6% in the second year, although it has been recorded that most (55%) cases have occurred in the two first decades of life, principally in first one (almost 30%), confirming prior findings on the transmission of this parasite in the Amazon region of Brazil.

Acknowledgments We are grateful for the technical assistance of the following people: Raimundo Nonato Pires, João Alves Brandão, Zuila Corrêa, Edna Leão, Domingas Everdosa, Roseli de Jesus, Raimundo Negrão, Antonio Júlio Monteiro, Raimundo Machado, João Batista Palheta, Antonio Martins, José Aprígio Lima, Iorlando Barata, Maria Suely Pinheiro, Fábio Medeiros, and Maria das Graças Soares Silva. This research was supported by the Instituto Evandro Chagas (Secretaria de Vigilância em Saúde, Ministério da Saúde, Brazil); Instituto de Medicina Tropical (Universidade Federal do Pará, Brazil); Wellcome Trust (London); Laboratório de Investigação Médica (LIM)-50 (Hospital de Clínicas (HC)-Faculdade de Medicina (FM)-Universidade de São Paulo (USP), Brazil); and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP: 06/56319-1, Brazil). This study was approved by the Ethics Committee in human research of the Instituto Evandro Chagas, from the Surveillance Secretary of Health, Ministry of Health, Brazil, with the protocol number CEP (Comitê de Ética em Pesquisa)/IEC 16/2003 and the CAPPesq (Comitê Avaliador de Programas em Pesquisa)/FMUSP (Faculdade de Medicina da Universidade de São Paulo) 0255/07.

Conflicts of interest statement The authors have no conflicts of interest concerning the work reported in this paper.

References

Alencar JE (1962) Kala-zar in Brazil. Sci Rep Inst Sup Sanitá 2:116–123

Awasthi A, Mathur RK, Saha B (2004) Immune response to *Leishmania* infection. Indian J Med Res 119:238–258

Ayres M, Ayres Jr M, Ayres D, Santos AS (2004) Bioestat 4.0: Aplicações estatísticas nas áreas das Ciências Biológicas e Médicas. Sociedade Civil Mamirauá-Brasília CNPq, Belém, Pará, Brasil

Badaró R, Jones TC, Lorenço R, Cerf BJ, Sampaio D, Carvalho EM, Rocha H, Teixeira R, Johnson WD Jr (1986) A prospective study of visceral leishmaniasis in an endemic area of Brazil. J Infect Dis 154:639–649

Blackwell JM, Mohamed HS, Ibrahim ME (2004) Genetics and visceral leishmaniasis in the Sudan: seeking a link. Trend Parasitol 6:268–274

Brasil (2003) Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Manual de Vigilância e Controle da Leishmaniose Visceral. Brasília-DF, Ministério da Saúde pp 1–120

Caldas AJM, Silva DRC, Pereira CCR, Nunes PMS, Silva BP, Silva AAM, Barral A, Costa JM (2001) Infecção por *Leishmania (Leishmania) chagasi* em crianças de uma área endêmica de leishmaniose visceral americana na ilha de São Luis-MA, Brasil. Rev Soc Bras Med Trop 34:445–451

Caldas AJM, Costa JML, Silva AAM, Vinhas V, Barral A (2002) Risk factors associated with infection by *Leishmania chagasi* in north-east Brazil. Trans Roy Soc Trop Med Hyg 96:21–28

Costa O (1966) Calazar no município de Cachoeira do Arari, estado do Pará. Rer Serv Esp Saúde Pública, Rio de Janeiro 12:91–98

Chagas E, Cunha AM, Ferreira LC, Deane L, Deane G, Guimarães FN, Paumgartten MJ, Sá B (1938) Leishmaniose visceral americana (Relatório dos trabalhos realizados pela Comissão Encarregada do Estudo da Leishmaniose Visceral Americana em 1937). Mem Inst Oswaldo Cruz 33:89–229

Cunha AM, Chagas E (1937) Nova espécie de protozoário do gênero *Leishmania* patogênico para o homem. *Leishmania chagasi* n.sp. Nota prévia. Hospital (Rio de Janeiro) 11:3–9

de Jesus RCS, Corrêa ZC, Everdosa DR, Martins AP, Eliseu LS, Campos MC, Jenning YAA, Ishikawa EAI, de Souza AAA, Silveira FT (2003) Comparação das técnicas de RIFI (ag. IEC x ag. Bio-Manguinhos) e ELISA no sorodiagnóstico da leishmaniose visceral canina (LVC), Estado do Pará, Brasil. Rev Soc Bras Med Trop 36:323

Gama MEA, Costa JML, Gomes CMC, Corbett CEP (2004) Subclinical form of the American visceral leishmaniasis. Mem Inst Oswaldo Cruz 99:889–893

Instituto Brasileiro de Geografia e Estatística (2004) Contagem nacional de populações. Superintendência de estudos geográficos e sócio-econômicos, Rio de Janeiro, RJ, Brasil

Jamieson SE, Miller EM, Peacock CS, Fakiola M, Wilson ME, Bales-Holst A, Shaw MA, Silveira F, Shaw JJ, Jerônimo SM, Blackwell JM (2007) Geome-wide scan for visceral leishmaniasis susceptibility genes in Brazil. Genes Immun 8:84–90

Jeronimo SMB, Holst AK, Jamieson SE, Francis R, Bezerra FL, Ettinger NA, Nascimento ET, Monteiro GR, Lacerda HG, Miller EN, Cordell HJ, Duggal P, Beaty TH, Blackwell JM, Wilson ME (2007) Genes at human chromosome 5q31.1 regulate delayed-type hypersensitivity responses associated with *Leishmania chagasi* infection. Genes Immun 8:539–551

Lainson R (1988) Ecological interactions in the transmission of the leishmaniases. Philos Trans Roy Soc Lond B Biol Sci 321:389–404

Lainson R (1989) Demographic changes and their influence on the epidemiology of the American leishmaniasis. In: Service M (ed) Demographic and vector borne disease. CRC Press, Boca Raton, pp 85–106

Lainson R, Rangel EF (2003) Ecologia das leishmanioses: *Lutzomyia longipalpis* e a eco-epidemiologia da leishmaniose visceral americana (LVA) no Brasil. In: Rangel EF, Lainson R (eds) *Flebotomíneos no Brasil*. Fiocruz, Rio de Janeiro, pp 311–336

Lainson R, Rangel EF (2005) *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil—a review. Mem Inst Oswaldo Cruz 100:811–27

Lainson R, Shaw JJ (2005) Leishmaniasis in the New World. In: Collier L, Balows A, Sussman M (eds) Topley & Wilson's microbiology and microbial infections, 10th ed., vol 5, Parasitology. Arnold, London, pp 313–349

Lainson R, Shaw JJ, Ryan L, Ribeiro RSM, Silveira FT (1984) Presente situação da leishmaniose visceral na Amazônia, com especial referência a um novo surto da doença ocorrido em Santarém, Estado do Pará, Brasil. Bol Epidemiol, Fundação SESP, Rio de Janeiro 1:1–8

Lima LVR, de Souza AAA, Jennings YL, Corrêa Z, de Jesus R, Everdosa D, Ayres M, Silveira FT (2003) Comparison of the reactivity between antigens of *Leishmania (L.) chagasi*, *L. (L.)*

amazonensis e *Leishmania sp*. (Bio-Manguinhos) in the serodiagnosis of visceral leishmaniasis by the indirect fluorescent antibody test (IFAT). Rev Inst Med Trop São Paulo 45(Supl 13):147

Nascimento MDSB, Souza EC, da Silva LM, da Cunha Leal P, de Lima Cantanhede K, de Barros Bezerra GF, de Castro Viana GM (2005) Prevalência de infecção por *Leishmania chagasi* utilizando os métodos de ELISA (rK39 e CRUDE) e intradermorreação de Montenegro em área endêmica do Maranhão, Brasil. Cad Saúde Pública, Rio de Janeiro 21:1801–1807

Pampiglione S, Manson-Bahr PEC, La Placa M, Borgatti MA, Musumeci S (1975) Studies in Mediterranean leishmaniasis: 3. The leishmanin in skin test kala-azar. Trans Roy Soc Trop Med Hyg 69:60–68

Peacock CS, Sanjeevi CB, Shaw MA, Collins A, Campbell RD, March R, Silveira F, Costa J, Costa CH, Nascimento MD, Siddiqui R, Shaw JJ, Blackwell JM (2002) Genetic analysis of multicase families of visceral leishmaniasis in northeastern Brazil: no major role for class II or class III regions of HLA. Genes Immun 3:350–358

Penna HA (1934) Leishmaniose visceral no Brasil. Bras Med 48:949–950

Pereira EA, Chagas EJ, Jesus R, Carneiro LA, Jennings YL, Lima LV, Campos MB, Banhos AP, Brandão JA, Monteiro AJO, Negrão R, Machado R, Ishikawa EA, Souza AA, Silveira FT (2006) A eliminação regular de cães soropositivos para leishmaniose visceral reduz a transmissão da enzootia canina em área endêmica. Rer Soc Bras Med Trop 39(Supl 1):115

Secretaria Executiva de Saúde do Estado do Pará (SESPA) (2004) Leishmanioses visceral e tegumentar americana. Boletim Epidemiológico, Departamento de Controle de Endemias, Belém, Pará, Brasil, pp 6–8

Silveira FT, Lainson R, Shaw JJ, de Souza AA, Ishikawa EAI, Braga RR (1991) Cutaneous leishmaniasis due to *Leishmania (Leishmania) amazonensis* in Amazonian Brazil, and the significance of a negative Montenegro skin-test in human infections. Trans R Soc Trop Med Hyg 85:735–738

Silveira FT, Shaw JJ, Bichara CNC, Costa JML (1997) Leishmaniose visceral americana. In: Leão RNG (ed), Doenças Infecciosas e Parasitárias: Enfoque Amazônico. Belém, PA, CEJUP: 631–644.

Silveira FT, Blackwell JM, Ishikawa EA, Braga RR, Shaw JJ, Quinnell RJ, Soong L, Kima P, McMahon-Pratt D, Black GF, Shaw M-A (1998) T cell responses to crude and defined leishmanial antigens in patients from the lower Amazon region of Brazil infected with different species of *Leishmania* of the subgenera *Leishmania* and *Viannia*. Parasite Immunol 20:19–26

ANEXO-15

Further evidences on a new diagnostic approach for monitoring human *Leishmania (Leishmania) infantum chagasi* infection in Amazonian Brazil

Silveira, F.T.; Lainson, R.; de Souza, A.A.A.; Campos, M.B.; Carneiro, L.A.; Lima, L.V.R.; Ramos, P.K.S.; Gomes, C.M.C.; **Laurenti, M.D.;** Corbett, C.E.P.

Parasitology Research 2010; 106: 377-386

Parasitol Res (2010) 106:377–386
DOI 10.1007/s00436-009-1672-x

ORIGINAL PAPER

Further evidences on a new diagnostic approach for monitoring human *Leishmania (L.) infantum chagasi* infection in Amazonian Brazil

Fernando Tobias Silveira · Ralph Lainson · Adelson Alcimar Almeida De Souza · Marliane Batista Campos · Liliane Almeida Carneiro · Luciana Vieira Rego Lima · Patrícia Karla Santos Ramos · Cláudia Maria de Castro Gomes · Marcia Dalastra Laurenti · Carlos Eduardo Pereira Corbett

Received: 6 August 2009 / Accepted: 21 October 2009 / Published online: 28 November 2009

Abstract This was a prospective study carried out during a period over 2 years (May/2006–September/2008) with a cohort of 1,099 individuals of both genders, aged 1 year old and older, from an endemic area of American visceral leishmaniasis (AVL) in Pará state, Brazil. The object was to analyze the prevalence and incidence of human *Leishmania (L.) infantum chagasi* infection as well as the dynamics evolution of its clinical-immunological profiles prior identified: (1) asymptomatic infection (AI); (2) symptomatic infection (SI=AVL); (3) sub-clinical oligosymptomatic infection (SOI); (4) sub-clinical resistant infection (SRI) and; (5) indeterminate initial infection (III). The infection diagnosis was performed by using both the indirect fluorescent antibody test and leishmanin skin test with amastigotes and promastigotes antigens of *L. (L.) i. chagasi*, respectively. A total of 187 cases of infection were recorded in the prevalence (17%), 117 in the final incidence (6.9%), and 304 in the accumulated prevalence (26.7%), which provided the following distribution into the clinical-immunological profiles: AI, 51.6%; III, 22.4%; SRI, 20.1%; SOI, 4.3%; and SI (=AVL), 1.6%. The major finding regarding the dynamics evolution of infection was concerned to III profile, from which the cases of infection evolved to either the resistant profiles, SRI (21 cases, 30.8%) and AI (30 cases, 44.1%), or the susceptible SI (=AVL; 1 case, 1.5%); the latter 16 cases remained as III till the end of the study. These results provided the conclusion that this diagnostic approach may be useful for monitoring human *L. (L.) i. chagasi* infection in

F. T. Silveira (✉) · R. Lainson · A. A. A. De Souza · M. B. Campos · L. A. Carneiro · L. V. R. Lima · P. K. S. Ramos
Parasitology Department, Evandro Chagas Institute (Surveillance Secretary of Health, Ministry of Health), Belém, Pará, Brazil
e-mail: fernandotobias@iec.pa.gov.br

R. Lainson
e-mail: ralphlainson@iec.pa.gov.br

A. A. A. De Souza
e-mail: adelsonsouza@iec.pa.gov.br

M. B. Campos
e-mail: marlianecampos@iec.pa.gov.br

L. A. Carneiro
e-mail: lilianecarneiro@iec.pa.gov.br

L. V. R. Lima
e-mail: lucianavrlima@iec.pa.gov.br

P. K. S. Ramos
e-mail: patriciaramos@iec.pa.gov.br

F. T. Silveira
Tropical Medicine Institute, Federal University of Pará, Belém, Pará, Brazil

C. M. de Castro Gomes · M. D. Laurenti · C. E. P. Corbett
Pathology Department, Medical School of São Paulo University, São Paulo, São Paulo, Brazil

C. M. de Castro Gomes
e-mail: gomescla@usp.br

M. D. Laurenti
e-mail: mdlaurent@usp.br

C. E. P. Corbett
e-mail: ccorbett@usp.br

endemic area and preventing the high morbidity of severe AVL cases.

Introduction

Recently, the interaction between *Leishmania (L.) infantum chagasi* (Lainson and Rangel 2005) or *Leishmania chagasi* (Cunha and Chagas 1937), the etiological agent of American visceral leishmaniasis (AVL), and the human immune response has received reasonable attention in viewing of its importance regarding the clinical and immunological spectrum that may result from this interaction. In this way, a better understanding on the repertory of immune responses which can give rise to this clinical-immunological spectrum of human *L. (L.) i. chagasi* infection is also of interest. Taking these considerations into account, it has been suggested that the clinical spectrum may range from an asymptomatic stage of infection in resistant individuals, which have an efficient T-cell immune response (delayed-type hypersensitivity, lymphocyte proliferation, and high interferon-gamma response) towards to a symptomatic stage in susceptible ones, in which a specific immune-suppression of these T-cell responses may lead to classic AVL (Holaday et al. 1993; Vinhas et al. 1994). Nevertheless, between these two polar stages of infection, there are some individuals showing an intermediary immune-genetic profile which has been considered as a sub-clinical oligosymptomatic stage, in which the clinical and immunological features have not yet clearly defined (Pearson and Souza 1996; Costa et al. 1999).

In Brazil, although some studies have been carried out with the aim of a better understanding on the clinical-immunological spectrum of human *L. (L.) i. chagasi* infection, these investigations have, unfortunately, been based either on the specific antibody response, or on the delayed-type hypersensitivity response of infected individuals, which has raised some difficulties concerning a complete view of the immune response against infection (Badaró et al. 1986a; Gama et al. 2004; Jeronimo et al. 2000). In other words, these studies have generally used either a serological, such as the enzyme-linked immunosorbent assay, or a T-cell method, like the leishmanin skin test (LST), for diagnosing active *L. (L.) i. chagasi* infection, which has underestimated the possibility that some infected individuals living in endemic areas can express both immune responses, the humoral and T-cell responses, against infection.

Recently, however, we have shown the capacity of both indirect fluorescent antibody test (IFAT) and LST for diagnosing human *L. (L.) i. chagasi* infection in AVL-endemic area (Silveira et al. 2009a). This diagnostic approach was based in a high specificity of *L. (L.) i. chagasi* antigens used for IFAT (amastigotes) and LST (promastigotes), which also provided the identification of the largest clinical-immunological spectrum of human *L. (L.) i. chagasi* infection in Amazonian Brazil, consisted in the following profiles of infection: (1) asymptomatic infection (AI), (2) symptomatic infection (SI=AVL), (3) sub-clinical oligosymptomatic infection (SOI), (4) sub-clinical resistant infection (SRI) and, (5) indeterminate initial infection (III) (Crescente et al. 2009).

Regarding the above findings, we considered of interest to present further evidences on this diagnostic approach for monitoring human *L. (L.) i. chagasi* infection, which resulted from a prospective study over a 2-year period realized in AVL-endemic area in Cametá municipality, Pará state, Brazil; it reinforced the efficacy of this diagnostic approach, mainly for diagnosing the clinical-immunological profile III, which consists in early asymptomatic cases of infection but with potential for developing active AVL. The relevance in diagnosing early cases of infection for preventing the high morbidity of severe AVL cases is discussed here.

Materials and methods

Study area and population

This study was carried out in four small villages (Ajó, Vacaria, Vacajó, and Enseada) in Cametá municipality (01°56′S:54°45′W), northeastern Pará state, Brazil, which is situated on the border of the river Tocantins (Fig. 1). The climate is typically equatorial, with an average temperature of 28°C and high humidity. The annual rainfall in the region is of 2.500 mm or more, with the period from January to June forming the principal rainy season. Following extensive destruction of the primary forest, the area now consists mainly of plantations, with occasional patches of developing secondary forest. Approximately 70% of the inhabitants occupy wooden houses in the non-flood land, which is surrounded by a secondary forest, while the rest lives in the "várzea", an area of low vegetation which is flooded twice daily by waters of the river Tocantins. Thus, the climate and environment conditions of this area are very similar to those found in Barcarena municipality, Pará state, at about 150 km distant from this study area in Cametá municipality, where we have prior studied the transmission dynamics of human *L. (L.) i. chagasi* infection (Silveira et al. 2009a).

The population enrolled in this study consisted in a cohort of 1,099 individuals (92.2% of total population), being 596 males and 503 females aged between one (min.) to 84 (max.) years old, with a median age of 24.4 years old,

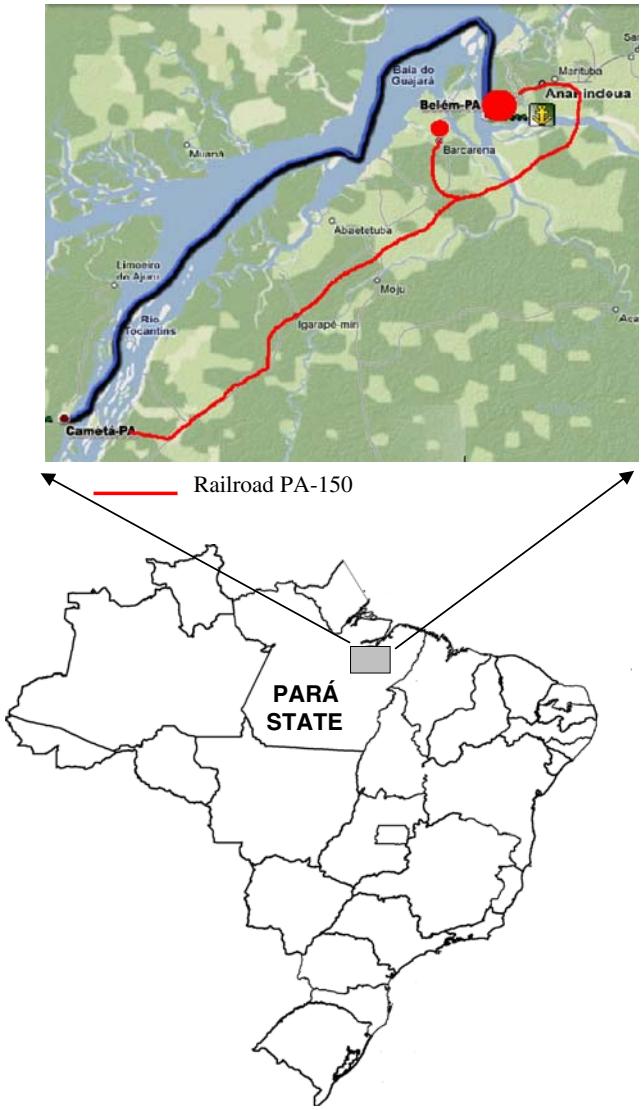

Fig. 1 Geographic localization of Cametá municipality, Pará state, Amazonian Brazil

characterizing to be a relatively younger population. When the study began, the number of inhabitants in the area was estimated to be 1,192 (Instituto Brasileiro de Geografia e Estatística 2004).

Study design

Regarding that the present study had been performed to analyse the prevalence and incidence of human *L. (L.) i. chagasi* infection as well as the dynamics evolution of its clinical-immunological profiles of infection, it was necessary to design a prospective study to follow-up a cohort (1,099 individuals) during a period of over 2 years (May/2006–September/2008). Thus, the IFAT and LST were chronologically used at the same time points in the prevalence and incidence surveys; i.e., for all individuals previously selected for the prevalence and, for the following two incidences, at 12 and 24 months, these tests were only performed on those individuals that were negative either in the prevalence or in prior incidence surveys. In this way, in cases of reactivity by LST alone, which represents a genetic characteristic of immunological resistance to infection (Jeronimo et al. 2007), these individuals were removed from subsequent LST surveys, similar to that proposed in a longitudinal study in Sudan (Zijlstra et al. 1994). Moreover, in cases revealing reactivity for both tests, the individuals were tested only by IFAT. Finally, in cases of reactivity by IFAT alone which, in contrary to LST, represents an immunological status of susceptibility to infection, the individuals remained under investigation by both tests, with the aim of analyzing the evolution of both immune responses. For a number of different reasons, such as holidays or travel, a loss of almost 5% (54 individuals) of original sample occurred over the 2-year follow-up period. In addition, the total population was also divided into three age groups; 1–10, 11–20, and ≥21 years old, consisting of 303, 252, and 544 individuals, respectively, with the aim of analyzing the age distribution of infection.

Clinical evaluation of infected individuals

All individuals presenting any type of immune reaction, either by LST and/or by IFAT, were clinically examined (a physical examination) in order to identify any signs and/or symptoms that could be recognized as classical features of AVL, as well as those symptoms (fever, asthenia, pallor, and slight hepatomegaly or spleenomegaly) prior associated to sub-clinical oligosymptomatic infection (Crescente et al. 2009); only cases with typical features of AVL received conventional antimony therapy, as recommended by the Brazilian AVL control program (Brasil 2003). The sub-clinical oligosymptomatic cases were, in principle, only followed-up during 2- to 3-month period to confirm their spontaneously clinical resolution, which has also been observed in a prospective study carried out in Maranhão state, Brazil (Gama et al. 2004).

Criteria for identification of human infection

Regarding that IFAT evidences, humoral response (susceptibility) and LST T-cell response (resistance; Awasthi et al. 2004), the definition of human *L. (L.) i. chagasi* infection case was assumed to be the reactivity to either one or both immunological tests. However, considering that human "HIV" co-infection could interfere with this diagnostic approach, it is important to state that up to the onset of the

study, no case of human "HIV" infection had been recorded in the study area by the Health Care Secretary of Cametá municipality.

Moreover, considering the importance in revealing the specificity of IFAT and LST, a scale of semi-quantitative results was used with scores varying from + to ++++, as follows: for IFAT, serological titers (IgG) with 80–160 and 320–640 received + and ++ and those with 1,280–2,560 and 5,120–10,240, +++ and ++++, respectively. For LST, exacerbated skin reactions (≥16 mm) were regarded as ++++, strongly positive (13–15 mm) as +++, moderately positive (9–12 mm) as ++, and weakly positive (5–8 mm) as + (Silveira et al. 2009a). Thus, it was assumed that serological reactions with 80 (IgG) titer and skin reactions forming indurations with ≥5 mm in diameter were regarded as positive cut-off for IFAT and LST, respectively (Lima et al. 2003; Silveira et al. 1991, 1998). In addition, combining the clinical status of infected individuals with this semi-quantitative scale of scores for LST and IFAT, it was possible to identify the following clinical-immunological profiles of infection: (1) AI (LST+/++++ and IFAT-), (2) SI=AVL, and (3) SOI with the same immune profile (LST− and IFAT+++/++++), (4) SRI (LST+/++++ and IFAT+/++), and (5) III (LST− and IFAT+/++; Crescente et al. 2009).

Immunological tests procedures

The proceedings for LST and IFAT were the same as those prior described (Crescente et al. 2009; Silveira et al. 2009a).

Data analysis

The data obtained were analyzed by Bio-Estat 4.0 software (Ayres et al. 2004) and the X^2 and binomial tests were used to determine the significance of differences between the clinical-immunological profiles of infection with a confidence interval of 95% (p value <0.05).

Results

Distribution of clinical-immunological profiles of human *L. (L.) i. chagasi* infection regarding the prevalence

The actual prevalence of infection was 17% (187 cases/1,099 individuals), which means 90 cases of infection diagnosed only by LST (AI profile), 54 by IFAT (SI profile, four; SOI profile, nine; and III profile, 41), and 43 by both LST and IFAT (SRI profile). The distribution of clinical-immunological profiles revealed a greater ($p<0.05$) frequency of AI profile (48.1%) over other profiles; SRI (23%), III (22%), SOI (4.8%), and SI=AVL (2.1%; Table 1), and showed that frequencies of SRI and III profiles were also higher ($p<0.05$) than those of SOI and SI, although there were no differences ($p>0.05$) between the frequencies of SRI versus III, and SOI versus SI profiles. These results have also shown that within the prevalence survey, the great majority (93%/174) of infected individuals was asymptomatic (AI, SRI, and III profiles).

Distribution of clinical-immunological profiles of human *L. (L.) i. chagasi* infection regarding the incidences

The first incidence (12 months) of infection was 7.2% (64 new cases/892 non-infected individuals from the prevalence), which represented 28 cases of infection diagnosed only by LST (AI profile), 21 by IFAT (SOI profile, four and III profile, 17), and 15 by both LST and IFAT (SRI profile). The distribution of these profiles revealed again a greater ($p<0.05$) frequency of AI profile (43.7%) over other profiles; III (26.6%), SRI (23.4%), and SOI (6.3%; Table 1).

Table 1 Frequency rates of clinical-immunological profiles of human *L. (L.) i. chagasi*-infection in the prevalence, incidence, accumulated prevalence and final evolution of infection in Cametá municipality, Pará state, Amazonian Brazil

Surveys	Clinical-immunological profiles				
	Number (%)				
	AI	SI	SOI	SRI	III
Prevalence (n=187 cases)	90 (48.1)	4 (2.1)	9 (4.8)	43 (23.0)	41 (22.0)
Incidence (12 months) (n=64 cases)	28 (43.7)	–	4 (6.3)	15 (23.4)	17 (26.6)
Incidence (24 months) (n=53 cases)	39 (73.6)	1 (1.8)	–	3 (5.7)	10 (18.9)
Final incidence (n=117 cases)	67 (57.3)	1 (0.8)	4 (3.4)	18 (15.4)	27 (23.1)
Accumulated prevalence (n=304 cases)	157 (51.6)	5 (1.6)	13 (4.3)	61 (20.1)	68 (22.4)
Final evolution (n=304 cases)	238 (78.3)	1 (0.3)	3 (1.0)	46 (15.1)	16 (5.3)

AI asymptomatic infection, *SI* symptomatic infection (AVL), *SOI* sub-clinical oligosymptomatic infection, *SRI* sub-clinical resistant infection, and *III* Indeterminate initial infection

There was no case of SI profile (=AVL) amongst new cases of infection within the first year of study. These findings have also shown that frequencies of III and SRI profiles were higher ($p<0.05$) than that of SOI profile, although there was no difference ($p>0.05$) between the frequencies of III versus SRI profiles ($p>0.05$).

The second incidence (24 months) of infection was 6.6% (53 new cases/763 non-infected individuals from prior incidence); this also represented 39 cases of infection diagnosed only by LST (AI profile), 11 by IFAT (SI profile, one and III profile, ten), and three by both LST and IFAT (SRI profile). The distribution of these profiles showed again a greater ($p<0.05$) frequency of AI profile (73.6%) over other profiles; III (18.9%), SRI (5.7%), and SI (1.8%; Table 1). Thus, there was no case of SOI profile amongst new cases of infection within the second year of study. These findings have also shown that frequency of III profile (18.9%) was higher ($p<0.05$) than those of SRI (5.7%) and SI (1.8%), and finally, that frequency of SRI profile was also higher ($p<0.05$) than that of SI.

In summary, these surveys have recorded a final incidence of 6.9% for both two years period with 117 new cases of infection which were classified in a decreasing order, as follows: AI profile recorded the greatest ($p<0.05$) frequency (57.3%), followed by III (23.1%), SRI (15.4%), SOI (3.4%), and SI=AVL (0.8%; Table 1). Thus, these results have shown again that the great majority (95.7%/112) of infected individuals in the incidence surveys were also asymptomatic (AI, III, and SRI profiles).

Distribution of clinical-immunological profiles of human *L. (L.) i. chagasi* infection regarding the accumulated prevalence

Following these three surveys (prevalence and two incidences), a total of 304 cases of human *L. (L.) i. chagasi* infection were recorded with an accumulated prevalence of 27.6%; AI profile was the most frequent (51.6%), followed by III (22.4%), SRI (20.1%), SOI (4.3%), and SI=AVL (1.6%) profiles (Table 1).

Age distribution of clinical-immunological profiles of human *L. (L.) i. chagasi* infection within the prevalence and incidence surveys

Regarding the prevalence (187 cases), it was noted that within the 1–10-year age group (23%/43 cases) there was no difference ($p>0.05$) amongst the frequencies of AI (30.2%/13), SRI (30.2%/13), and III (25.6%/11) profiles, which were higher ($p<0.05$) than those of SI (9.3%/4) and SOI (4.7%/2) profiles. Besides this, it was also observed that within the 11–20-year age group (23.5%/44 cases), there was no difference ($p>0.05$) between the frequencies of AI (47.7%/21) and III (34.1%/15) profiles, which were higher ($p<0.05$) than those of SRI (13.6%/6) and SOI (4.6%/2) profiles. At last, within the ≥21-year age group (53.5%/100 cases), it was noted that the frequency of AI profile (56%/56) was higher ($p<0.05$) than those of other profiles; SRI (24%/24), III (15%/15), and SOI (5%/5).

With regards to the incidence (117 cases), it was observed that most cases were recorded within the two smallest age groups; (1) in the 1–10-year age group (43.6%/51 cases) the AI profile presented a higher ($p<0.05$) frequency (58.8%/30 cases) than those of III (27.5%/14 cases), SRI (5.9%/3 cases), SOI (5.9%/3 cases), and SI=AVL (1.9%/1 case) profiles; (2) in the 11–20-year age group (37.6%/44 cases) the AI profile showed again a higher frequency (68.2%/30 cases) than those of SRI (18.2%/8 cases) and III (13.6%/6 cases) profiles.

Dynamics evolution of clinical-immunological profiles of human *L. (L.) i. chagasi* infection

The dynamics evolution of infection was based in the clinical-immunological profiles recorded in all three surveys, but only those from the prevalence and first incidence (12 months) were really follow-up, once those from the second incidence (24 months) could not be follow-up. Thus, regarding III profile (IFAT+/++ and LST−) as the earliest stage of infection, it was observed that amongst 68 (22.4%) cases recorded (41 in the prevalence and 27 in the incidence), 21 (30.9%) evolved to SRI profile, 30 (44.1%) to AI, one (1.5%) to SI (=AVL) and, 16 (23.5%) have conserved their original profile till the end of study. With regards to 61 cases (20.1%) of SRI profile (IFAT+/++ and LST+/++++), 43 in the prevalence and 18 in the incidence, it was noted that 47 (77%) changed to AI profile and, 14 (23%) have maintained their original profile; however, considering that 21 cases from III, eight from SOI, and three from SI (=AVL cases successful treated) profiles have also evolved to SRI profile, this provided a final evolution rate of infection of 15.1% (46 cases) to SRI profile. In relation to SOI profile (IFAT+++/++++ and LST−), which recorded 13 (4.3%) cases of infection (nine in the prevalence and four in the incidence), it was observed that eight (61.5%) cases developed to SRI, two (15.4%) to AI, and three (23.1%) have kept their original profile. Moreover, amongst five (1.6%) AVL (SI profile) cases (IFAT+++/++++ and LST−), four in the prevalence and one in the incidence, the antimony treatment carried out three (60%) cases to SRI profile, one (20%) to AI, and the last one (20%) although has retained its original immune response has also became clinically asymptomatic. Finally, with regards to 157 (51.6%) cases of AI profile (LST+/++++ and IFAT−), 90 in the prevalence and 67 in the incidence, it was noted that even these cases did not change their clinical status once they