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

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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*

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 Leishmania 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 Leishmania 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 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 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 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 Institut 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 aseptically 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 (NH₄Cl 8.29 g, K₂HCO₃ 1.0 g; EDTA 0.0372 g for 11 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% CO₂ 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 × 10⁶ 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 micro-litre of r-phycocerythrin-conjugated rat antimouse Pan-NK cell (DX5) antibody, 4 μl of FITC-conjugated rat antimouse CD3ε (CD3ε chain) antibody, 4 μl of FITC-conjugated rat antimouse CD4 (RM4-5) antibody and 4 μl of FITC-conjugated rat antimouse CD8α (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 10⁶ cells/ml in 20 ml of RPMI-1640 and was labelled with sodium chromate Na₂¹⁵⁵CrO₄ (IPEN-CNEN/SP, São Paulo, SP, Brazil). After incubation at 37 °C in 5% CO₂ atmosphere for 60 min, the cells were washed in RPMI 1640 to remove the unlabelled sodium chromate, and the concentration was adjusted to 5 × 10⁶ cells/ml. Hundred micro-litre 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% CO₂ atmosphere for 18 h in a humid chamber. Then, the plate was centrifuged at 200 × 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 × 10⁶ 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
mice were inoculated subcutaneously with promastigotes of *L. (L.) amazonensis* Csg about 40 days after inoculation, as described by Pinho et al. 2005. The viable parasites were evaluated in 100 μl of RPMI medium supplemented with 10% fetal bovine serum, 2% human urine, 10 mg/ml of gentamicin and 100 IU/ml of penicillin. The culture plate was maintained in a 25°C incubator for 7 days. The pathogenic forms of the parasite were quantified using a Neubauer chamber.

**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 pathogenic forms of the parasite were quantified using a Neubauer chamber.

**NO determination.** The NO production was determined through the concentration of nitrates 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% nafthlenodiamine dithidroclor 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 NO₃⁻ 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 × 10⁵ cells/ml was placed in 96-well plates (Costar), and the culture was kept at 5% CO₂ 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, KMC04021) and 50 μl for IL-12 (Mouse IL-12 + p40 Immunoassay Kit, KMC04121) measurements.

**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 ⁵¹Cr-release microcytotoxicity assay showed that the percentage of lysis of targets (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.

**Results**

**Characterization of A-NK cells**

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**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).
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-$\gamma$ and IL-12 levels

Both at 0 and 24 h, the IFN-$\gamma$ 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-$\gamma$ levels were directly proportional to the concentration of A-NK cells used. The presence of rIL-2 in the cultures further increased IFN-$\gamma$ 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 cell : 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...
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 tumoureal 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-γ 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 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).

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.
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 \textit{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 \textit{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 \textit{in vitro}.

The direct role of A-NK cells in control of the promastigote forms of \textit{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 \textit{Leishmania} before they can enter the macrophages.

Figure 5 Measurement of interferon-γ by ELISA in the supernatant of the BALB/c peritoneal macrophage cultures infected with promastigotes of \textit{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).

Figure 6 Measurement of IL-12 by ELISA in the supernatant of the BALB/c peritoneal macrophage cultures infected with promastigotes of \textit{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).
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 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).

**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*.

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

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

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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. (Leishmania) 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.
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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

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focus of this review on these two Leishmania parasites. Following infection by these species, some naturally resis-
tant 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 fre-
quent 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 ther-
apy.

From the center of the spectrum, some infections not
totally under the control of T-cell immune mechanisms
evolve to one of the two polar forms of disease: either
the T-cell hypersensitivity pole, represented by mucocuta-
neous 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 Amer-
ica. It has now been determined that this deviation of T-cell
immune response during infection to either MCL (hypersen-
sitivity) 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 mis-
understanding 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 dis-
tinguish 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 nasopharyn-
geal 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 dissemi-
nates 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,
IL-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 leish-
maniasis (BDCL), which may be caused by either L. (V.)
braziliensis or L. (L.) amazonensis. However, even this
condition presents certain differences that should be consid-
ered: 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 dissemina-
tion 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.) amazon-
ensis, who present greater T-cell response inhibition than
those infected by L. (V.) braziliensis. Despite this, suffi-
cient 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 hypersen-
sitivity (DTH) reactions between LCL patients infected
by L. (L.) 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 individu-
als 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 dis-
ease 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 lym-
phocyte proliferation assay for all clinical forms of ACL
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-}, 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 cytoplasmic 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).
The most interesting finding was that associated with the group of LCs than that of two other groups: L. (V.) braziliensis reactions, which showed a higher (\(L\)) 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 (ADCLDTH−>BDCLDTH−>LCLDTH−>LCLDTH+) (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 (DHT−) 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 (DHT−) 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-1α, 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 ACL 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 (Favalli et al., 2007).

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

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**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.

**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 *Chiropterus satanas* monkeys, *Choanepus didactylus* sloth, edentate *Bradypus tridactylus*, cetain mice *Nasua nasua*, and sandfly *Lutzomyia whitmani* 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)*
The opinion is that in the body (Shaw et al. 1991). More recently, however, 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-piperazinethanesulfonic 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 distilled 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 10⁵ 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% CO₂ 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.

Stationary phase promastigote forms were washed three times in PBS, and the parasites concentration was adjusted to 2×10⁷ promastigotes/ml. Fifty microliters was injected subcutaneously into the hind footpads of the mice (around 10⁶ 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 1µg/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.

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...
mononuclear cell, and moderate 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
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

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**Fig. 4** Histopathology of the skin lesion produced in C57BL/6 mice by *Leishmania (Viannia) shawi*. a Two weeks pi \((x>200)\), b 4 weeks pi \((x>200)\), c 6 weeks pi \((x>200)\), and d 8 weeks pi \((x>200)\); details are immunohistochemistry reactions showing amastigote forms
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...
understanding of immunopathogenesis of American Tegumentar Leishmaniasis, caused by this parasite species.

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

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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.) linder-begi* 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.
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, 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) 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 1×10⁶ 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 puncture and biopsies of skin and popliteal lymph nodes were collected. Sera were collected and stored at −20 °C to study the humoral immune response. The biopsies were used for histopathological examination and estimation of parasite load by 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 37 °C) to prevent nonspecific binding. Mouse sera (1:100) were added and the plates were incubated for 1 h at 37 °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 10⁶ cells/90 µl of elution buffer [PBS plus 0.5% bovine serum albumin (BSA, Boehringer, Germany)] with 10 µl of anti-mouse CD4 and CD8 microbeads. After 20 min of incubation, the 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 and 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 1×10⁶ CD4⁺ T and dnCD4CD8⁻ cells and from 2×10⁶ 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 MgCl₂), 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 OptiPlex 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 monitoring. 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-β3 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
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 ( \( p_{\text{week } 3 \text{pi}} = 0.0381; \ p_{\text{week } 4 \text{pi}} = 0.0021; \ p_{\text{week } 5 \text{pi}} = 0.0014 \) ), 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 \( \times \) 10^5 promastigotes/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.

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 (\( p_{\text{CD4+}} = 0.0294; \ p_{\text{dnCD4CD8}} = 0.0256 \)), 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 (\( p_{\text{CD4+}} = 0.0056; \ p_{\text{CD8+}} = 0.0401; \ p_{\text{dnCD4CD8}} = 0.0001 \)); 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 populations 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.
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.

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

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**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).

**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.

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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 αβ+ dnCD4CD8 T cells contribute to the control of human cutaneous leishmaniosis caused by *L. braziliensis*, while γδ+ 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

![Fig. 4](image-url)  
**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](image-url)  
**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.
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.

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

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ARTIGO ORIGINAL

REVISÃO SOBRE A PATOGENIA DA LEISHMANIOSE TEGUMENTAR AMERICANA NA AMAZÔNIA, COM ENFASE À DOENÇA CAUSADA POR Leishmania (V.) braziliensis E Leishmania (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

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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 inmune 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 pólio imunológico hiperreativo, representado pela leishmaniose cutâneo-mucosa (LCM), com exacerbação da hiperesensibilidade e perfil da resposta CD4 tipo-Th1. A L. (L.) amazonensis mostra o oposto, dirigindo a infecção para o pólio imunológico hiperreativo, representado pela leishmaniose cutânea atípica difusa (LCAD), com forte inibição da hiperesensibilidade e perfil da resposta CD4 tipo-Th2. Entre as formas central LCL e as formas polares LCM e LCAD a infecção passa por uma fase intermediária, a leishmaniose cutânea disseminada bordeline (LCDB), com inibição parcial da hiperesensibilidade 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 inmune do homem infectado.

A título de esclarecimento, hoje são reconhecidas,

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na referida região, sete espécies de *Leishmania* que podem produzir sintomatologia da LTA, sendo seis do subgênero *Vinia* e uma do subgênero *Leishmania*. 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 longo 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 aguda em forma diffusa (LCAD); as duas formas clínicas de maior gravidade no espectro clínico-imunológico da LTA; a primeira, associada ao polo imunológico hiporeativo, com forte hipersensibilidade celular e, a segunda, ao polo imunológico hiperreativo, com fraça (ou ausente) hipersensibilidade celular. Além disso, as duas espécies podem, ainda, ser agentes causais da leishmaniose cutânea disseminada borderline (LCDB), uma forma intermediária, hiperreativa, 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) lindenbergeri* 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 (Sb5), o antimonialio 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 hospedeiro, fortemente associado à resposta imune celular, e da virulência da espécie de *Leishmania* infectante. Nesse sentido, a patogenia da doença será definida em consequência 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.

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 lírica do complemento. Entretanto, algumas das formas promastigotas inoculadas, promastigotas metacíclicas, são resistentes à ação lírica do complemento; apresentam na membrana plasmática moléculas de glicococonjuga-dos, lipofosfoglicanos e glicoproteínas, as quais, não só favorecem a fixação de componentes do complemento, C3b e iC3b, como também, representam uma barreira de proteção contra a lise pelo complemento.

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. Vários receptores presentes na membrana do macrófago já foram identificados; receptor para a molécula de manose-fucose em L. (L.) donovani e para manose-6-fosfato em L. (L.) amazonensis. Receptores para lectina, glicoconjuga- dos e lipofosfoglicanos (LPG), estão envolvidos na adesão de L. (L.) major e de L. (L.) donovani. Receptor para a principal glicoproteína de superfície, a gp63, foi identificado em L. (L.) mexicana.

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, facilitando a adesão e posterior fagocitose através dos receptores CR3 e CR1. Essa ligação promove também, aumento da sobrevida do parasito no macrófago, pela inibição da produção do óxido nítrico.

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. Desse modo, após a fagocitose, o parasito transforma-se em amastigota dentro do vacúulo parasitóforo; este funde-se com grânulos lisossômicos para formar os fagolisossomos, onde as formas amastigotas deverão se multiplicar. Entretanto, nesse microambiente, 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. Desse modo, considerando que a forma amastigota é capaz de superar os mecanismos microbicidas do macrófago, a sua sobrevida 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. 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. 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. 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, receptoresFc e C3b, CD1, ICAM-1, ICAM-3, LFA-3, CD4, receptores de IL-2 e atividade de ATPase de membrana. Contém 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 estímulo das células T. 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 CD4, Th1 e Th2, cujas citocinas não regular positivamente (IL-2, INF-γ e TNF-α) 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.

Recentemente, porém, estudos realizados sobre o papel da CL na imunopatogênesis 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, são as células dendríticas da derme as verdadeiras responsáveis pela estimulação antígeno-específica dessas células. Além disso, foi observado, também, que a hiperсенsibilidade do tipo tardio (delayed type hypersensitivity) mostrava-se visivelmente exacerbada em camundongo geneticamente deficiente (knock-out) para CL, sugerindo que, quanto menor a presença da CL na infeção por Leishmania, mais forte seria a resposta de hiperсенsibilidade. 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-se em células T reguladoras (“Treg cells”), sugerindo que a CL poderia inibir os eventos inflamatórios na infeção pela L. (L.) major. 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-inunológico 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 de 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, indica a existência de forte correlação negativa, espécie-específica, entre a densidade da CL e das células CD4 e CD8 no espectro clínico de LTA. Em outras palavras, enquanto a densidade da CL aumenta ao longo do espectro clínico-inunológico da infeção por L. (L.) amazonensis, no sentido das formas imunesuprimidas 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 infeção por L. (L.) amazonensis cursar com supressão da resposta imune e, consequentemente, desenvolver as formas imunesuprimidas LCDB e LCAD.

EVENTOS INERENTES À LTA

Conforme referido antes, a patogenia da LTA é fortemente influenciada pelo perfil imunogenético do homem, ao qual, encontrando-se usualmente associada a resposta imune mediada por célula T, e, ainda, pelo perfil de vitulência 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 infeção pode manter-se assintomática em indivíduos naturalmente resistentes, com resposta imune inata capaz de controlar a progressão da infeção ou, como acontece em indivíduos com susceptibilidade imunológica, resultar em um espectro de manifestações clínicas na pele e/ou mucosas naso-faringeia, 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 infeção, 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.) lindenberghi* e *L. (V.) naffii*, além da *L. (L.) amazonensis*, único representante do subgênero *Leishmania*.

Saindo do centro do espectro, as infeções não controladas pela resposta imunológica podem evoluir para um dos polos imunológicos do espectro: i) para o polo de hiporeatividade, caracterizado por forte hiperesensibilidade e representado pelas formas LCM ou LM, nas quais, a necrose de tecido muscular é a principal manifestação do estado de hiperesensibilidade dessas formas; ou ii) para o polo de hipereatividade, caracterizado por inibição parcial ou total da hiperesensibilidade e representado pela forma LCAD. Ainda que a grande disseminação das lesões nodulares no pêlo demonstre a fragilidade da resposta imune celular em controlar a infecção. Essa dicotomia da resposta imune, de caráter subgênero-específico, tem papel fundamental na patogenia da LTA; somente infeções por espécies do subgênero *Viannia*, como a *L. (V.) braziliensis*, evoluem para o polo imunológico de hiporeatividade, enquanto somente infeções por espécies do subgênero *Leishmania*, como a *L. (L.) amazonensis*, evoluem para o polo imunológico de hipereatividade. 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 infeção para o polo de hiperesensibilidade, marcado por forte resposta CD4 tipo-Th1, a *L. (L.) amazonensis* mostra o anterior, dirigindo a infeção para o polo de hiperesensibilidade, associado à resposta CD4 tipo-Th2.

Entre as formas polares LCM ou LM (DTH++) e LCAD (DTH+) e a forma central LCL (DTH0), a infecção pode passar, ainda, por uma fase intermediária de disseminação, a LCDB, durante a qual a resposta imune celular apresenta-se parcialmente atenuada (DTH+). 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 decenas ou até centenas de lesões cutâneas pápulo-ularosas (ectimatôdikes), enquanto nos casos por *L. (L.) amazonensis*, a disseminação é lenta, resultando em número limitado (cerca de dez) de lesões cutâneas eritemato-inflamatórias. 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 inmadérica de Montenegro (DTH) e a proliferação de linfócitos são sempre negativas. No entanto, 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 antimonioté de meglumina, o dobro do que é utilizado para a forma LCL.[5,7,34,35]

**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, histioinflamplasmonciária, acompanhada ou não de necrose dos tecidos e reação granulomatosa.[5,7,34,35,56,60,61,62,63,64]

Contudo, existem evidências demonstrando que em determinadas formas clínicas e dependentes 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, raramente parasitados, junto ao infiltrado celular linflamplasmonciário. Além disso, fazia parte do quadro áreas inipientes de necrose cercadas por células epitelióides.[50] 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 linflamplasmonciária, 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 hiperesensibilidade 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...
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 suscetível (Th2), respectivamente, contra a infecção.

Desse modo, tendo a forma LCL representando a maioria dos casos de LTA (c.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.

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*; 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.

Nas formas LCM ou LM, associadas à forte resposta de hipersensibilidade e proliferação de linfócitos, o perfil de linfócitos CD4 e CD8 no infiltrado das lesões tem mostrado predomínio de CD4+34,78,82,83,84,85,86, o que também foi encontrado nessas formas da LTA na Amazônia, reiterando a importância do linfócito CD4 na patogênese da 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 histioplasmocitário 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 predominio 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 epitelioïdes 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 epitelioïdes; a cura ocorreu também por cicatrização das lesões. 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 epitelioïde. Nos casos da forma LCBD por *L. (L.) amazonensis*, o predomínio é dos macrófagos vacuolizados, ricamente parasitados, com raros grupos de células epitelioïdes. Nas formas LCM ou LM (hiperreativas), o quadro é caracterizado por infiltrado linfoplasmocitário denso e difuso, entremeado por áreas de necrose e reação granulomatosa epitelioïde; em casos de longa evolução (mais de 5 anos) esta reação granulomatosa pode ser tuberculóide, refletindo o estado de hipersensibilidade celular desta forma. Na forma LCAD (hipoativada), o quadro é caracterizado por infiltrado macrófagico vacuolizado, ricamente parasitado e com escassa reação linfoplasmocitária. Como não há hipersensibilidade, o macrófago está inativo, não há necrose e não reação granulomatosa; existe apenas intensa reação macrófagica, conhecida como nódulo macrófagico ou histiocitoma, ricamente parasitado. Em casos de LCAD muito avançados, com lesões ósseas de extremação, foi descrito, recentemente, um tipo de osteomielite causada por *L. (L.) amazonensis*. 
LCM/LM. Desse modo, parece significativo que na forma LCM/LM tenha sido identificado um perfil de células CD4 maior que nas outras formas da LTA (LCI, LCDB e LCAD), sugerindo a possibilidade de níveis significativos de IFN-γ e TNF-α nessas formas, já que essas 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 e no sudoeste do Brasil, 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. 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; esse estímulo prolongado culminaria com a produção de altos níveis de IFN-γ e TNF-α, considerados importantes na gênese da lesão mucosa. Contudo, foi evidenciado, recente, que um fator genético do hospedeiro, o polimorfismo do promotor II,6-174 G/C, poderia também contribuir na patogênese da LCM/LM.

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. 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, veio confirmar que a resposta imune celular é claramente Th2, o que explica as frequentes recidivas nesses pacientes após tratamento com diferentes quimioterápicos.

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 antimônio pentavalente o dobro da dose total usada para L. (V.) braziliensis.

**CONSIDERAÇÕES FINAIS**

Com base nesses comentários, parece indiscutível a importância da espécie de *Leishmania* na patogênica 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 parasite, 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* evoluída, 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 hiporetativo, representado pela forma LCAD, com inibição parcial ou total da hipersensibilidade, conforme demonstrado, estes desvios da resposta imune celular, com exacerbação ou supressão dos
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.

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40. SCHMITZ D. La présentation antigénique au niveau de la peau. Ann Dermatol Venere; 1990; 117: 405-413.
### Tabela 1. Patogenia da LTA determinada por *L. (V.) braziliensis* e *L. (L.) amazonensis* na Amazônia, Brasil.

<table>
<thead>
<tr>
<th>Formas clínicas</th>
<th>LCAD</th>
<th>LCDB</th>
<th>LCL</th>
<th>LCDB</th>
<th>LCM</th>
</tr>
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<tbody>
<tr>
<td><strong>Parasito</strong></td>
<td><em>L. (L.)</em> a</td>
<td><em>L. (L.)</em> a</td>
<td><em>L. (L.)</em> a / <em>L. (V.)</em> b</td>
<td><em>L. (V.)</em> b</td>
<td><em>L. (L.)</em> b</td>
</tr>
<tr>
<td><strong>Carga parasitária</strong></td>
<td>++</td>
<td>++</td>
<td>+++/+</td>
<td>+ / + / +</td>
<td>+</td>
</tr>
<tr>
<td><strong>Tipo de lesão</strong></td>
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<td>placa infiltrada</td>
<td>úlcera / úlcera</td>
<td>pápula ulcerada</td>
<td>necrótica</td>
</tr>
<tr>
<td><strong>Distribuição</strong></td>
<td>disseminada</td>
<td>+</td>
<td>úlcera múltipla</td>
<td>disseminada</td>
<td>maso-bucal</td>
</tr>
<tr>
<td><strong>Macrófago</strong></td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Plasmócito</strong></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Linfócito</strong></td>
<td></td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Necrose</strong></td>
<td></td>
<td></td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Granuloma</strong></td>
<td></td>
<td></td>
<td>epitéliode</td>
<td>epitéliode / epitéliode</td>
<td>tuberculíde</td>
</tr>
<tr>
<td><strong>DTH</strong></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Linfoproliferação</strong></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Células T CD4</strong></td>
<td>Th2</td>
<td>Th1+Th2</td>
<td>Th1 / Th1</td>
<td>Th1 &gt; Th2</td>
<td>Th1</td>
</tr>
<tr>
<td><strong>Tratamento (Sbv)</strong></td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

LCAD = leishmaniose cutânea anêmica difusa; LCDB = leishmaniose e úlceras disseminadas; LCL = leishmaniose cutânea localizada; LCM = leishmaniose cutânea vascular; *L. (L.)* a = *Leishmania (L.) braziliensis*; *L. (L.)* b = *Leishmania (L.) amazonensis*; *L. (V.)* b = *Leishmania (V.) braziliensis*; DTH = hipersensibilidade do tipo retardada; IFN-g = interferon gama; IL-4 = interleucina 4; Th1 = linfócito T auxiliar 1; Th2 = linfócito T auxiliar 2; Tratamento (Sbv) - terapêutica com antimônio pentavalente.
A cross-section study on the clinical and immunological spectrum of human *Leishmania (L.) infantum chagasi* infection in the Brazilian Amazon region.

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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, Fernando T. Silveira, Ralph Lainson, Claudia M.C. Gomes, Márcia D. Laurenti, Carlos E.P. Corbett

**Key Words**

*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—10240, 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%.

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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 Sudan1,2 and Ethiopia3–5, 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 India6 and Nepal7, 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 Italy8, Spain9 and Greece10, the clinical and immune responses to human infection with Leishmania (L.) 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.) 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.) 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.) 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.) 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.) 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.) 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 ≥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 (1/100,000), 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 χ² 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 23.8% 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 ≥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%).
A cross-sectional study on the clinical and immunological spectrum of human Leishmania (L.) infantum chagasi

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, 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. 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, northeastern 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. 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-

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, they were limited to children up to 15 years old, which neglected the importance of older individuals in the epidemiology of infection.

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

<table>
<thead>
<tr>
<th>Susceptible immunological pole</th>
<th>Indeterminate initial infection (III)</th>
<th>Resistant immunological pole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic infection (AVL)</td>
<td>LST−</td>
<td>Asymptomatic infection (AI)</td>
</tr>
<tr>
<td>LST−</td>
<td>LST−</td>
<td>LST+/++++</td>
</tr>
<tr>
<td>IFAT++++/++++</td>
<td>IFAT+/++</td>
<td>IFAT+−</td>
</tr>
<tr>
<td>Subclinical oligosymptomatic infection (SOI)</td>
<td>IFAT+++/++++</td>
<td>Subclinical resistant infection (SRI)</td>
</tr>
<tr>
<td>LST−</td>
<td>LST+/++</td>
<td>LST+/++</td>
</tr>
<tr>
<td>IFAT+++/++++</td>
<td>IFAT+/++</td>
<td>IFAT+/++</td>
</tr>
</tbody>
</table>

IFAT++++: 5120–10240 (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.
In the 1980s, the epidemiological situation of human infection of *L. (L.) amazonensis* for LST and a soluble extract of *L. (L.) i. chagasi* for ELISA revealed infection prevalence rates of 18.6% by LST and 13.5% by ELISA, higher than 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. The data revealed a 16.6% prevalence rate of infection in individuals of different ages by LST. 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. 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 Umu–Salala, respectively, using a promastigote-antigen suspension of the same parasite, demonstrating a higher infection transmission level than found in the Brazilian Amazon region.

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.

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. 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. 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.

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. 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 or 5 years old. 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 Barcarena) in the northeast region of Pará State, with a higher incidence of AVL than the municipality of Barcarena. The prevalence rate of infection was 18.4% and the frequency rates of the clinical-immunological profiles were as follows: AVL: 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.

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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


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.

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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

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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%.

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 Abacetuba and Moju 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 Caezal 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 Caezal 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 Caezal village, which is situated on the banks of the river Caezal, only 7 km from the administrative center of Barcarena municipality (01° 30′ 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
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 (RPMM 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 × 10⁶ parasites per millilitre. 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 χ² 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>
<thead>
<tr>
<th>Surveys</th>
<th>LST</th>
<th>IFAT</th>
<th>LST/IFAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence</td>
<td>11.2/106</td>
<td>3.4/32</td>
<td>12.6/120</td>
</tr>
<tr>
<td>Incidence 1</td>
<td>4.7/38</td>
<td>0.6/5</td>
<td>5.2/42</td>
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<tr>
<td>Incidence 2</td>
<td>4.7/34</td>
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<td>6.3/46</td>
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<td>Incidence 3</td>
<td>2.9/19</td>
<td>0.8/5</td>
<td>3.6/23</td>
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<td>Incidence 1st</td>
<td>9.0/72</td>
<td>3.1/25</td>
<td>11.5/88</td>
</tr>
<tr>
<td>Incidence 2nd</td>
<td>2.9/19</td>
<td>0.8/5</td>
<td>3.6/23</td>
</tr>
<tr>
<td>Accumulated</td>
<td>197 (20.8)</td>
<td>62 (6.5)</td>
<td>231 (24.4)</td>
</tr>
</tbody>
</table>

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
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 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).

Regarding the LST and IFAT specificity, it was revealed that, among the 91 IFAT 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* 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* 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* 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* 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* 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*, 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-domestic or peri-domestic, 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) 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.

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

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


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

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

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 (9.9%/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...