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Biological behavior of *Leishmania (L.) amazonensis* isolated from a human diffuse cutaneous leishmaniasis in inbred strains of mice

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Summary. After a subcutaneous injection of 10^4 purified amastigotes of an isolate from a diffuse case of cutaneous leishmaniasis caused by the MHOM/BR/76/Ma-5 strain of *Leishmania amazonensis*, three inbred mouse strains developed a progressive nodular lesion, which evolved to an ulcerated lesion. Based on these data, mice of BALB/c, C57BL/6 or C57BL/10 could be classified as susceptible. The majority of mice developed metastases in the footpads, ear, tail, nose and oral mucosa. Amputation of the members related to the primary lesion was frequent. Experiments using the limiting dilution analysis showed that there was no correlation between lesion and parasite load. It has been demonstrated that these mouse strains could be considered excellent models for mucocutaneous leishmaniasis when infected with *L. amazonensis*.

Metastatic lesions caused destruction of the nasal region with many parasitized macrophages under the epithelial surface of the nasal mucosa. Bone destruction occurred with an extensive inflammatory reaction presenting macrophages heavily parasitized by amastigotes. The parasites also spread to the periodontal ligament and other structures of the oral cavity, which could induce a severe inflammatory process. This study indicates that both nasal and oral lesions in mice infected by *L. amazonensis* were characterized by an inflammatory reaction with the presence of a high parasite load within macrophages.

Key words: Inbred mice, *Leishmania amazonensis*, Oral and nasal mucosa, Pathology, TNFα

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Introduction

Leishmaniasis is a complex of diseases caused by the intracellular protozoan parasites of the genus *Leishmania*, in which the clinical manifestations of each one have been determined by the species of leishmania and by the host's immune response to the parasite. It has been established that cutaneous leishmaniasis is chiefly caused by *Leishmania major* or *Leishmania tropica* in the Old World and by *L. braziliensis*, *L. mexicana* or *L. amazonensis* in the New World. Species like *L. donovani*, *L. infantum* and *L. chagasi* have been associated with visceral leishmaniasis in the Old and New World respectively, while *L. braziliensis* has usually been associated with mucocutaneous disease. Recently, *L. amazonensis* has been isolated from various clinical forms of leishmaniasis including cutaneous, diffuse, mucocutaneous and visceral clinical forms (Schotellius and Gonçalves da Costa, 1982; Sampaio et al., 1985; Barral et al., 1986). *L. tropica* has been isolated from the bone marrow of soldiers in the Middle East (Magill et al., 1993). Inbred strains of mice have been employed to investigate the importance of T-cell and lymphokines in the control of the multiplication and dispersion of *Leishmania* in murine leishmaniasis. Strains of mice with different susceptibilities have shown a very definite pattern for some *Leishmania* species (Handman et al., 1979; Scott, 1991) but not for *L. braziliensis*. Most inbred strains of mice have been shown to be susceptible to *L. amazonensis* infection (Calabrese et al., 1992), presenting a progressive primary lesion and multiple metastatic lesions. In contrast with *L. major*-infected mice, in which the paradigm Th1/Th2 cells explains the differences between resistant strains (C57BL/6J and CBA) and susceptible strains (BALB/c), *L. amazonensis*-infected susceptible mice did not show an enhanced Th2 interleukine pattern (Liew, 1989; Heinzel et al., 1991; Afonso and Scott, 1993; Soong et al., 1997). It is

interesting to emphasize that the rhinopaties are perhaps the oldest lesions for which we have evidence, being present in old ceramics and statuettes from Peru and Ecuador. In 1571, Pedro Pizarro reported the San Antonio disease, characterized by lesions in the nose of people living in the Peruvian mountains, which now we know to be leishmaniasis (Leon and Leon, 1976).

The present report shows the behavior of one strain of *L. amazonensis* isolated from a diffuse human case in BALB/c, C57BL/6 and C57BL/10 which have become excellent models for studying mucocutaneous lesions.

Materials and methods

Animals

Female C57BL/6, C57BL/10 and BALB/c mice, 6 to 8 weeks old, were used. These mice were originally obtained from the Jackson Laboratory, Bar Harbor, Maine (USA) and afterwards have been propagated in the animal facilities of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil.

Parasites

The MHOM/BR/76/Ma-5 strain of *Leishmania (L.) amazonensis*, isolated from a patient with diffuse cutaneous leishmaniasis (Schottelius and Gonçalves da Costa, 1982) has been maintained in vivo by serial passages in BALB/c mice. These mice were originally obtained from the Jackson Laboratories, Bar Harbor, Maine (USA) and afterwards have been propagated in the animal facilities of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil. Purified suspensions were obtained from the primary lesion of BALB/c mice, 4 months after subcutaneous injection with *L. amazonensis*. Parasites were harvested and purified after the rupture of the tissue nodules in a Potter-Elvehjens homogenizer with a teflon pestle in PBS. Cells were then filtered through gauze using a 10ml syringe. The filtered suspension was generally free from intact host cells. Evaluation of the percentage of damaged promastigotes cells was made using Erythrosin B stain, as described elsewhere (Hodgkinson et al., 1980).

Limiting dilution assay (LDA) for the quantification of L. amazonensis

Mice were killed in a CO_2 chamber prior to the removal of the footpad lesions. Each point of LDA was made by a pool of three mice. The lesions were pressed in a sterile filter paper to remove the blood, washed twice in a tube containing PBS and then dried by pressing in a sterile paper. The fragment of the lesion was then weighed. The tissue was disrupted in Poter homogenizer containing 5 ml of LIBHIT media with antibiotics; cellular debris was then eliminated by a passage through a stainless steel mesh screen. The resulting suspension was centrifuged to remove cellular debris. Ten-fold serial dilutions of the lesion homogenate were made in LIBHIT media and 100 μl of each dilution was distributed in groups of 16 replicate microculture in round-bottomed microplate wells, each group with a different dilution. Control plates for determining the efficiency of the procedure consisted of serial dilution of a known number of *Leishmania*. All plates were cultured in humidified 5% CO_2 in air atmosphere. The reading was evaluated following culture growth of the parasite on the 4th day. The number of positive wells for parasite growth was scored using an inverted microscope. By correlation between the tissue weight and the percentage of negative cultures, the number of parasites was estimated, following the method described by Taswell (1981, 1984).

Serum TNF-α level determination

Serum TNF-α levels were measured with commercial ELISA kits for these cytokines (Pharmingen International, Becton Dickson Company) using a three-mice sera pool for each time of the kinetic. Kits were used according to the manufacturer's specifications.

Statistical analysis

Results were expressed as arithmetic means of 8 mice for the analysis of the local reaction and the standard error of the mean (SEM) was calculated. Statistical significance was determined by Student's T test for non-paired data. For the limiting dilution assay, the parameter estimation was based upon the single –hit Poisson model (Taswell, 1984). A standard curve was obtained by plotting the concentrations of TNFα standards versus their resulting absorbances. The mTNFα concentrations in experimental samples were then determined using the standard curve. A calculator capable of performing linear regression analysis was used.

Histopathology

The draining lymph nodes, spleen, liver, lung, kidney and thymus were removed and fixed in neutral-buffered formalin. Part of this material was then routinely processed for paraffin embedding. Sections (5 μm thick) were stained with Hematoxylin-Eosin (HE).

Experimental schedule

Separate groups of inbred BALB/c, C57BL/6 and C57BL/10 mice were subcutaneously injected with 10^4 amastigotes in a volume of 0.04 ml through the plantar surface with a 30-gauge needle. Time course of infection was accompanied during 18 months. At intervals thereafter, 2 mice from each group were necropsied. Primary lesions from 3 mice were excised and used for parasite burden quantification obtained from experimentally separated duplicate groups. The acute

phase of infection was studied at intervals of 15, 30, 45, 60, 75 and 90 days. Chronic phase was investigated at intervals of 6, 9, 12 and 18 months according to the survival rate of the various groups.

Local reaction

Eight mice were utilized to analyze the kinetics of footpad swelling. At varying times after infection the footpad swelling was measured with a dial gauge caliper (Schnelltaster, H.C. Kröplin, GRBH, Hessen, Germany), as described previously (Gonçalves da Costa et al., 1988).

The size of the lesions was expressed as the difference in thickness between the footpads of mice in the experimental infected group compared to the normal control group.

Results

Evaluation of mice susceptibility to L. amazonensis

Inoculation of 10^4 amastigotes into the footpads of BALB/c, C57BL/6 and C57BL/10 mice resulted in a reproducible progressive infection. These mouse strains developed lesions in the footpads at the site of inoculation after 20 days.

Local lesions continued to grow in all three-mouse strains studied (Fig. 1) and became ulcerated after 15 to 20 weeks. Metastatic lesions began to appear in all strains in contralateral footpad, forelimbs, ears, tail and nose.

At the beginning of the infection the primary lesion of the C57BL/6 showed a fast growth curve in comparison with BALB/c mice. A direct correlation was observed between the kinetics of the lesion and the level of TNFα (Fig. 2a,b). A peak of TNFα was also observed 4 hours after (1100 pg/ml of serum) infection in C57BL/6 whereas it was negative in BALB/c mice.

Kinetics of parasite load by LDA

In a parallel set of experiments, the inbred strains of mice were infected with 10^4 amastigotes and lesion pieces were collected 45, 60, 75 and 90 days after infection for quantification of *L. amazonensis* by LDA. The experiments showed that growth of *L. amazonensis*, at low density in round-bottomed microplate wells containing LIBHIT medium, was more efficient than those containing LIT or RPMI, because the yellow color of LIBHIT medium favors the detection of low-density positive wells. Figure 3 shows that there was no correlation between lesion progression and parasite load. These findings were more unstable when C57BL/6 mice were analyzed.

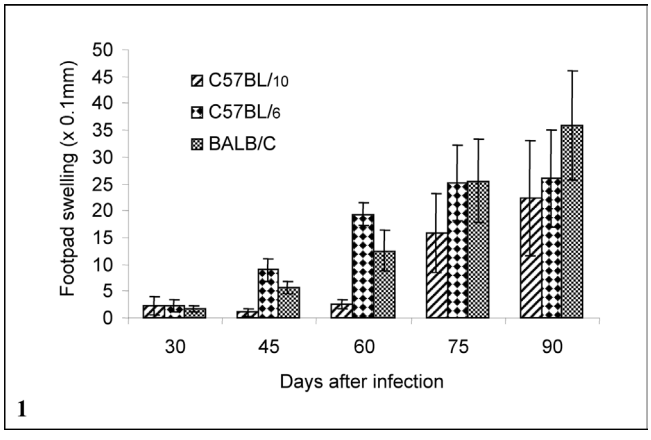
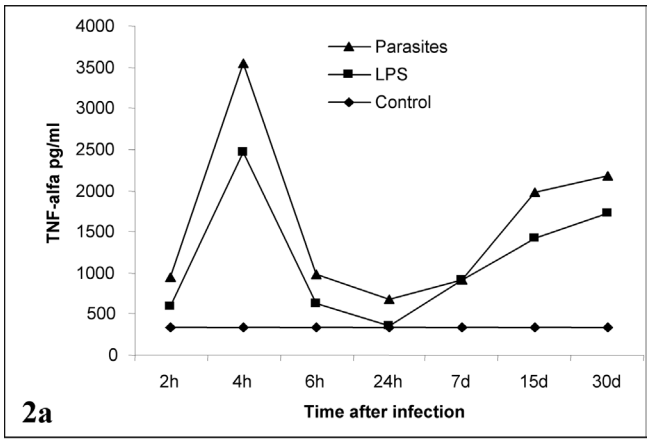


Fig. 1. Kinetics of footpad swelling in three inbred strains of mice infected with 10^4 amastigotes in the high hind footpad in a volume of 0,04 ml. Primary lesion size of BALB/c, C57BL/6 and C57BL/10 mice was represented as a mean of three mice * SEM.



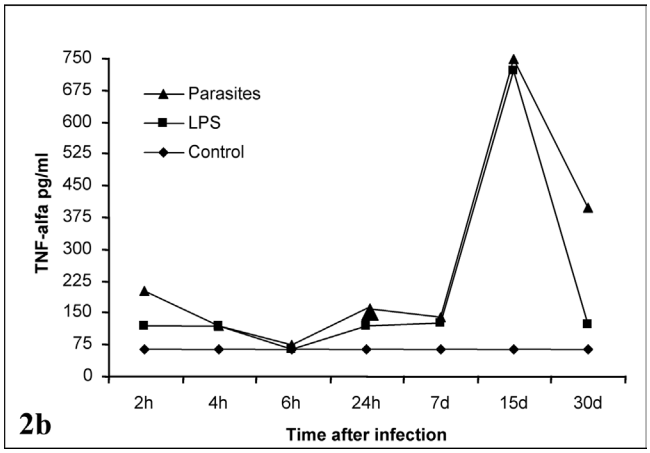


Fig. 2. Sera TNFα levels were determined by ELISA assay, at different intervals. In **a**, C57BL/6 production reaches a peak 4 hours after the challenge compared with BALB/c **b**, which starts in production two weeks later.

Histopathology of the primary lesion

Fig. 4a,b show the histopathological analysis of the primary lesion two months after infection in BALB/c mice. The primary lesion showed a clear predominance of heavily infected macrophages with few lymphocytes. Some zones of necrosis could be found and free parasites could be observed in the lesion. Decreasing parasite burden was observed during the course of infection in C57BL/6 mice (Fig. 4c,d).

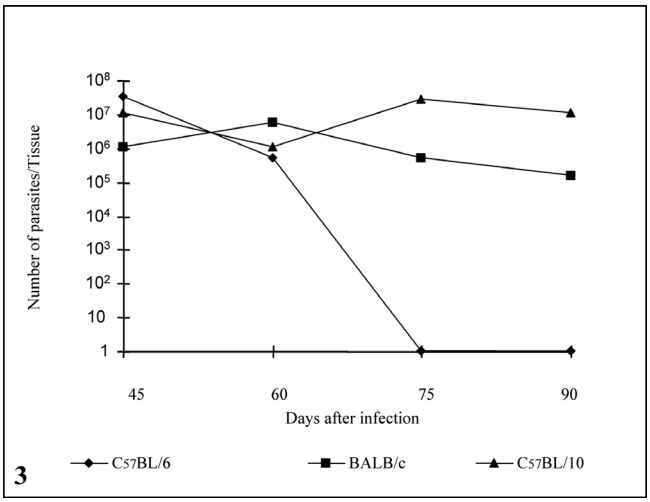


Fig. 3. Parasite load was estimated by Limiting Dilution Analysis, using LIBHIT media culture 45, 60, 75 and 90 days after infection of different mice strains. While the load is maintained higher in BALB/c and C57BL/10 during the experiments, a dramatic reduction is observed after sixty days in C57BL/6, showing no correlation with the lesion size progression.

Histopathology of oral and nasal lesions in experimental cutaneous leishmaniasis

A remarkable feature in the late-phase of the leishmaniasis infection was the outcome of oral and nasal lesions. Both lesions were characterized by an extensive inflammatory infiltrate in which a large number of vacuolated macrophages heavily laden with amastigotes was observed (Fig. 5a).

In BALB/c mice, the nasal lesions presented inflammatory infiltrates composed mainly by macrophages; many parasitized macrophages were observed under the epithelial surface of the respiratory mucosa (Fig. 5b). In addition, these mice showed involvement of periodontal ligament, which exhibited an intense inflammatory process rich in macrophages filled with parasites (Fig 5c).

C57BL/10 mice, 10 months post-infection, showed granuloma with a great number of macrophages filled with parasites, and massive necrosis. These mice also presented bone destruction and inflammatory reaction around the periosteum of alveolar bone (Fig. 5d).

Discussion

In the present study we have shown that BALB/c, C57BL/6 and C57BL/10 mice developed metastatic lesions in the naso-oral region. These lesions occurred in the late-phase of infection by *L. amazonensis*. These results confirm previous reports from our laboratory showing that almost all inbred strains are susceptible to

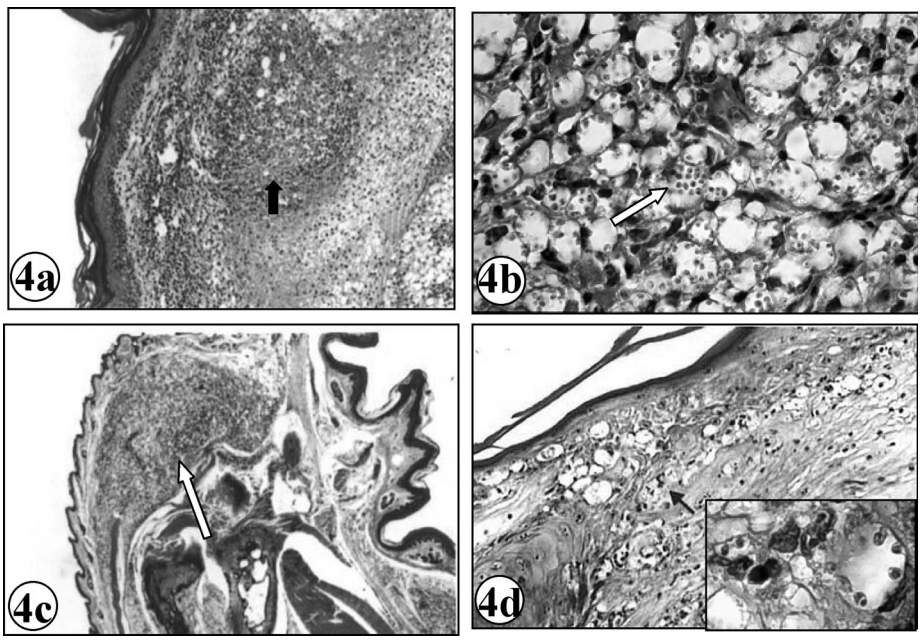


Fig. 4a. Histopathology of primary lesion of BALB/c mice after 60 days of infection with 10^4 amastigotes. Low magnification showing inflammatory reaction with intense macrophage colonization (thin arrow) and a focal necrosis zone (large arrow). Haematoxylin and Eosin stain (HE). Figure 4a An intense inflammatory infiltrate with parasite load in macrophages (arrow) is observed in the dermis. x 100. ∫ **b. A** high magnification of this lesion showing amastigotes inside macrophage cells (arrow), x 400. **c.** Histopathological aspect of primary nodular lesion in the left hind footpad of C57BL/6, 60 days after infection showing a granuloma formation (arrow) in which macrophage was filled by amastigotes. x 40. **d.** Histopathological analysis showing a late-stage (14 months) primary lesion from C57BL/6 mice infected with *L. amazonensis*. The lesion presents dermal fibrosis; the persistence of the parasite is consistently observed in the dermis (arrow) and epidermis, x 200. Window shows amastigotes within macrophage cells. x 1700

infection with *L. amazonensis* (Calabrese and Gonçalves da Costa, 1992), but that they show a different time course pattern. This contrasts with other authors who consider the C57BL/6 mice as being resistant to *L. amazonensis* and L. mexicana (Perez et al., 1978, 1979; Vasconcelos and Sotto, 1997).

C57BL/6 mice infected with *L. mexicana* have been considered cleared of infection after 20 weeks (Perez et al., 1978) or showing a few preserved parasites associated with cell-mediated response expressed by a well-organized granuloma after 51 weeks (Vasconcellos and Sotto, 1997). In the present investigation it was discovered that, unlike those observations in several murine models of Old-Word cutaneous and visceral leishmaniasis (Bradley and Kirkley, 1977), only a relative resistance to *L. amazonensis* was observed in mice presenting different genetic backgrounds when the late-infection phase was considered.

The data presented in this paper show that the parasite reaches the mucose, cartilage and bone structure, leading to their destruction. It has been shown that C57BL/6 mice are a suitable model for mucocutaneous disease, while BALB/c which present rapid progressive, metastatic lesions, are a model resembling diffuse cutaneous leishmaniasis (Barral et al., 1983). This provides a good correlation with the TNFα protecting role, mainly at the first infection events. We described here that although some differences were observed in the clinical course of infection in BALB/c C57BL/6 and C57BL/10 mice, all of them presented intense parasitism and inflammatory infiltrate in the soft-nasal and oral structures. This may occur independently from external ulceration of the oral metastatic lesions in some cases.

The loss of hair was observed around the nose with progressive destruction of the nasal region in BALB/c as well as C57BL/6 or C57BL/10 mice, culminating in most cases by ulceration. Mucosal lesions, however, looked very similar when histopathological analysis was developed. In BALB/c mice these lesions appeared early, usually after 6 months, while in C57BL/6 and C57BL/10 these mucocutaneous lesions appeared later, typically 9 months after infection. Destruction of bone tissues occurs with inflammatory infiltrate in conjunctive tissue of the oral zone presenting numerous vacuolated and parasitized macrophages.

Leishmania has the ability to evade from the host immune system and persists in the host after the resolution of the lesion either as a self-healing infection or by drug treatment. The persistence of the parasite induces a premunition condition that protects against reinfection, since the patient is immunocompetent. Previous reports from our laboratory have shown that scar patients, who had clinical cure by healing the lesion, presented amastigotes or parasite antigen (Schubach et al., 2001). Amastigotes in the scar were demonstrated through isolation in culture (Schubach et al., 1998). Resting parasites in tissues allow relapses, which occur after alterations in the immune system, as in AIDS (Modabber, 1989; Coura et al., 1987; Mattos et al., 1998) or during chemotherapy for leukemia (Hanteville et al., 1980; Gastant et al., 1981). In such cases the spread of *Leishmania* infection became atypical, leading to a visceralization of normally dermotropic parasites (Hernandez et al., 1993) or to mucosal lesions with high parasite load and atypical colonization of oro-nasal mucosa by viscerotropic leishmania strain (Rioux et al., 1980; Borzoni et al., 1991; Michiels et al., 1994; Bañuls

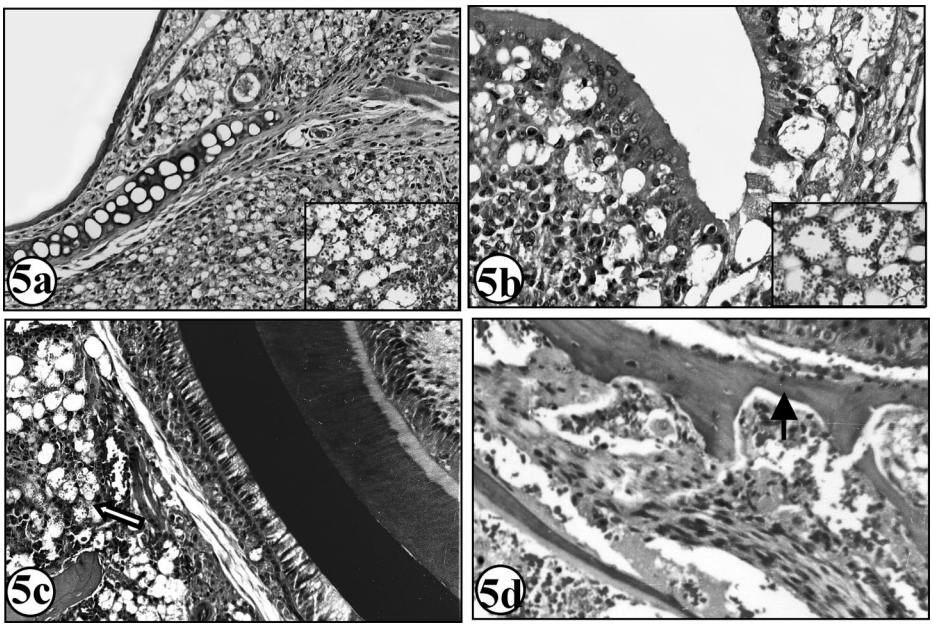


Fig. 5. BALB/c mice infected with 104 amastigotes of *L. amazonensis* one-year post infection. Haematoxylin and Eosin stain (HE). **a.** Metastatic lesion in nasal mucosa, showing an inflammatory reaction in which amastigotes within macrophages are observed. x 200. Window shows amastigotes within macrophage cells. x 400. **b.** Nasal lesion where it can see inflammatory infiltrates with macrophages parasitized under the epithelial structure of the respiratory mucosa can be seen. x 400. Window shows amastigotes within macrophage cells. x 1000. **c.** Metastatic mucocutaneous lesion showing the inflammatory infiltrate in the oral mucosa. The figure shows heavily parasitized macrophages (white arrow) in the periodontal ligament. x 200. **d.** C57BL/10 mice, 10 months after infection, present bone lesions, which are characterized by fibrous connective formation and bone destruction (black arrow). x 400

et al., 1995; Sasaki et al., 1997; Echevarria et al., 1993).

Even mucosal lesions with different clinical episodes have been found in the same patient in which two different zymodemes of *L. infantum* were isolated. In this last case, chronic destructive nasal ulcers were noticed and the subsequent dissemination of the parasites for oral mucous membrane occurred as a consequence of the impairment immune system by HIV-coinfection (Córtes et al., 1997). This picture is similar to the *L. amazonensis* mouse model and contrasts with the classical mucocutaneous leishmaniasis caused by *L. braziliensis*. In these cases *L. braziliensis* parasites are rarely found in mucosal lesions and are not easily isolated in culture media. The present data show that mice could be an appropriate model for mucocutaneous leishmaniasis if bone structure destruction by inflammatory infiltrate is analyzed or when mucocutaneous lesions observed in immuno-compromised host are discussed. It seems to be useful to follow the evolution of destructive nasal lesions and, particularly, aspects of osteochondritis as well as the mechanisms of nasal lesion restoration after anti-leishmanial treatment.

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