Research Article

Susceptibility of different mouse strains to *Leishmania amazonensis* infection

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Abstract

Background. In the most studied mouse model produced by Leishmania major, association was found between Th1 and Th2 immune responses, and resistant (C57BL/6) and susceptible (BALB/c) strains, respectively. This dichotomy is not observed in models developed with other Leishmania species. Therefore, advancing in the study of experimental models involving dominant species in our region represents an important challenge. The purpose was to reproduce the disease in different mouse strains after infection with L. amazonensis.
Methods. To study the effect of the mice strain variable on susceptibility for L. amazonensis infection, a constant parasite inoculum was applied to the studied strains. Response to infection was characterized in C57BL/6, BALB/c, and Swiss strains by measuring lesions, estimating parasite load and histological analysis. Serum antibodies and cytokines were determinated by ELISA. Statistical analysis: ANOVA test.
Results. BALB/c showed maximum susceptibility to infection, Swiss demonstrated an intermediate phenotype, and C57BL/6 was the less susceptible strain. We obtained murine models reproducing different clinical forms comparable to human disease.
Conclusions. The results will be useful to extrapolate the conclusions of future therapeutic and prophylactic analysis in experimental models to human pathology (Dermatol Argent 2009; 15(5):334-339).

Key words: *L. mexicana, animal models, mice.*

Introduction

Leishmania genus protozoan parasites cause a broad spectrum of diseases known as leishmaniasis. In our setting, *L. braziliensis* is the main etiological agent of American tegumentary leishmaniasis (ATL), but not the only one: *L. amazonensis* and *L. guyanensis* have also been described as circulating species in the province of Salta.[1-3]

Murine models of cutaneous leishmaniasis (CL) are valuable tools to study different disease-related mechanisms. The broad variety of *Leishmania* species causing human ATL, combined with host immune mechanisms, produce a great spectrum of clinical, histopathological, and immunopathological manifestations, as well as different therapeutic responses to conventional drugs. The most studied murine model is produced by *L. major*, where specific mouse strains are more resistant to infection (C57BL/6) and others are more susceptible (BALB/c). In this model, there is correlation between a Th1 type cell-mediated immune response profile predominant in the more resistant strains and a Th2 type profile in the more susceptible strains.[4-6] This dichotomy is not identified in other models developed with other *Leishmania* species. For example, models infected by *L. braziliensis* prove that both BALB/c and C57BL/6 strains may heal spontaneously.[7] In the case of experimental in-

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fection by the *L. mexicana* complex strains, such as *L. amazonensis*, no relation is found between resistance and susceptibility with predominant Th1 or Th2 responses. However, the observations obtained from different groups do not always coincide, thus indicating different degrees of susceptibility and resistance between the mentioned mouse strains.[8-10] In addition, human CL caused by *L. major* is usually bening, producing a localized skin lesion eventually healing spontaneously and promotes life-long immunity against reinfection. In contrast, the disease caused by *L. amazonensis* is characterized by chronicity, latency and tendency to develop metastatic foci in nasal mucosa.[11]

Murine models may reflect clinical and histopathological features similar to those appearing in human infection. Therefore, and due to the controversies found in different studies on this matter, it is highly relevant to move forward in the study of experimental models with dominant species in our setting. Our objective was to reproduce the disease in different mouse strains, in order to demonstrate different susceptibility to infection by *L. amazonensis*, the circulating species in the Province of Salta.

Materials and methods

Design. In order to recognize the effect of the mouse strain variable upon susceptibility to *L. amazonensis* infection, a consistent parasite inoculum was applied to the mice of each studied strain sample.

Population. Two 4-months-old male inbred (BALB/c and C57BL/6) and one outbred (Swiss) mouse strains were used.

Sample. Each experimental group consisted of 6 mice, and 6 non-infected mice of each strain were used as controls.

Parasite culture, subcutaneous inoculation, and measeurement of lesions. Parasites of a *L. amazonensis* strain (Biomedicine Institute, Universidad Central de Venezuela), Ministry of Health and Social Development, Caracas, Venezuela) were kept by serial passages on BALB/c mice right footpad (RFP). Isolation was performed by inoculating the RFP homogenate in modified USMARU medium (blood-base agar as solid phase containing 15 percent of defibrinated and hemolyzed rabbit blood, and 1 percent glucose saline). First through eighth passages were used. Parasites were harvested during the exponential phase, after 3 days culture (at room temperature, in the dark) and washed (sterile phosphate-buffered saline [PBS] 1′, pH=7.2 with 100 IU/ml penicillin/streptomycin). Parasites as promastigotes in the exponential phase were resuspended in PBS at a 2×10^7/ml concentration. A 50 µl volume containing 10^6 parasites was subcutaneously inoculated in the RFP of each experimental animal. Progressive swelling (granuloma) caused by the infectious inoculum was assessed from day 7 to day 78 post-infection by measuring both mouse hind footpads with a special sub-millimeter caliper and computing the difference (delta, Δ).

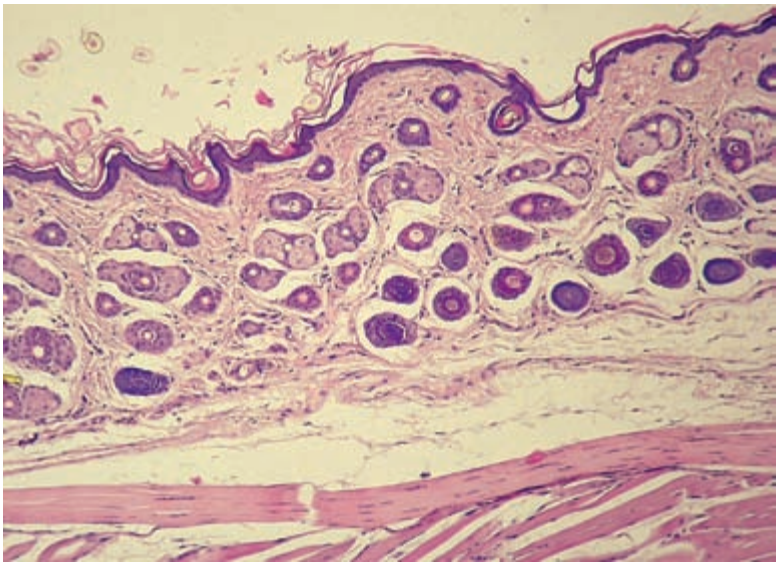

Figure 1. *Normal BALB/c mouse footpad skin. Aspect of epidermis and dermis is visualized, with numerous sweat glands. Part of muscle tissue underlies dermis (H-E, ×10).*

Spleen parasite load estimate: splenic index. It was calculated by spleen weight/body weight ratio. The presence of amastigotes was assesed by stained spleen histology sections (May-Grünwald-Giemsa).

Histology. It was performed 11 weeks post-infection. Primary infection sites (RFP) were removed, fixed with 10 percent formaldehyde, processed (after 12-24 hours) and included in paraffin. Sections 5 µm thick were stained with hematoxylin-eosin.

Protein Leishmania parasite homogenate. Promastigote forms of *L. amazonensis* were cultured at 25°C in LITHSP culture medium containing 20 percent fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 g/ml). Parasites were concentrated by centrifugation, 10 minutes at 5000 rpm. Pellet was treated with 0.1 percent PBS-BSA (phosphate saline buffer - bovine serum albumin), three times. Soluble fraction extract was obtained by pellet lysis using lysis buffer (50 mM Tris base pH=8, 1 mM EDTA, 1 mM 0.1 percent phenylmethyl sulfonyl fluoride [PMSF] Triton ×100), with sucrose addition (final concentration 10 percent). The parasite lysate was centrifuged 20000 ×g for 60 minutes at 4°C. An aliquote was separated from the supernatant (soluble homogenate) for quantification by the Bradford method.[12] Supernatant was preserved at –20°C until used.[13]

Enzyme-Linked Immunosorbent Assay (ELISA). Two mg *L. amazonensis* protein homogenate per well (in polystyrene plates) were used. For plate adhesion, antigens were diluted in sodium carbonate buffer (pH=9.6) and incubated at 4°C (12 hrs). Blockade was done with PBS-5 percent low fat milk (1 hour, room temperature). Serum was added diluted 1/25 in PBS-1 percent milk for 1 hour (room temperature). All samples were analyzed in duplicate. Secondary mouse anti-

body (total biotin marked anti-IgG, SIGMA) was used diluted 1:2500, incubating plates for 30 minutes. After washing 3 times (0.1 percent PBS-Tween), 1:2000 diluted conjugate was added for 30 minutes. Ortho-phenylene diamine (OPD) was used as chromogen and 30 percent H_2O_2 (SIGMA) as substrate diluted in citrate buffer (pH=5.3). After 30 minutes incubation at room temperature and the dark, the reaction was stopped with 2M sulfuric acid. Microplates were read at 490 nm.[14-16]

Determination of serum pro-inflamammatory and regulatory cytokines. Serum from the different experimental groups were obtained on days 15, 22, and 80 post-infection and kept at –20ºC until used. Dosage of IFN-γ (15-2000 pg/ml), IL-12 (15-2000 pg/ml) and IL-4 (4-500 pg/ml) in said samples was done by commercial enzyme-immunoassay (ELISA), following manufacturer's instructions (ebioscience, San Diego, U.S.A.).

Statistical analysis. Progressive footpad swelling was analyzed by variance analysis (ANOVA) for iterated measurements, including statistical significance for treatment effect (strain) and swelling progress (time), as well as interaction of both. A comparison of means by orthogonal contrasts was performed to determine the time when swelling differed according to strain. Splenic index and strain factor serology data were compared by one-way analysis of variance (ANOVA). It was fixed at $\alpha = 0.05$ for all inferences, and the null equality hypothesis was rejectd below that value; in such case, Tukey's mean comparison test was used ($\alpha = 0.05$) to check where differences were concentrated. Distributive data normality was verified by the Shapiro-Wilk test.

Results

Swelling comparison on different post-infection days. Significant differences ($p < 0.0001$; ANOVA for iterated samples) were found between the different studied strains. BALB/c strain mice showed mean swelling significantly larger in diameter than C57BL/6 and Swiss strains (**Chart 1**). This difference started to be significant as from day 49 post-infection, and extended to the end of study (day 78 post-infection). In contrast, the progressive swelling developed by C57BL/6 and Swiss strains was similar throughout the analyzed period of time.

Comparison of splenic index (SI). Splenic index was used as disease progression indicator, and thus of the degree of infection susceptibility. Significant differences ($p < 0.0001$) between average SI of the different studied groups was demonstrated by ANOVA test in analyzing the various groups of experimentally infected mice and their respective control groups

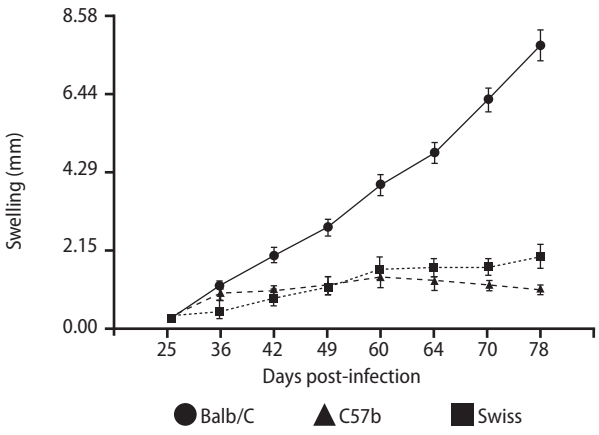

Chart 1. *RFP swelling progression in time in each strain of L. amazonensis infected mice.*

(healthy mice). However, by using Tukey's test, this difference was only found in the BALB/c strain infected mouse group in relation to the remaining animals (C57BL/6 and Swiss-infected experimental groups, and healthy controls of the three strains; see **Table 1**). On the other hand, in coincidence with these results, the BALB/c-infected mouse group was the only showing presence of amastigotes in spleen, although in small amounts.

Spleen and primary infection site histopathology. In the BALB/c group, RFP showed wide skin ulceration, abscess formation and chronic moderately exacerbated inflammatory infiltrate invading the dermis, with abundant parasite-filled macrophages (**Figures 1 to 6**; Figure 1 is a microphotography of normal skin as comparison). The spleen had abundant chronic inflammatory reaction foci, and a moderate number of amastigotes. In the Swiss group, RFP showed skin abscess formation with *Leishmania* in a large amount, while spleen showed macrophages with few parasites. In contrast, in the C57BL/6 group, RFP showed granuloma with few or no amastigotes. The spleen of these mice showed macrophages with little or no *Leishmania*.

Comparison of serology data. This analysis demonstrated that average optical density values (OD) were significantly different ($p = 0.029$) between some of the three studied mouse strains. The Tukey's test determined that average OD found in Swiss strain mice was significantly higher than C57BL/6, while average OD of BALB/c did not express comparative differences. All control group mice showed negative results (**Chart 2**).

Serum proinflammatory and regulatory cytokines. In all analyzed cases and time periods, the obtained results were below the used technique detection range.

TABLE I. AVERAGE SPLENIC INDEX VALUES (MEAN ± STANDARD ERROR) FOUND IN DIFFERENT MOUSE STRAINS (INFECTED BY L. AMAZONENSIS AND NOT INFECTED).

Swiss	Swiss (control)	C57BL/6	C57BL/6 (control)	BALB/c	BALB/c (control)	p
2.51a ± 0.24	3.02a ± 0.26	2.66a ± 0.10	2.45a ± 0.11	7.34b ± 1.42	2.67a ± 0.19	< 0.0001
Letras distintas indican diferencias significativas de acuerdo con la prueba de Tukey (alfa = 0,05).						

Comments

Histopathological findings correlate with clinical findings, with reference to lesion site swelling degree and determined splenic indexes. In infected BALB/c mice we found massive inflammatory reaction and great amount of parasites in RFP, clinically correlated with large and progressive swelling. In spleen, we also identified massive inflammatory reaction with moderate number of parasites, consistent with the higher splenic index found in comparison with the other studied strains. These data indicate a remarkable *L. amazonensis* infection susceptibility of the BALB/c strain. In analyzing the Swiss strain results, we also found inflammation and a large number of parasites in RFP, consistent with progressive evolution of lesions,[a] but less intense than the BALB/c strain. In addition, Swiss mice spleen contains scarce *Leishmania*, correlating with a lower splenic index. Finally, the C57BL/6 group shows few parasites in RFP and spleen, regressing swelling, and a low splenic index. These clinical and histological findings in the C57BL/6 strain are consistent with a lower susceptibility to *L. amazonensis* infection.

On the other hand, in different studies carried out by infection by different *Leishmania* species in humans and experimental mouse models, an increase in total IgG levels may correlate not only to protection failure, but may also contribute to disease progression, while titration reduction may be associated with improvement or effective treatment.[17,18] The critical role of circulating antibodies in the pathogenesis was also identified in *L. amazonensis* infection.[19] In this work, serology results support clinical findings, since the two strains with disease progression (Swiss and BALB/c) have higher average OD values than C57BL/6 sera. Even though these differences are statistically significant in the Swiss strain, this trend also appears in BALB/c. With reference to Th1 and Th2-type serum cytokine production levels in the suggested model, they may result too low for detection with the technique used (serum detection), not only in early, but also in extended infection periods of time.

In conclusion, by characterizing infection in each mouse strain, we obtained murine models with different degrees of resistance and susceptibility to *L. amazonensis* infection. In turn, these models reproduced different clinical forms which are

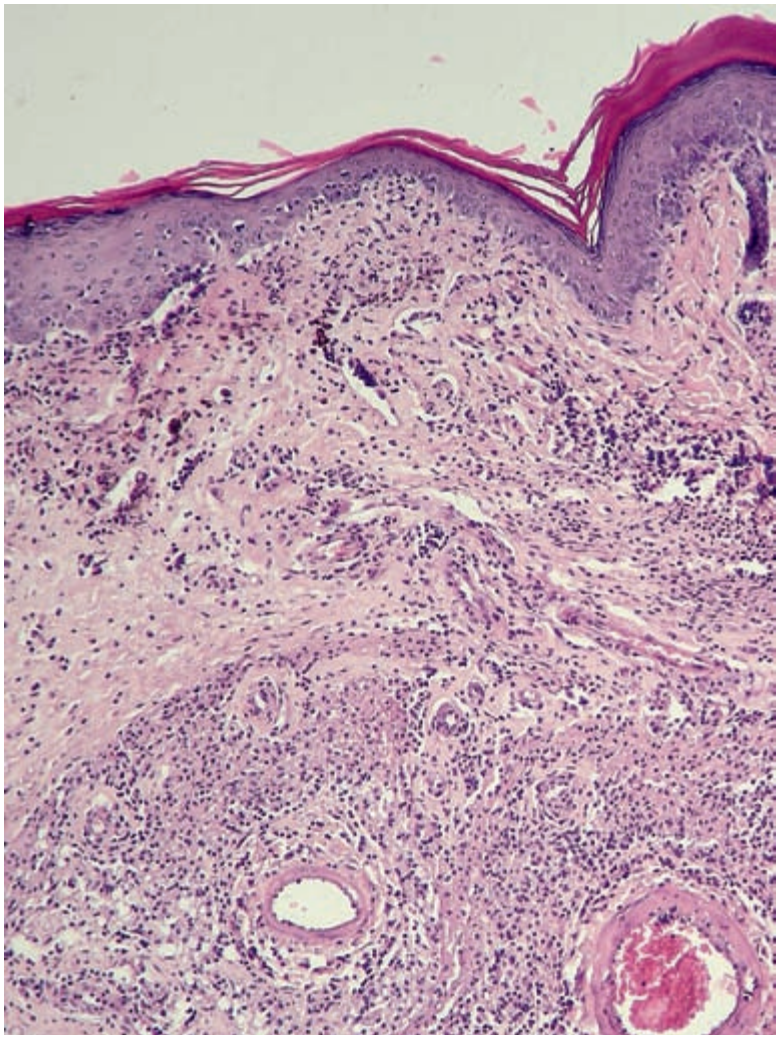

Figure 2. *BALB/c mouse footpad skin inoculated with L. amazonensis and eutanized 11 weeks post-infection. Chronic inflammatory infiltrate is identified in dermis, with signs of vasculitis (H-E, ×10).*

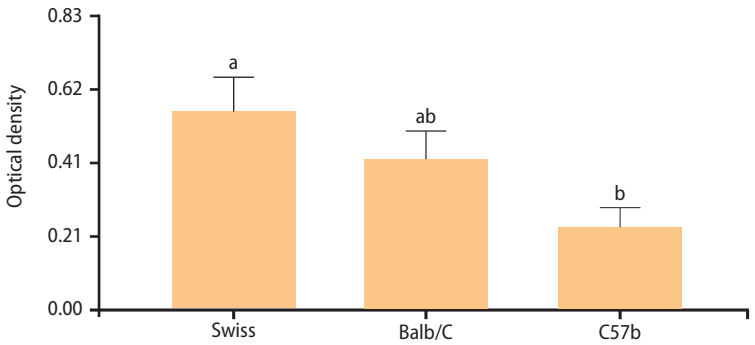

Chart 2. *Average OD values (± standard error) found in different L. amazonensis inoculated mouse strains. Different letters indicate significant differences according to Tukey's test (alfa = 0,05).*

[a] Although RFP swelling progression in Swiss does not show significant differences with C57BL/6, the curve trend differs, as seen in Chart 1: progression trend in Swiss, and regression trend in C57BL/6. This might have been significantly expressed in a later analysis.

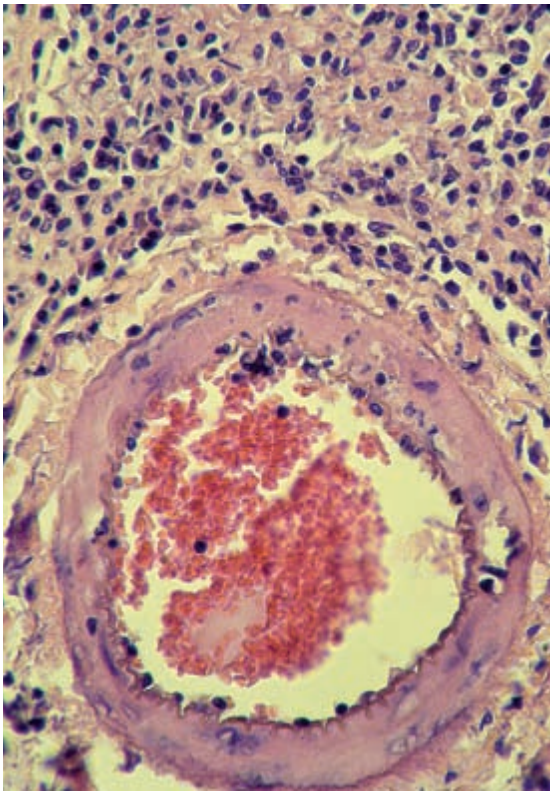

Figure 3. *Zoom-amplified view of a segment of right lower part in Figure 2, observing dermal and pertiarteriolar inflammatory infiltrate with greater detail.*

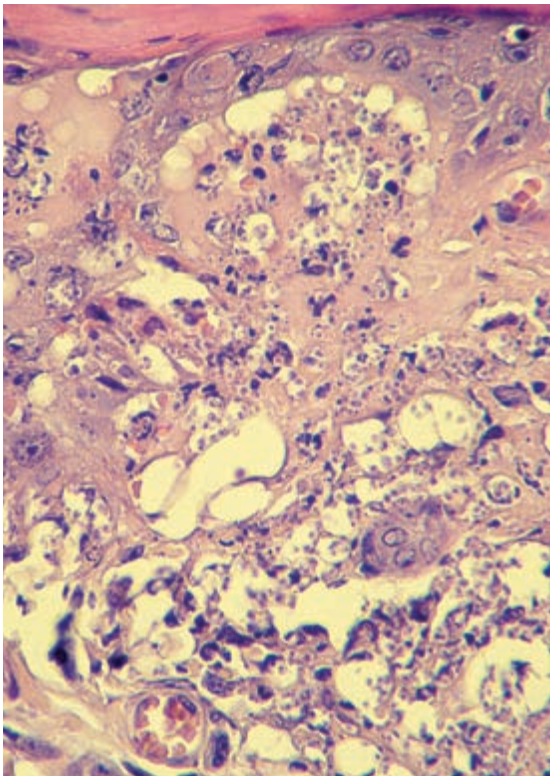

Figure 5. *Zoom-amplified view of the Figure 4 segment enclosed in a circle. Macrophages loaded with Leishmania are identified with greater detail.*

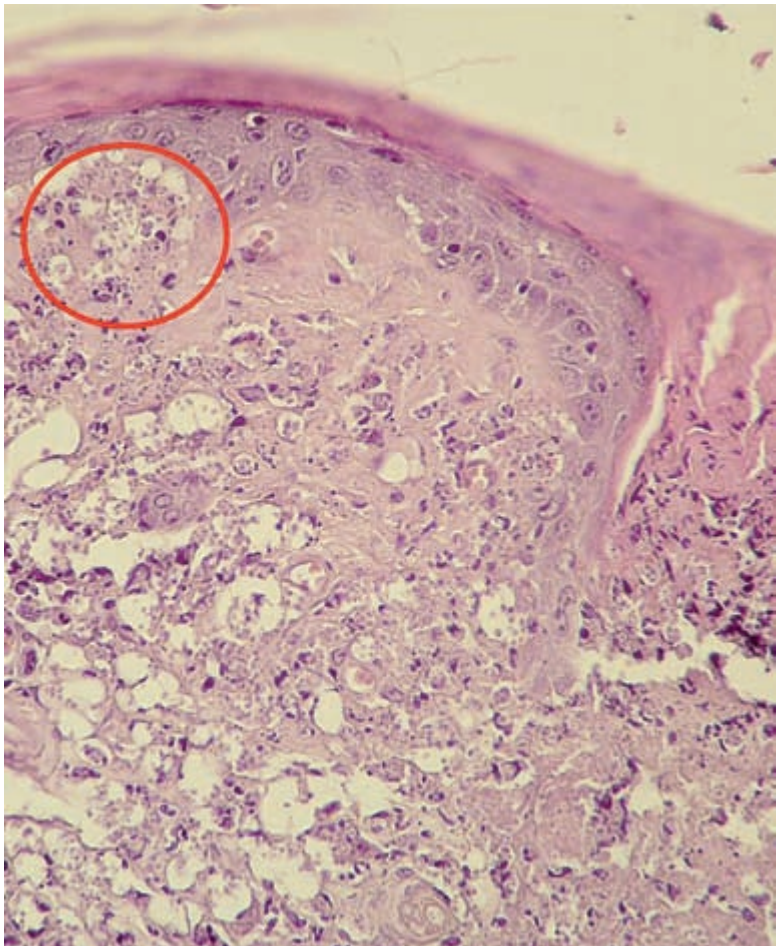

Figure 4. *BALB/c mouse footpad skin, inoculated with L. amazonensis and eutanized 11 weeks post-infection. Chronic dermal inflammatory infiltrate is seen in this section, with a large number of Leishmania amastigotes.* ***Circle:*** *subepidermal focus with macrophages loaded with amastigotes (H-E, ×40).*

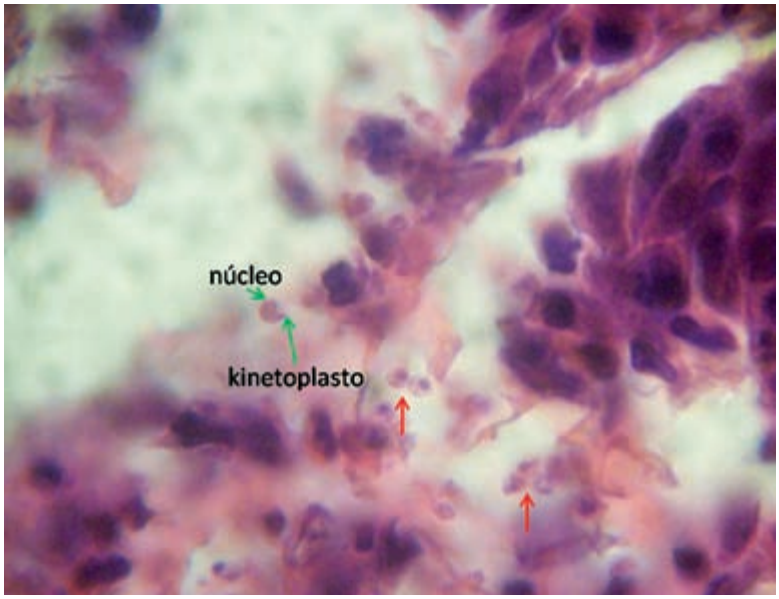

Figure 6. *Subepidermally located L. amazonensis amastigotes in BALB/c infected mouse footpad* ***(red arrows).*** *Morphology is clearly visualized, especially nucleus and kinetoplast* **(green arrows)** *(H-E, ×100).*

comparable to human disease. These results shall be useful to explore in depth host mechanisms capable of regulating infection response, and to extrapolate subsequent therapeutic and prophilactic assays in experimental animals to human pathology.

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