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Different isolates from *Leishmania braziliensis* complex induce distinct histopathological features in a murine model of infection

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ABSTRACT

The aim of this study was to evaluate the histopathological features in tissues of mice infected by human isolates (I, II, and III) or the reference M2903 strain of *Leishmania braziliensis* complex. BALB/c and C57Bl/6 mice were infected in the hind footpad with 10^6 stationary-phase promastigotes of *L. braziliensis* complex. The evolution of lesions was observed for 10 weeks and the animals were then euthanized and liver, spleen and popliteal lymph nodes were collected. Tissues were stained with hematoxylin and eosin and analyzed by immunohistochemistry assay. Increased thickness of infected footpads was observed in all animals, lesions were nodular and non-ulcerated. Mice infected with isolate I presented inflammatory infiltrates consisting predominantly of mononuclear cells in all tissues examined, and also a great number of megakaryocytes, compared with other isolates. Infection with isolate II led to an infected footpad enlargement not seen in other isolates. In addition, mononuclear infiltrates in the liver and hemosiderin in spleen were noted. Conversely, mice infected with either isolate III or M2903 strain only showed an increased number of megakaryocytes in spleen. All tissues examined had detectable amastigote forms of *Leishmania* by immunohistochemistry in all groups. Taking together, our results showed an unforeseen behavior of different isolates of *L. braziliensis* complex that led to diverse pathological findings.

1. Introduction

Leishmaniasis is an infectious disease caused by parasitic protozoans of the *Leishmania* genus. Clinical manifestations of tegumentary leishmaniasis can range from skin ulcers to mucocutaneous erosive forms with progressive destruction of the nasopharyngeal tract. The disease also manifests as a severe systemic infection with liver and spleen enlargement, cachexia and persistent fever, called visceral leishmaniasis. American tegumentary leishmaniasis (ATL) is a serious zoonosis, endemic throughout considerable areas of Latin America. *Leishmania (Viannia) braziliensis* is the main etiological agent of this disease in Brazil, and the involvement of mucosa, is the most serious complication in *L. braziliensis* infection, which can develop disfiguring injuries commonly named "espundia" to a variety of patients (Pirmez, 1992; Gontijo and Carvalho, 2003). These clinical signs have been attributed to the ability of the parasite to spread through lymphatic and blood vessels (Marsden, 1986; Martinez et al., 1992).

In endemic areas of leishmaniasis caused by *L. (V.) braziliensis*, the tissue manifestations are characterized by a series of reactions that are the results of successive events

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caused by the parasite persistence in tissues (Magalhães et al., 1986). Histopathological findings reveal alterations in dermis and epidermis that depend primarily on the *Leishmania* strain but also on disease stage. In dermis, it is common to see a mononuclear inflammatory infiltrate, consisting mainly of lymphocytes and macrophages containing Leishman-Donovan bodies. These findings, though not typical of mucosal and visceral leishmaniasis, are more common in recent lesions of cutaneous and post-kalazar dermal leishmaniasis, respectively, as well in disseminated cutaneous leishmaniasis (Magalhães et al., 1986; Andrade-Narvaez et al., 2005). Langerhan's epithelial giant cells may be present within granuloma and numerous plasma cells are found especially in mucosal leishmaniasis. The infiltrate extends from the upper to lower dermis, sometimes around a central necrotic zone where fibrinoid degeneration of vessels may be present (Choi and Lerner, 2001).

Susceptibility to disease can be measured by the macroscopic aspect of lesions, evidence healing, persistence of parasites at the site of inoculation, and visceralization to the liver or spleen following inoculation in noses (Childs et al., 1984). The murine model reproduces many aspects of cutaneous leishmaniasis of the Old and New World, including different degrees of susceptibility depending on the strain of mice used. The subcutaneous infection of susceptible BALB/c mice by *L. major* is one of the best studied models (Sacks and Trauth, 2002; Gumy et al., 2004). These mice strain were ranked as most susceptible to *Leishmania* infection, although *L. (V.) braziliensis* does not produce severe or lasting cutaneous lesions in these animals. This resistance phenotype in BALB/c responsible for killing the parasite could be IFN-γ mechanism-dependent, and the weak infectivity of *L. (V.) braziliensis* in this mouse strain might be due to the inability of the parasite to elicit a strong and sustained IL-4 production (Dekrey et al., 1998).

BALB/c mice receiving intra-dermal injection of *L. (V.) braziliensis* on the ear shows parasite replication in the site of injection accompanied by the development of an ulcerated lesion which heals spontaneously. Histological analysis of lesions shows the presence of a mixed inflammatory infiltrate consisting of both mononuclear and polymorphonuclear cells (Moura et al., 2007). BALB/c and C57BL/6 mice infected with *L. (V.) braziliensis* developed only small, nodular lesions that completely resolved within 4–6 weeks, in spite of the fact that there was long-term persistence of parasites in the draining lymph nodes of both strains of mice (Rocha et al., 2007).

Taking together, the data available in the literature show that the disease progression and the histopathological findings are related to the *Leishmania* strain. On the other hand, few data are available in the literature concerning infection and histopathological alterations promoted by different isolates from the same complex of *L. (V.) braziliensis*. In this way, here we evaluated the histopathological features in liver, spleen and lymph nodes of both BALB/c and C57BL/6 mice lineages experimentally infected with different parasites of *L. braziliensis* complex. Our data showed that different isolates, although from the same complex, may behave differently regarding their ability to develop lesions in strains of mice.

2. Material and methods

2.1. Animals

Isogenic female BALB/c and C57BL/6 mice aged 8–10 weeks were used for experimental trials. The animals were bred and maintained in the animal facilities, Universidade Federal de Uberlandia, with water and food *ad libitum*.

2.2. Parasites

We used three *Leishmania* isolates belonging to the *L. braziliensis* complex (I, II, and III) characterized by multiplex PCR (Harris et al., 1998) and a reference strain of *L. (V.) braziliensis* (MHOM/BR/75/M2903 strain) was obtained from the Infectious Diseases Center of the Universidade Federal do Espírito Santo, Vitória, Brazil. Isolate I was obtained from the lesion of a male patient, 79-year-old with skin lesion in the elbow; isolate II was isolate of a male patient, 17-year-old with a single skin lesion in the right arm; isolated III was obtained from a male patient, 35-year-old with skin lesions in the leg (Ethical Committee No. 058/06). Parasites were kept and isolated in golden hamsters (*Mesocricetus auratus*). To obtain the infective promastigote forms, infected popliteal lymph nodes of infected hamsters were cultivated in BHI medium (Brain Heart Infusion, Oxoid Ltd., Basingstoke, Hampshire, UK), supplemented with 10% fetal bovine serum (Cultilab, Campinas, BR), 100 μg/ml gentamycin, 1 mM L-glutamine (Gibco BRL-Life Technologies, New York, USA) at 26 °C until the stationary phase.

2.3. Experimental infection

Promastigote forms in stationary phase of growth were harvested, centrifuged for 10 min at 1000 × *g*, and then resuspended in phosphate buffered saline (PBS, 0.15 M, pH 7.2). The parasite concentration of each isolate (I, II, and III) and *L. (V.) braziliensis* M2903 reference strain was adjusted to 2×10^6/50 μL in PBS, and inoculated subcutaneously in the hind left footpad of mice in both strains BALB/c and C57BL/6 ($n = 5$/isolate/strain). Negative control animals were inoculated with PBS only. The evolution of the infection was monitored weekly through the volume measurement of footpad swelling using a manual caliper (Mitutoyo, Tokyo, Japan), for 10 weeks. The measurements between the infected footpad and the contralateral uninfected footpad refer to differences in the lesion size, in millimeters (mm).

2.4. Histopathological and morphometric analyses

Ten-weeks post infection, the mice were sacrificed in accordance to the ethical principles in research of the Brazilian College of Animal Experimentation. Fragments of popliteal lymph nodes and spleen were removed, fixed in 10% buffered formalin solution, dehydrated and embedded in paraffin. The paraffin blocks were sectioned, stained with hematoxylin and eosin (HE) and analyzed by light microscopy to determine megakaryocyte count in spleen, presence of parasites in spleen, popliteal lymph nodes and