CENTRAL NERVOUS SYSTEM INVOLVEMENT IN EXPERIMENTAL INFECTION WITH *LEISHMANIA (LEISHMANIA) AMAZONENSIS*

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Abstract. We describe the pathologic alterations of the central nervous system (CNS) observed in experimental tegumentary leishmaniasis in BALB/c and Swiss mice. The mice were subcutaneously infected with 10^4 amastigotes of *Leishmania (Leishmania) amazonensis*. Animals were killed and brains were removed for histologic and immunocytochemical studies. Histologic examination showed that 66.6% of infected mice had a discrete hyperemia and inflammatory infiltrate in the meninges, composed of mononuclear cells and neutrophils with no detectable parasites. However, parasitized macrophages were detected in the cerebral parenchyma, as well as mast cells, lymphocytes, and polymorphonuclear cells. Necrosis in the cerebral parenchyma was also observed. Confocal fluorescence microscopy showed that CD8+ T lymphocytes are the major component of the inflammatory infiltrate in the CNS. In addition to these cells, CD4+, CD11b, and dendritic cells are present, in small numbers, in the inflammatory processes of the CNS. Thus, *L. amazonensis* is able to cross the blood-brain barrier and cause significant pathologic changes in the CNS.

INTRODUCTION

*Leishmania (Leishmania) amazonensis*, the etiologic agent of human tegumentary leishmaniasis, is an obligatory intracellular parasite of mammals. *Leishmania* promastigotes are introduced by sand flies into the skin of mammalian host where they infect cells of the mononuclear phagocytic system, as well as fibroblasts.1 In these cells, they transform into amastigotes within parasitophorous vacuoles, causing broad spectrum diseases in humans, ranging from cutaneous to mucosal and diffuse leishmaniasis.2-5 *Leishmania (L.) amazonensis* has also been implicated in non-cutaneous forms of the disease such as mucosal, visceral, and post-kala-azar dermal leishmaniasis.4,5 Visceral leishmaniasis can also be caused by *L. (L.) amazonensis* in patients with associated infections with human immunodeficiency virus.6 It has been shown that BALB/c mice are highly susceptible to *L. (L.) amazonensis*, and show a progressive infection that may disseminate to lymph nodes, spleen, and liver, and promote metastasis in almost all extremities of the animal body.

The detection of *Leishmania* antigens in the central nervous system (CNS) of patients with acquired immunodeficiency syndrome7 led us to carry out the present study in which we examined the spread of *L. (L.) amazonensis* to the CNS in the susceptible inbred BALB/c and outbred Swiss mice. These mouse strains are excellent models for investigating the development of encephalitis, since the parasite causes the formation of significant inflammatory infiltrates, recruiting several cell types that appear to be involved in the pathogenesis of the encephalitis.

MATERIALS AND METHODS

Animals. Six- to eight-week-old female BALB/c and Swiss mice were obtained from the animal facilities of Instituto Oswaldo Cruz. Six animals were used per experimental group.

Parasite. The H21 MHOM/BR/76/MA-76 strain of *L. (L.) amazonensis* was isolated from a patient with diffuse cutaneous leishmaniasis and maintained by serial passages in mice in our laboratory.

Experimental design. Mice were subcutaneously infected in the left footpad by injecting 10^4 *L. (L.) amazonensis* amastigotes. Eight-months post infection, the experimental group was killed and the brains were removed for histologic and immunocytochemical studies. These experiments were conducted in accordance with guidelines for experimental procedures of Fundação Oswaldo Cruz (Process no. P0062-00) and were performed three times with comparable results.

Histopathology and immunocytochemistry. Three brains of each mouse strain were fixed in 10% buffered formalin, routinely processed for embedding in paraffin, sectioned in the frontal plane, and stained with hematoxylin and eosin and Lennert’s Giemsa. Three other brains were embedded in Tissue Tek (OCT Compound-embedding medium for frozen specimens; Miles, Inc., Elkart, IN) and immediately frozen; 5-μm sections were used for cell subset identification. Briefly, cryostat sections of brain were picked up on slides covered with γ-methacryloxypropyl-trimethoxysilane, fixed in acetone for 15 minutes, and dried at room temperature. Subsequently, the sections were blocked in phosphate-buffered saline (PBS) containing 0.25% gelatin and 0.1% sodium azide (PGN) and incubated in a humid chamber for 15 minutes. The slides were then incubated for 40 minutes with human polyclonal anti-*Leishmania* serum obtained from a patient with visceral leishmaniasis that was diluted 1:600 in PGN with 0.1% saponin. After the sections were washed three times in PBS (pH 7.2) with gentle agitation, they were covered with secondary antibodies conjugated with Cy3 (Sigma, St. Louis, MO), incubated for 40 minutes, and washed as described earlier. The slides were then incubated for 40 minutes with either anti-mouse CD4, anti-mouse CD8, or anti-mouse CD11b (all rat monoclonal antibodies, Pharmingen, San Diego, CA), or monoclonal antibody DEC205 (SeroTec, Oxford, United Kingdom) diluted 1:10 in PGN-saponin. After three washings, the specimens were incubated for 40 minutes with secondary antibodies (goat anti-rat immunoglobulin conjugated to fluorescein isothiocyanate; Sigma). The nuclei were stained with 4,6-di-amin-2-phenylindole fluorescent DNA-binding probe (Molecular Probes, Eugene, OR). The slides were then washed three times in PBS and mounted in glycerol containing 0.1% p-phenylenediamine (Sigma). The slides were ex-
amine on a Bio-Rad (Hercules, CA) 1024 (UV) confocal scanning system coupled to a Zeiss (Wetzlar, Germany) Axiovert 100 microscope, using a 40× 1.2 N.A. PlanApochromatic water-immersion objective.

RESULTS

Both strains of mice used showed apathy, weight loss, an ulcerated lesion at the inoculation site, and metastasis in the nose at clinical examination. The brains did not show macroscopically detectable lesions. Histologic examination showed that 66.6% of studied mice had a discrete hyperemia and inflammatory infiltrate in the meninges composed of mononuclear cells and neutrophils, with no detectable parasites. One animal had small hemorrhagic areas and inflammatory infiltrates in the white matter that were rich in lymphocytes, and contained some polymorphonuclear cells, parasitized macrophages, and mast cells (Figure 1). Another animal showed in cortical region of the brain temporal lobe intense inflammatory infiltrates composed of neutrophils and rare macrophages containing few parasites. Necrosis in the cerebral parenchyma was also observed (Figure 2).

Confocal microscopy showed that CD8+ T lymphocytes, as demonstrated by their reactivity with anti-mouse CD8, are the major component of the inflammatory infiltrate in the CNS (Figure 3B). In addition to these cells, CD4+ (Figure 3A), CD11b (macrophages) (Figure 3C), and dendritic cells

**FIGURE 1.** A. Histopathologic analysis of the brain of a BALB/c mouse eight months post-infection with 10^4 amastigotes of Leishmania (L.) amazonensis, showing areas of hemorrhage and inflammatory reaction (arrow) (hematoxylin and eosin stained, bar = 50 μm). B. Higher magnification showing polymorphonuclear cells, plasma cells, and infected macrophages (arrow) (hematoxylin and eosin stained, bar = 50 μm). C. Mast cells and amastigote forms within macrophages (arrow) (Lennert’s Giemsa stained, bar = 25 μm).

**FIGURE 2.** Histopathologic analysis of the brain of a BALB/c mouse eight months post-infection with 10^4 amastigotes of Leishmania (L.) amazonensis. A. Intensive inflammatory infiltrate in the cortical region, consisting mainly of neutrophils and a few amastigotes within macrophages (hematoxylin and eosin stained, bar = 100 μm). B. Necrosis of the cortical region associated with an inflammatory reaction (hematoxylin and eosin stained, bar = 50 μm).
DISCUSSION

The healthy brain is considered to be an immunologically privileged site due to the low expression of major histocompatibility complex antigens, the low number of antigen-presenting cells, and an efficient blood-brain barrier. This barrier is composed of an endothelium lining, with intercellular tight junctions, pericytes within the capillary basement membrane, perivascular macrophages, and astrocytic foot processes. However, several pathogens, such as bacteria of the genus *Brucella*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*, are able to cross the blood-brain barrier and cause lesions.

Several possible routes of pathogen entry into the CNS have been proposed. These include pathogen-directed invasion of epithelial cells from the choroid plexus or cerebral capillary endothelial cells, passage between cells of the blood-brain or blood-cerebrospinal fluid barriers, and transportation across these barriers within infected leukocytes.
In this study, we report an unexpected inflammatory process in the CNS, with detectable amastigotes of *L. (L.) amazonensis*, a species known to cause tegumentary leishmaniasis. Migration of *Leishmania* to the CNS has been described in human and canine visceral leishmaniasis caused by *L. infantum*. Analysis of bone marrow of the animals of this study showed the presence of amastigotes within macrophages. Furthermore, kidneys and liver showed extramedullar hematopoiesis where free amastigotes could be observed among immature cells. This suggests that parasites could have arrived in the CNS via infected leukocytes. This route was suggested to occur in encephalitis caused by bacteria.

Confocal fluorescence microscopy of CNS from infected mice showed that CD8+, CD4+ T lymphocytes, macrophages, and dendritic cells participated in the pathogenesis of the cerebral lesions caused by *Leishmania*.

It has been reported that CD4+ T cells are associated with pathogenesis and ulcer formation during infection with *L. (L.) amazonensis*. Mice deficient in CD4 cells are able to control infection by *Leishmania*, which shows that these cells are involved in pathogenesis of cutaneous leishmaniasis.

Many studies have shown that the ability to survive and regulate toxoplasmic encephalitis during this phase of infection is dependent upon T cell-mediated immunity involving both CD4+ and CD8+ T cell responses, with CD4+ cells and interferon-γ playing a crucial role.

CD8+ T cells play a protective role in immunity to cutaneous leishmaniasis. However, it is not clear how these cells execute this function. In our model, we observed an increase in CD8+ T cells in the CNS compared with CD4+, macrophages, and dendritic cells. The granulocytes present in the inflammatory site could promote further recruitment of neutrophils, as well as the subsequent accumulation and activation of monocytes, macrophages, and lymphocytes. In our study, a large number of neutrophils was observed, which could be further recruiting CD8+ cells to the site of the infection.

Dendritic cells were found within the CNS inflammatory cells infiltrates. Such cells could also be important in the onset and progression of the lesion caused by *Leishmania*. This hypothesis has been suggested in an experimental model of autoimmune encephalomyelitis. Evidence has been reported that *M. tuberculosis* can parasitize immature dendritic cells and stimulate them to secrete cytokines and alter their surface adhesion molecules in ways that contribute to cell migration and aid pathogen dissemination within the host.

Dendritic cells also appear to play a role in cerebral ischemia since there is an increase of these cells in permanent middle cerebral artery occlusion.

Studies have shown that mast cells are found in brains of several mammalian species. In rats, it was demonstrated that these cells migrate into the CNS during embryonic development through blood vessels. In the present study, the presence of mast cells in the inflammatory infiltrates is an indication that mast cells play a role in host defense during pathogen invasion, which corroborates the results of previous studies. Mast cell–derived products may be influencing capillary permeability, thus facilitating the access of inflammatory cells to nervous tissue.

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