Experimental Parasitology 133 (2013) 156–161

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Ocular experimental leishmaniasis in C57BL/10 and BALB/c mice induced by *Leishmania amazonensis* infection

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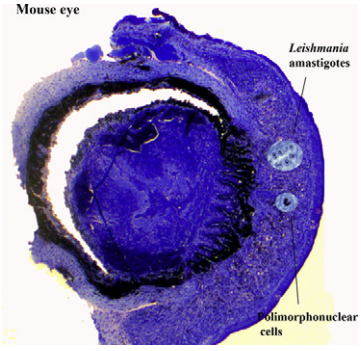
HIGHLIGHTS

- There are few studies on human ocular leishmaniasis found in the literature.
- We describe experimental ocular leishmaniasis, induced by *L. amazonensis* in murine model.
- Ocular lesions observed in mice are similar to those seen in dogs from endemic areas.

GRAPHICAL ABSTRACT

Mouse eye.



ARTICLE INFO

Article history:
Received 29 May 2012
Received in revised form 12 September 2012
Accepted 14 November 2012
Available online 29 November 2012

Keywords:
Leishmaniasis
Eye
Mice
Leishmania amazonensis
Experimental model

ABSTRACT

There are few studies on human ocular leishmaniasis found in the literature. The purpose of this study was to describe experimental ocular leishmaniasis, caused by *Leishmania amazonensis* evaluating two different infection routes: intravitreal and instillation in C57BL/10 and BALB/c mice. In this work all animals presented low anti-*Leishmania* IgM and IgG titers regardless of the infection route or mouse strain. The histopathological eye analysis showed that the mice inoculated by the intravitreal route developed more severe lesions, presenting parasites in the anterior region of the eye 60 days after infection. The C57BL/10 mice presented cells containing parasitophorous vacuoles associated with pigmented cells and inflammatory infiltrate, which included mast cells. Ninety days after infection no parasites could be found in either mouse strain, which led us to hypothesize that parasites had been eliminated. In this context, we show that both intravitreal and instillation routes were effective in promoting ocular leishmaniasis infections in C57BL/10 and BALB/c mice. There were no differences in the parasite infection between the two mouse models and it mimicked the ocular lesions described in symptomatic dogs in endemic areas of visceral leishmaniasis.

1. Introduction

Ocular lesions are one of the various manifestations of leishmaniasis and in dogs they have a predisposition for the anterior segment of the eye (Puchol and Gonzalez, 1989). In dogs, ocular manifestations are often described and generally occur concomitantly with other systemic signs (Ciaramella et al., 1997; McConnel

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0014-4894
http://dx.doi.org/10.1016/j.exppara.2012.11.008

et al., 1970; Molleda et al., 1993; Peña et al., 2008; Roze, 1986; Slappendel, 1998). In cat and human leishmaniasis, although less frequently described, these ocular lesions are usually associated with systemic signs and rarely independent of them (Ciaramella et al., 1997; El Hassan et al., 1998; Navarro et al., 2010; Oliveira-Neto et al., 2000).

The relative prevalence of ocular lesions in dogs with leishmaniasis ranges from 16% to 80% (Peña et al., 2000; Slappendel, 1998). Some of the most important ophthalmic manifestations in this disease are conjunctivitis (Slappendel, 1998), keratoconjunctivitis, keratouveitis and blepharitis (Boldy and Clerc 1989). Molleda et al. (1993) showed that 96.7% of dogs with leishmaniasis presented ocular lesions, attributed to this disease. Later, Peña et al. (2000) in a retrospective study of canine leishmaniasis showed that periocular and ocular tissues are affected in approximately 25% of the animals. In cats, leishmaniasis generally appears as the cutaneous form of the disease. Recent research into the prevalence of leishmaniasis points to the involvement of cats as a reservoir host, but only sporadic cases of feline leishmaniasis have been reported (Navarro et al., 2010). In humans, there is growing evidence that the eye can be involved in *Leishmania* infection, principally in immunocompromised individuals (Meenken et al., 2004). Ocular leishmaniasis is usually associated with either cutaneous or post-kala-azar leishmaniasis (Khalil et al., 2011; Sadeghian et al., 2005).

Eyelid lesions caused by *Leishmania* have been observed rarely, probably because of their movements that prevent the sting of the vector (Morgan, 1965). Studies of human ocular leishmaniasis epidemiology show that eyelid lesions represent 1.93–2.9% of cutaneous leishmaniasis (Gurel et al., 2002; Satici et al., 1998) and from 2% to 5% of facial lesions (Satici et al., 1998). The pathological pattern of leishmaniasis in eyelid lesions is comparable to skin lesions on other parts of the body, but the fragility of the eyelids presents a special risk of local spreading (Aouchiche, 1981).

Animals with larger eyes are preferably used in ocular disease studies. In experimental ocular toxoplasmosis studies for example, rabbits are often chosen as models because visualization of their eyes is better than those of smaller animals (Nozik and O'Connor, 1971); however other models such as mouse have also been used (Calabrese et al., 2008). In the literature only a few studies described ocular lesions in experimental leishmaniasis and the model chosen was the hamster or the guinea pig (Abboud et al., 1970).

The purpose of the present study is to describe experimental ocular leishmaniasis, induced by *Leishmania amazonensis* in C57BL/10 and BALB/c mice, comparing two different infection routes: intravitreal and instillation. Also the internal structures of the mouse eye are studied and described.

This work brings basic research in parasitology to the eye clinic and intends to help professionals to identify ocular lesions associated with leishmaniasis in dogs and humans. The data presented here could aid professionals make an accurate and early diagnosis and consequently the appropriate treatment of this disease.

2. Materials and methods

2.1. Animals

Healthy C57BL/10 and BALB/c female mice, 4–6 weeks old, weighing from 15 to 18 g were used. During the experiments, all mice were maintained under controlled temperature, receiving food and water *ad libitum*.

2.2. Parasites

The *L. amazonensis*, MHOM/BR/76/Ma-5 strain, isolated from a man with diffuse cutaneous leishmaniasis (DCL), was characterized by isoenzymes and lectins (Grimaldi et al., 1991, Schottelius and Gonçalves da Costa, 1982). Promastigotes were maintained by serial passages in our laboratory, in LIBHIT medium (Gonçalves da Costa and Lagrange, 1981).

2.3. Experimental design

All experiments with animals were conducted in accordance with the guidelines for experimental procedures of Oswaldo Cruz Foundation (Ethical Committee, Process Number P0315/06) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The inoculations were performed after intraperitoneal administration of diazepam 20 mg/kg (COMPAZ® – Cristália Ltda.) and fentanyl 0.15 mg/kg (Fentanyl® – Janssen-Cilag Ltd.), associated with topical anesthesia with citrate proxymetacaine 0.5% (ANESTALCON® – Acon of Brazil Ltda.) using one drop in each eye.

The C57BL/10 mice were divided into four groups with 12 animals in each group. In Group 1 (G1) the mice were infected intravitreally, according to Garweg et al. (1998), with 10^6 promastigote forms of *L. amazonensis*; animals from G2 were instilled with 10^6 promastigote forms of *L. amazonensis* in one drop, according to Gil et al. (2002); G3 was composed of non-infected control mice that received PBS pH 7.2 intravitreally and G4 was composed of non-infected control mice that were instilled with PBS pH 7.2. The same experimental design was used for BALB/c mice.

Four animals of each experimental group were killed by CO_2 inhalation 60, 90 and 120 days after infection, the eyes were enucleated and sectioned in a sagittal plane, dividing the bulb into two hemispheres. Both hemispheres were fixed in Bouin and routinely processed for historesin embedding medium (hydroxyethylmethacrylate, Leica – Heidelberg, Germany). The processed tissue was cut in a rotary microtome (Micron HM360, Germany) into sections of 1 μm which were stained using 0.25% toluidine blue, in 1% sodium tetraborate. Upper eyelids were obtained by incision and removal of the skin and processed as described above. The results presented here are representative of three independent experiments.

2.4. Antibody assay

Serum was obtained from the blood of the animals by intra-cardiac punction on days 30, 60, 90 and 120 after infection and was analyzed for IgM and total IgG (SIGMA) antibodies against *Leishmania* sp. by indirect immunofluorescence assay (IIFA) (Camargo and Leser, 1976).

3. Results

3.1. Antibody assay

Independent of the route indirect immunofluorescence analysis of BALB/c mice showed low levels of IgM and IgG antibodies (1/10) throughout the whole experiment. A discreet elevation of IgM antibodies (to 1/40) was observed 30 days after infection in BALB/c mice intravitreally infected. Low titers of antibodies were also observed when C57BL/10 mice were analyzed (Table 1). Normal non-infected mice were non reagent.

3.2. Histopathological findings

The histopathological eye analysis showed, at the beginning of the infection, similar patterns of response characterized by collagen fibers dissociation in corneal stroma of both mice strains, however, 60 days after intravitreal infection the mice displayed an

Table 1
L. amazonensis antibody titers in BALB/c and C57BL/10 mice serum infected by ocular instillation (IT) or intravitreally (IV) with 10^6 promastigotes 30, 60 and 90 days after infection.

	IgM			IgG		
	30	60	90	30	60	90
BALB/c IT	10	10	NR	NR	10	10
BALB/c IV	40	20	NR	NR	10	10
C57BL/10 IT	10	10	NR	NR	20	20
C57BL/10 IT	20	20	NR	10	10	10

NR = No Reaction.

intense inflammatory reaction associated with vacuolated parasitized cells in the corneal stroma (Fig. 1A) and sclerocorneal region (Fig. 1B). In the C57BL/10 mice besides these alterations, pigmented cells among the parasites (Fig 1C) and severe dissociation between the photoreceptor outer segment and epithelial pigmented retinal cells were also seen (data not shown).

At 90 days post infection no more parasites were seen in the eye, even in the intravitreally inoculated mice. Meanwhile, all mice presented a thickening of the anterior and posterior epithelium and dissociation of the corneal stroma (Fig. 2A) and the C57BL/10 mice also presented round pigmented cells in the ciliary body and in the sclerocorneal region (Fig. 2B).

Independent of the route, the C57BL/10 mice presented an intense inflammatory reaction and pigmented cells in the epithelium of the eyelids from the 30th day post infection as well as a lot of intact mast cells in the conjunctiva (Fig. 2C), but which decreased by the 90th day. On the other hand, BALB/c presented no lesions in the initial phase of infection other than an enhancement of intact mast cells in the conjunctival region 30 days after infection via the instillation route (Fig. 2D). On the 60th day post infection a discreet inflammatory infiltrate and the presence of degranulated mast cells were noted in the conjunctival region (Fig. 3A). On the 90th day a discrete inflammatory reaction was seen in the intravitreally inoculated mice (Fig. 3B).

4. Discussion

The *Leishmania* infection, as with other parasitic infections, takes place through the interaction of factors relating to the parasite and host, for example, animal genetic background, route of infection, *Leishmania* strain used for infection, parasite maintenance conditions, inoculum size and number of parasite passages. Thus, the ongoing clinical forms of leishmaniasis include cutaneous lesions ranging from self-resolution and disfiguring lesions of the mucosa to the visceral form, potentially lethal, if untreated, which are directly linked to the factors described above (Blackwell, 1996; Salam, 2009).

Parasites that cause eye diseases often include protozoa, helminths and arthropods. Among the protozoa, the most important are *Acantamoeba*, *Microsporidia*, *Toxoplasma* and *Leishmania* (Shoukrey and Tabara, 1986). Few studies have been carried out on experimental ocular leishmaniasis and natural infections observed in dogs. In humans, ocular leishmaniasis is associated with the presence of primary cutaneous leishmaniasis (Ferrari et al., 1990; Roizenblatt, 1979) or previous cases of American visceral leishmaniasis (Abdel-Hameed et al., 1990; El Hassan et al., 1998).

To the best of our knowledge there are no descriptions in the literature of a murine model for ocular leishmaniasis studies. A small number of studies reporting ocular lesions in leishmaniasis have used hamsters (*Mesocricetus auratus*) and guinea-pigs (*Cavia porcellus*) as the experimental models (Abboud et al., 1970). So, in this paper, we described a murine model to study ocular leishmaniasis, comparing two routes of infection: ocular instillation and intravitreal.

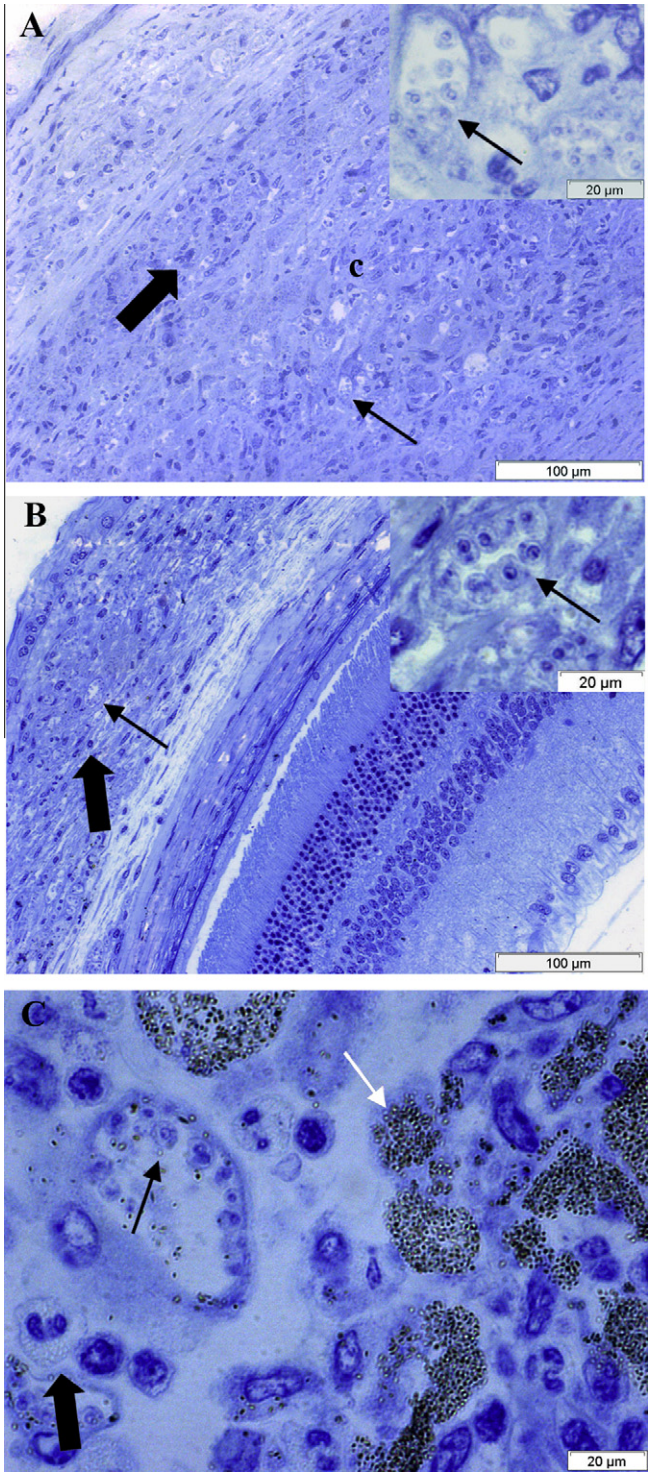

Fig. 1. Eye sections of BALB/c (A and B) and C57BL/10 (C) mice on the 60th day after intravitreal injection with 10^6 promastigotes of *L. amazonensis*. (A) The corneal stroma (c) with intense inflammatory infiltrate (large arrow) and the parasites inside vacuoles (thin arrows). Window show a lot of amastigotes inside vacuoles. (B) An intense inflammatory infiltrate (large arrow) associated with amastigotes inside vacuoles (thin arrows) was observed in the sclerocorneal region. Window shows numerous amastigotes inside vacuoles. (C) Amastigotes (thin arrows) associated with polymorphonuclear cells (outline arrows) and pigmented cells (white arrows) were observed in the sclerocorneal region. Toluidine blue staining was used.

Although the intravitreal route is more commonly used for large animals to study ocular diseases (Garweg et al., 1998) we employed it in this study to establish a direct contact between the

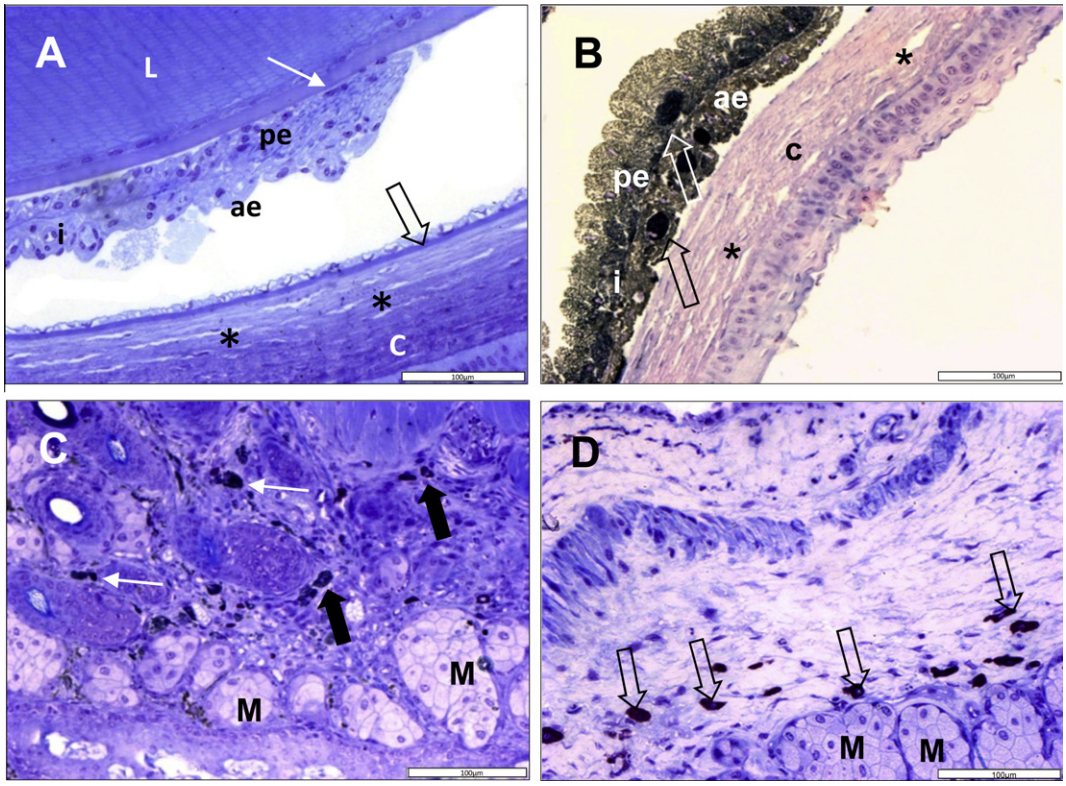

Fig. 2. Histopathology of eyes after instillation of 10^6 promastigotes of *L. amazonensis*. (A) A BALB/c mouse eye on the 90th day post infection: a discreet thickening of the iris (i) and the anterior epithelium (ae) and posterior epithelium (pe) besides an enhancement of interstitial space characterized by a dissociation (∗) can be seen. The lens (L), capsule (c) (white arrow) and corneal endothelium (outline arrow) present normal morphology. (B) In a C57BL/10 mouse on the 90th day post infection a thickening of the iris (i) and the anterior epithelium (ae) and posterior epithelium (pe) associated with pigmented cells (outline arrows) are observed. The cornea presented dissociation of collagen fibers which forms the stroma (∗). (C) A C57BL/10 mouse eyelid on the 30th day post infection shows an intense inflammatory infiltrate (large arrows) in conjunctive tissue around the Meibomian gland (M) where pigmented cells (white arrows) were also seen. (D) A BALB/c mouse eyelid, on the 30th day after infection, with a conjunctival region with numerous intact mast cells (outline arrows) near the Meibomian gland (M) can be seen. Toluidine blue staining was used.

parasite and the retina. Our results showed that both intravitreally and instillation infection routes were able to produce ocular leishmaniasis in mice. No differences were observed in parasitism when mice strains were compared. Parasites were observed when the intravitreal route was used in both mice strains. However even when no parasites were observed in the instillation infected mice, an inflammation was noted. In conclusion, we suggest that the intravitreal route should be preferentially used in experimental leishmaniasis in the murine ocular model, to obtain an ocular infection in a shorter time.

We compared BALB/c and C57BL/10 mice, which are both susceptible to leishmaniasis (Cupolilo et al., 2003). The former is more sensitive, but lacks pigmentation in the epithelium of the retina, iris, choroidal and ciliary body; the latter already has pigmented melanocytes, allowing the evaluation of these cells in the *L. amazonensis* infection, as has been reported by Tedesco et al. (2004, 2005) in murine ocular toxoplasmosis. In addition to ocular structures we evaluated the eyelid, since there are reports of its involvement in cutaneous leishmaniasis although rare, ranging from 2% to 5% of human cutaneous leishmaniasis (Abboud et al., 1970; Baddini-Caramelli et al., 2001; Satici et al., 2004; Tomkins and Bryceson, 1972).

Humoral response was also evaluated to verify possible changes in anti-*Leishmania* antibody levels depending on the inoculation route. A large number of studies have shown that antibody response is less important than cellular response in the development of protective immunity against leishmaniasis (Liew and Odonnell, 1993). It seems that the role of the antibodies in determining the cutaneous leishmaniasis course in the murine model is small, whereas cellular immunity is essential for protection (Olobo et al., 1980). However, Kima et al. (2000) showed that antibodies play a critical role in the pathogenesis of infection caused by parasites of the *Leishmania mexicana* complex, such as *L. amazonensis*. These authors reported that the maintenance of infection by these parasites was impaired with the absence of circulating antibodies in the murine model, which was not observed in our model. Our results showed that although both strains of mice had low titers of IgM and IgG in serum, independent of the inoculation route used, significant eye injuries were still observed.

Evaluating uveitis immunopathology in canine leishmaniasis, Garcia-Alonso et al. (1996) showed that there was no correlation between the level of antibodies circulating in animal serum and the level observed in the aqueous humor. The detection of antibodies in the anterior chamber of animal eyes was independent of antibody levels in serum from the same animal and we believed that the presence of these antibodies could be related to the uveitis processes in the infected animal.

According to Peña et al. (2000), ocular manifestations are common in canine leishmaniasis, occurring in approximately 25% of the cases. Anterior uveitis is the most frequent clinical sign, which was confirmed by our results using the murine model. In humans there is much discussion about the possible origin of the eye infection. The parasite can, according to Nandy et al. (1991), be taken to the eyelid and eyeball by a patient's contaminated finger after scratching a preexisting lesion in another area of the body. Some authors have pointed out the main mechanisms that could explain the involvement of the eyes and eyelids in infection by *Leishmania* spp. inoculation, or by hematogenous contamination. However, the

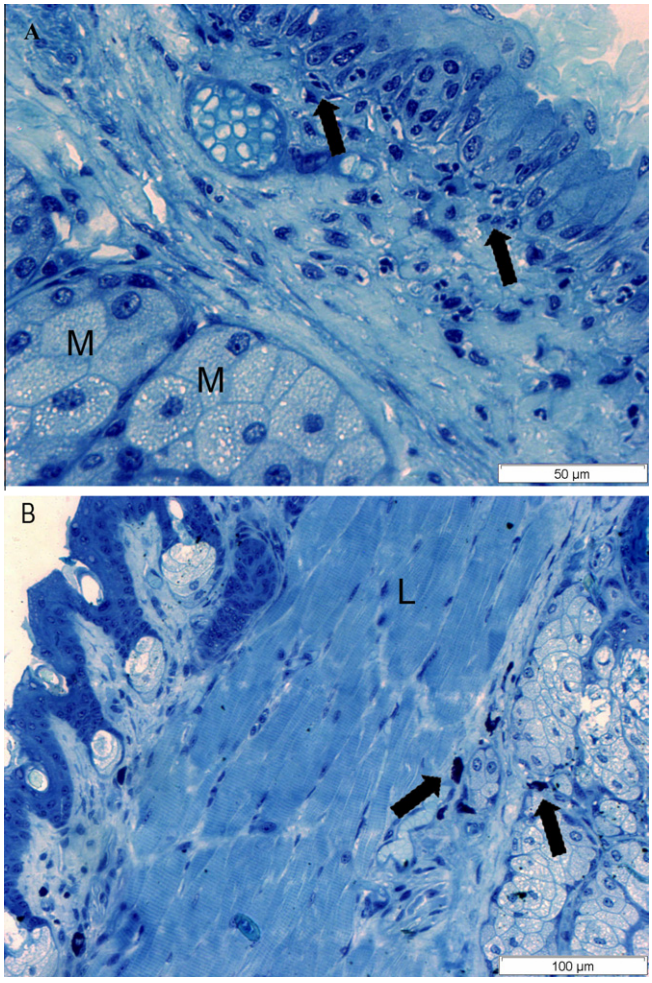

Fig. 3. Histopathology of BALB/c mice eyelid infected with 10^6 promastigotes of *L. amazonensis*. (A) Mild inflammatory infiltrate (large arrow) in the conjunctival tissue of the region and around the Meibomian glands (M) was noted 60 days after infection by instillation. (B) Intact and degranulated mast cells in the conjunctival region, just below the eyelid elevator muscle (L) were observed 90 days after intravitreal infection.

possibility of a hematogenous route is less likely, since other body parts would be involved (Roizenblatt, 1979).

The role of mast cells in *Leishmania* spp. infections has been widely discussed in the literature. It is believed that mast cells aggravate the infection (Ben-Sasson et al., 1990). Experiments using mast cell deficient C57BL/6 mice showed that these animals developed smaller lesions and healed more rapidly than the non-deficient control ones (Galli et al., 1992). However, some authors suggest that the role of mast cells varies with the strain of mice. Saha et al. (2004) demonstrated that in *Leishmania* susceptible animals there was a rapid influx of mast cells to the infection site, which was confirmed by our results, while in resistant mice, this influx does not occur. Also mast cells in resistant hosts have been shown to eliminate parasites at the beginning of the infection, while in a susceptible host they do not kill *Leishmania*. In this case, mast cells are involved in the fibrosis regulation and granuloma formation in the liver, restricting the spread of the parasite (Ashman et al., 1991; Saha et al., 2004). In our model the presence of mast cells in *Leishmania* susceptible animals was noted even though the parasites were not.

Although cutaneous leishmaniasis is considered as self-limiting, if eyelid lesions are not treated, the infection could be spread and engage the conjunctiva, sclera and cornea and even develop interstitial keratitis (Abrishami et al., 2002).

The eyelid may be involved in 2–5% of patients with cutaneous leishmaniasis and ocular lesions in 10–20% of patients with the mucocutaneous form (Morgan, 1965). Different authors have studied the involvement of the eyelid leishmaniasis. Oliveira-Neto et al. (2000) reported five cases of patients with mucocutaneous leishmaniasis, in which the presence of the parasite was not found histologically in the eyelid, but it was subsequently isolated in culture. The same results were reported by Brito et al. (2006) in dogs.

Analyses of the control non-infected C57BL/10 and BALB/c eyelids, instilled with PBS, showed the presence of rare intact mast cells in the conjunctival region. In mice infected via either ocular instillation or intravitreally, an enhancement of intact and also degranulated mast cells was observed, which could explain the absence of parasites in these tissues.

The histopathological evaluation of animal eyes showed the presence of parasites only in the eyes of those intravitreally inoculated, 60 days after infection. Infection by ocular instillation despite being the non-invasive route, caused the discontinuity layer of outer segments of photoreceptors in the retina and dissociation of collagen fibers of the corneal stroma, but less intense than that observed in animals intravitreally inoculated. The damage seen in animals infected by ocular instillation is probably a consequence of the response mediated by hypersensitivity type III, derived from *Leishmania* antigens and deposition of specific and nonspecific immunoglobulins (Garcia-Alonso et al., 1996). Our findings of the dissociation of collagen fibers confirmed the data of Brito et al. (2006). These authors only detected parasites, in dogs naturally infected by *Leishmania* spp., in the eyelid and bone marrow aspirate. Possibly, if the infection via ocular instillation was accompanied over a longer period of time, in the present study, the hypothesis of the appearance of parasites could not be ruled out. In experimental studies in hamsters inoculated with *Leishmania donovani* in the retrobulbar region, Abboud et al. (1970) observed ocular changes consistent with those found in our model, but the presence of amastigotes within macrophages was seen only in the lacrimal gland. Garcia-Alonso et al. (1996) observed the presence of parasites in the ciliary body and iris of dogs naturally infected with *Leishmania infantum*.

We observed in some animals the presence of spaces between collagen fibers and fibroblasts in the corneal stroma, which characterized an edema as previously reported by Brito et al. (2004). Fibroblasts have been described as being susceptible to infection from various species of *Leishmania* (Chang, 1978; Schwartzman and Pearson, 1985), and the presence of this parasite inside these cells in latent disease is frequent. The results of Bogdan et al. (2000) suggest that fibroblasts may form a less hostile environment to *Leishmania* than macrophages, allowing it to remain in the host. Our results clearly show, as observed by Peña et al. (2008), the existence of parasite tropism to the anterior region of the eye, especially the cornea and sclera regions with a significant number of fibroblasts.

In experimental ocular toxoplasmosis Tedesco et al. (2004) showed that there is an immediate reaction of the retinal pigmented epithelium by promoting the migration of its cells to the infection site, which seems to be an immunological phenomenon that could result in parasite elimination through phagocytosis. In our study we did not observe migration of this epithelium in the C57BL/10 animals infected by *L. amazonensis*, however we observed the presence of pigmented cells in the ciliary body and in the iris, associated to the parasite, suggesting a migration of the retinal pigmented epithelium or the differentiation of this epithelium in inflammatory phagocytic cells.

Thus, in the present paper we described ocular leishmaniasis lesions, caused by *L. amazonensis*, in a murine model similar to ocular leishmaniasis lesions observed in symptomatic dogs in visceral leishmaniasis endemic areas.

Acknowledgments

This work was supported by a grant from the Instituto Oswaldo Cruz. Kátia da Silva Calabrese (CNPq no 308630/2008-4) and Ana Lucia Abreu-Silva (CNPq no 306218/2010-0) are senior researchers. The authors thank the instrumental support given by Carl Zeiss of Brazil.

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