The susceptibility of domestic cats (Felis catus) to experimental infection with Leishmania braziliensis


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Abstract

Over the last few years, several cases of feline leishmaniasis (FL) with cutaneous and visceral forms have been reported around the world. Nonetheless, the real susceptibility of cats to infection with Leishmania spp. and the outcome of leishmaniasis in these animals are poorly understood. Experimental studies on feline models will contribute to the knowledge of natural FL. Thus, in order to determine the susceptibility of domestic cats (Felis catus) to experimental infection with Leishmania braziliensis, 13 stray cats were infected with 10⁷ promastigotes by the intradermal route in the ear and nose simultaneously and followed up for 72 weeks. Soon after infection, the earliest indication of a lesion was a papule on the ear at 2 weeks post-infection (w.p.i.). The emergence of satellite papules around the primary lesion was observed about 4 w.p.i. Two weeks later these papules coalesced and formed a huge and irregular nodule. Thereafter, there was lesion dissemination to the external and marginal surface of the ipsilateral ear, and later to the contralateral ear. At 10 w.p.i., some nodules became ulcerated. Nose lesions presented a similar evolution. At both sites, the largest lesion sizes occurred at 10 w.p.i. and started to decrease 15 days later. Ear and nose nodules healed at 32 and 40 w.p.i., respectively. Specific L. braziliensis IgG antibody titers (optical density ≥ 0.01 as positive result) were detected as early as 2 w.p.i. (0.09 ± 0.02) in only three animals (23%), and all cats had positive titers at 20 w.p.i. (0.34 ± 0.06). Only three animals (38%) continued to show positive serology at 72 w.p.i. (0.08 ± 0.02). Up to that time, none of the cats had lesion recurrence. In a feline model of cutaneous leishmaniasis, it seems that there is no correlation between active lesions and positive serology. The implications of these data are discussed.

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Keywords: Tegumentary leishmaniasis; Cat; Leishmania braziliensis; Experimental infection; Serology; Clinical manifestation

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1. Introduction

Leishmaniasis is a widespread zoonotic disease with a great impact on public health. It is endemic in 88 countries around the world (WHO, 2004). In Brazil, Tegumentary Leishmaniasis (TL) is primarily caused by the protozoon Leishmania braziliensis transmitted by the bite of phlebotomines of the genus Lutzomyia. Human TL may produce a variety of clinical syndromes ranging from a simple ulcer to destructive mucosal lesions that may be fatal.

Several vertebrate species are considered to be reservoir hosts, including wild and domestic animals. Among domestic animals, dogs are incriminated in the domestic transmission to human beings. However, due to the marked urbanization of leishmaniasis (Desjeux, 2002), the involvement of other domestic species in TL epidemiology in endemic foci may be possible. Although feline leishmaniasis (FL) is considered to be a rare finding (Costa Durão et al., 1994; Passos et al., 1996; Ozon et al., 1998), several cases of both visceral and cutaneous forms have been reported in the America, Europe, Africa and Asia (Simões-Mattos et al., 2004). Nonetheless, the real susceptibility of cats to infection by Leishmania spp. and the outcome of leishmaniasis in these animals are poorly understood (Shaw et al., 2001). On this basis, experimental studies on feline models will contribute to the knowledge of natural FL. Thus, the main aim of this study was to characterize the outcome of infection in cats experimentally infected with L. braziliensis in terms of clinical manifestations and serological responses.

2. Materials and methods

2.1. Parasites

The MHOM/BR/94/H-3227 strain of L. braziliensis was used to infect cats; originally isolated from a TL patient from Ceará State, Brazil, and typed by agarose gel electrophoresis using isoenzymes according to Momen et al. (1985), and by the indirect fluorescent antibody test (IFAT) using monoclonal antibodies provided by the World Health Organization (WHO). The parasites, stored in liquid nitrogen, were thawed and cultured as promastigotes to 26 °C in Schneider’s insect medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2% sterile normal human urine, 2 mM l-glutamine (Gibco BRL, Grand Island, NY), and antibiotics [100 U/ml penicillin, 100 µg/ml streptomycin sulfate (Sigma)]. Subcultures were prepared during the stationary phase of growth and parasites were used no later than after the fourth passage. Prior to infection, promastigotes were harvested from culture, washed in sterile saline, counted in a Neubauer chamber and adjusted to the appropriate concentration.

2.2. Animals

Thirteen young (3–4 months) female and male domestic cats (Felis catus) obtained from the Center of Zoonosis Control (CZC) of the city of Fortaleza, Ceará State (Brazil) were housed in groups of two in indoor–outdoor shelters (2 m²), and fed a dry maintenance cat food (ProPlan/Gatsy supplied by PURINA; Eukanuba Rice and Chicken supplied by IAMS Co. and Gireze Co., Brazil), with free access to water throughout the study. The animals were assessed clinically, dewormed (Endal Plus supplied by Shering Plough), vaccinated against feline panleukopenia, rhinotracheitis, calicivirus (Tricat supplied by INTERVET) and rabies infections before the experiment. The animals were left in quarantine for a period of 30 days. A survey for the presence of feline leukemia virus (FeLV) was performed in all animals by the indirect fluorescent antibody assay (IFA) using the FeLV kit Detection Set (VMRD, Inc.).

2.3. Experimental infection

The dose of inoculum consisted of $1 \times 10^7$ stationary phase L. braziliensis promastigotes in 20 µl of sterile saline. Before experimental infection, the cats were anesthetized with an intramuscular injection of 2% xylazine, 1 mg/kg body weight and 10% ketamine, 15 mg/kg body weight (Anasedan and Dopalan provided by Vetbrands Saúde Animal—Paulínia, São Paulo, Brazil). Thereafter, the cats were inoculated intradermally with 50-µl Hamilton disposable syringes in the center of the internal surface of the right ear and right side of the nose. Only one cat was not inoculated in the nose due to a small crusted
lesion at that site. Three hamsters (Mesocricetus auratus) were infected in the left foot as strain-infection controls under intramuscular anesthesia of 10% ketamine (Dopalen), 80 mg/kg body weight and 2% xylazine (Anasedan), 10 mg/kg body weight. The Animal Care and Utilization Committee of Universidade Federal do Ceará (Brazil) approved all the experimental procedures conducted on cats and hamsters in the present study.

2.4. Follow-up

All experimental animals were checked weekly by clinical examination. Signs of emergence and size of lesions, lymphadenopathy, visceromegaly, weight loss and nasal manifestation were evaluated up to 72 weeks post-infection (w.p.i.).

Ear lesion samples from nine cats were collected by aspiration under anesthesia and cultured in biphasic (agar–blood–Schneider) medium at 6 w.p.i. Cultures were incubated at 25°C and examined weekly by light microscopy (Nikon–Labophot, Japan, original magnification 400×) over a 4-week period. Imprints of spleen and liver and smears of bone marrow were obtained from four euthanized cats at 4, 12, 16 and 24 w.p.i. The slides were stained with May–Grunwald–Giemsa and evaluated by light microscopy (Nikon—original magnification 1000×). The animals’ euthanasia was performed with previous anesthesia and intravenous injection of 2 mL of 10% potassium chloride (procedure approved by Conselho Federal de Medicina Veterinária [CFMV] in Brazil, according to resolution number 714 of 06/20/2002).

2.5. Antibody determination

Blood was drawn directly from the jugular vein with disposable syringes before infection and at different times after infection for antibody titer determination. The sera were kept frozen at −20°C until the time for assay. The ELISA procedure used was a modification of a previously reported method (Evans et al., 1990). Briefly, a 96-well flat-bottom microtiter plate (Immulon II, Dynatech Laboratories, Inc.) was coated with 50 μl of whole L. braziliensis promastigotes, 10⁶ cells/well, diluted in 0.05 M carbonate–bicarbonate coating buffer (pH 9.6) and left to stand overnight at 4°C. The plates were then aspirated and blocked for 2 h at room temperature with 1.5% fetal calf serum (FCS; Laborclin, Brazil) in 0.01 M phosphate-buffered saline (PBS), washed three times with PBS containing 0.05% Tween-20 (PBS/Tween), and incubated for 1 h at 37°C with 50 μl of serum diluted 1:100 in PBS–1.5% FCS. The plates were washed in PBS/Tween three times and then incubated for 45 min at 37°C with 50 μl of 1:8000 peroxidase-conjugated Protein A (Sigma Chemical Co., St. Louis, MO). After three washes cycle, 200 μl/well of the substrate, 0.055% 2-azinodio-3-ethylbenzthiazoline ([ABTS], Sigma), was added in 0.1 M phosphate–citrate buffer (pH 5.0) containing 0.03% hydrogen peroxide (Sigma). The plates were incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 2N H₂SO₄ and the absorbance was read at 405 nm using an ELISA reader (Titertek Multiskan, Helsinki, Finland). According to previous studies (Simões-Mattos et al., 2001; Simões-Mattos, 2002), titers with optical density (OD) ≥ 0.100 (cut-off) were considered to be positive results.

2.6. Lesion size

Cats were observed daily until the emergence of the lesions, which were then measured (major and minor diameter) once a week. Lesion area (mm²) was calculated using the formula: \( \pi r_1r_2 \), where \( r_1 \) and \( r_2 \) are the major and minor radii of the lesion as previously described by Amaral et al. (2001).

2.7. Data analyses

Data on lesion development in weeks post-infection are reported as median lesion sizes and lesion areas data as standard error of the mean (S.E.M.). Linear regression between lesion size and optical density of specific Leishmania antibodies was performed. A \( p \) value < 0.05 was considered statistically significant.

3. Results

3.1. Establishment of infection and clinical evolution

The evolution of the lesions on the ears and nose is listed in Table 1. On the ear, the earliest lesion was a
single papule (Fig. 1) followed by the emergence of satellite papules around the primary lesion. These papules coalesced and formed a huge and irregular nodule (Fig. 2). Thereafter, there was lesion dissemination leading to the emergence of new lesions on the external and marginal surface of the ipsilateral ear, and later on the contralateral one (Fig. 3). At 10 w.p.i., three cats (25%) showed ulceration of some nodules.

Table 1
Evolution of the lesions in cats (*Felis catus*) experimentally infected with *Leishmania braziliensis*

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Frequency (%)</th>
<th>Median (min–max) in weeks post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Earb</td>
<td>Noseb</td>
</tr>
<tr>
<td>Papule</td>
<td>77</td>
<td>75</td>
</tr>
<tr>
<td>Nodule</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Satellites lesions</td>
<td>83.3e</td>
<td>45.5f</td>
</tr>
<tr>
<td>Huge lesion†</td>
<td>91†</td>
<td>9.1f</td>
</tr>
<tr>
<td>Disseminationd</td>
<td>42.6e</td>
<td>8.3f</td>
</tr>
<tr>
<td>Mucosal infiltration</td>
<td>–</td>
<td>88.9g</td>
</tr>
<tr>
<td>Ulceration</td>
<td>25e</td>
<td>33.3f</td>
</tr>
<tr>
<td>Healing</td>
<td>87.5h</td>
<td>100h</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Frequency (%)</th>
<th>Median (min–max) in weeks post-infection</th>
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<tbody>
<tr>
<td></td>
<td>Ear</td>
<td>Nose</td>
</tr>
<tr>
<td>Lesion onset</td>
<td>Ear</td>
<td>Nose</td>
</tr>
<tr>
<td>Papule</td>
<td>2 (1.5–3)</td>
<td>2 (1.5–3)</td>
</tr>
<tr>
<td>Nodule</td>
<td>3.5 (2.5–5)</td>
<td>3 (2.5–10)</td>
</tr>
<tr>
<td>Satellites lesions</td>
<td>4 (4–11)</td>
<td>6.5 (5–11)</td>
</tr>
<tr>
<td>Huge lesion†</td>
<td>6 (2–8)</td>
<td>10 (1–13)</td>
</tr>
<tr>
<td>Disseminationd</td>
<td>8.5 (7.5–22)</td>
<td>7.5 (7.5–7.5)</td>
</tr>
<tr>
<td>Mucosal infiltration</td>
<td>–</td>
<td>16 (6–22)</td>
</tr>
<tr>
<td>Ulceration</td>
<td>10 (9–11)</td>
<td>7 (6–7.5)</td>
</tr>
<tr>
<td>Healing</td>
<td>32 (30–33)</td>
<td>40 (33–44)</td>
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</table>

<table>
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<tr>
<th>Lesion</th>
<th>Frequency (%)</th>
<th>Median (min–max) in weeks post-infection</th>
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<tbody>
<tr>
<td></td>
<td>Ear</td>
<td>Nose</td>
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<tr>
<td>Lesion length</td>
<td>Ear</td>
<td>Nose</td>
</tr>
<tr>
<td>Papule</td>
<td>2 (0.5–3)</td>
<td>1 (0.5–2)</td>
</tr>
<tr>
<td>Nodule</td>
<td>28 (11–34)</td>
<td>41 (34–47)</td>
</tr>
<tr>
<td>Satellites lesions</td>
<td>2 (1–7.5)</td>
<td>2.5 (1–11)</td>
</tr>
<tr>
<td>Huge lesion†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Disseminationd</td>
<td>3 (2–6.5)</td>
<td>2 (2–2)</td>
</tr>
<tr>
<td>Mucosal infiltration</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ulceration</td>
<td>9 (4–16.5)</td>
<td>4.5 (4–21)</td>
</tr>
<tr>
<td>Healing</td>
<td>38 (34–72)</td>
<td>29 (43–72)</td>
</tr>
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</table>

<table>
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<tr>
<th>Lesion</th>
<th>Frequency (%)</th>
<th>Median (min–max) in weeks post-infection</th>
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</tr>
<tr>
<td>Healing</td>
<td>87.5h</td>
<td>100h</td>
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Fig. 1. Papule formed on the ear of a cat experimentally infected with $10^7$ promastigotes of *Leishmania braziliensis*.
and the ulcers closed and opened several times. The evolution of nose lesions was similar to that of ear lesions (Fig. 4). In addition, most animals had mucosal infiltration with partial nostril obstruction around 16 w.p.i. In both places, the lesion size peaked at 10 w.p.i., with a reduction occurring thereafter. Total resolution of the ear and nose lesions occurred at 32 and 40 w.p.i., respectively (Fig. 5). Only one cat had

Fig. 2. A huge and irregular nodule formed by several coalescing papules on the ear of a cat experimentally infected with 10⁷ promastigotes of *Leishmania braziliensis*.

Fig. 3. Ulceration (arrow) of the primary lesion, and dermal dissemination to the ipsilateral and contralateral ear.
lesion recurrence on the ear 4 months after self-healing. Up to 72 w.p.i., only one cat showed an apparent alopecic scar on the ear (Fig. 6).

Regional lymph node enlargement was observed at 11 w.p.i. in most animals (92.3%), ranging from 1.5 to 2.0 cm in diameter. Actually, enlargement of regional lymph nodes was detectable in two out of eight cats. However, at that time the disease was under control in all cats and all animals are apparently healthy. Four animals were euthanized at 4, 12, 16 and 24 w.p.i. for further histopathological study and one cat died at 24 w.p.i., but could not be submitted to post-mortem

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Fig. 4. Ulcerated lesion on the nose at 6 weeks post-infection in a cat experimentally infected with $10^7$ promastigotes of *Leishmania braziliensis*.

Fig. 5. Evolution of lesion size in mm$^2$ (mean ± S.E.M.) and of the humoral response in optical density (OD [mean ± S.E.M.]) throughout the trial in cats experimentally infected with $10^7$ promastigotes of *Leishmania braziliensis*.
evaluation due advanced autolysis. Eight cats are still kept under clinical observation to evaluate the probability of recurrence of cutaneous lesions.

3.2. Parasite detection

Parasite growth was obtained in NNN medium from aspirates of the primary ear lesion of 8 out of 9 animals (89%) at 6 w.p.i. Cultures and imprints from liver, spleen and bone marrow were negative for the four euthanized cats.

3.3. Behavior of the serological anti-Leishmania response and FeLV detection

Specific-Leishmania IgG antibody titers were first detected in only three animals (23%) at 2 w.p.i. (0.09 ± 0.02). The specific antibody concentrations in the serum of infected animals were low up to 12 w.p.i. At that time, serum conversion was observed in 50% of cats (0.10 ± 0.01). At 20 w.p.i., all cats were serologically positive (0.34 ± 0.06), and showed significant concentrations of specific antibody titers about 26 w.p.i. (0.45 ± 0.07). After that time, there was a significant fall in specific antibody production, and at 72 w.p.i., only 38% of the animals had detectable antibodies (0.08 ± 0.02). In all cats, positive antibody titers lasted 16 weeks (median). Regarding the feline leukemia virus, 9 out of 13 (69.2%) cats had a positive FeLV assay.

3.4. Correlation between lesion development and serology

Fig. 5 shows that the emergence of lesions preceded the occurrence of anti-Leishmania antibodies. In spite of a marked reduction in size and number of lesions at 32 w.p.i., antibody titers continued to be high. At 72 w.p.i., even with clinical recovery, three of eight cats (37.5%) still had detectable antibody titers. There was no correlation between anti-Leishmania antibody concentration in serum and ear lesion size throughout the experimental trial. However, there was a positive correlation between nose lesions and serology at 12 and 16 w.p.i. ($r^2 = 36.6\%, p = 0.037$ and $r^2 = 44.12\%, p = 0.0258$, respectively).

Fig. 6. Healing of the lesions of experimental feline leishmaniasis. Same cat as illustrated in Fig. 3. Note a small scar at the site of an old ulcer (arrow) at 72 weeks post-infection.
3.5. Non-specific signs

From the time of lesion manifestation until self-healing, 10 cats sneezed and/or had nasal discharge and were treated with 2.5% enrofloxacin, 5 mg/kg/IM. In addition, they showed lusterless, dry and brittle hair. However, up to 72 w.p.i., only one cat showed bilateral alopecia, especially on both legs, and another had slight sneezing. Also, weight loss and weakness were observed in five cats during the first 32 w.p.i.

4. Discussion

Although leishmaniasis is considered to be a rare occurrence in cats, several cases have been reported around the world in recent years (Bez, 1992; Marechal, 1993; Simões-Mattos et al., 2004). This may be associated in part with an increased incidence of Leishmania infection, with advances in diagnostic techniques, with increased breeding of cats in developed countries, and/or partly with greater health care devoted to pets.

Cutaneous lesions in cats naturally infected with Leishmania spp. occur mainly on the nose (Pennisi, 1999), followed by the ears (Craig et al., 1986), or at both sites (Machattie et al., 1931; Mello, 1940; Bonfante-Garrido et al., 1996). Therefore, we chose both sites for experimental infection. The time of evolution and the characteristics of the lesions observed in this study were similar to those of natural feline leishmaniasis (Barnes et al., 1993; Bonfante-Garrido et al., 1996; Laruelle-Magalon and Toga, 1996; Ozon et al., 1998; Hervás et al., 1999; Pennisi, 1999) as well as human cutaneous leishmaniasis (Grimaldi, 1982). Nonetheless, ulcers seem to be much more frequent in human beings (Costa et al., 1990) and dogs (Pirmez et al., 1988) than in the cats of this study.

The resolution of the lesions of the experimentally infected cats (Fig. 2) was slightly faster than observed in natural human cutaneous leishmaniasis (Costa et al., 1990). However, this self-healing may not signify the total absence of parasites. The presence of Leishmania antigens or amastigotes may persist in the cicatricial lesion, as already observed in the skin of dogs (Oliveira-Lima, 1996) and human beings (Schubach et al., 2001), and in a resistant model of murine leishmaniasis (Belkaid et al., 2001). This may explain the recurrence of lesions in a cat with leishmaniasis 7 years after surgical excision of the first lesion (Barnes et al., 1993).

In our study, about 40% of the cats had dermal dissemination to other sites. Apparently there was no visceral dissemination as indicated by the absence of Leishmania amastigotes in bone marrow, spleen and liver of the four cats that were examined. At least in this experimental model, the parasite seems to affect only the skin. However, dissemination of L. braziliensis protozoa to the viscera has been reported in natural human infection (Sousa et al., 1995), as well as in experimental infection in hamsters (Sinagra et al., 1997).

The immune response mediated by antibodies is not considered to be protective against the intracellular Leishmania parasites. It is known that, at least in canine visceral leishmaniasis, high antibody titers indicate active disease (Quinnell et al., 2003) and potential transmission of the protozoa to the vectors. In our study, the period during which the cats showed active lesions that harbored parasites and potentially acted as reservoirs could not be determined by conventional serological methods. The mean seroconversion of antibody titers occurred when lesions were in the resolution stage. In addition, the peak of the antibody titers was verified at 20 w.p.i., when lesions were significantly decreased in size. Similarly, there was a low correlation between lesion size and antibody titers. These data suggest that serology is not a good marker of the clinical course of feline TL and therefore, from an epidemic point of view, the absence of anti-Leishmania antibodies in the serum of cats with cutaneous lesions may lead to a clinical misdiagnosis in the differential diagnosis from other diseases. Thus, the lack of an early diagnosis of FL in endemic areas may imply that the animal will continue to represent a potential risk of Leishmania transmission to the vectors. In addition, it is important to emphasize that previous studies have shown that cats are more attractive (Johnson et al., 1993) and are more used as bloodmeal sources by biting phlebotomine sandflies (Ogosuku et al., 1994) by some phlebotomine species than dogs. In addition, in a previous assay with Lutzomyia migonei, one of the main vectors of L. braziliensis in Brazil, about 90% of the engorged female sandflies were recovered after contact with a cat in a cage (J.W. Oliveira-Lima, personal commu-
Another important finding of this study was the 38% rate of cats that continued to show positive serology at 72 w.p.i. Kirkpatrick et al. (1984) obtained similar findings in cats experimentally infected with L. chagasi and L. infantum. Apparently, this seems to indicate a condition of occult infection (silent phase) rather than sterile healing. In this respect, the remaining cats of this study should be currently under observation to determine the possible recurrence of cutaneous lesions. In addition to feline TL, the high rate of infection with feline leukemia virus (69.2%) among the experimental cats of this study should be considered regarding the probability of recurrence. According to previous studies on murine models, co-infection with murine leukemia virus (MLV) and L. major showed an important worsening of the outcome of the disease (Barral-Netto et al., 1995). It is possible that, like MLV and HIV, feline immunodeficiency virus (FIV) and FeLV as well as other feline myeloproliferative diseases and senility may be also able to promote TL recurrence in cats.

In conclusion, domestic cats provide an animal model that is partially susceptible to experimental infection with L. braziliensis. Cats showed chronic clinical manifestations, anti-Leishmania antibody titers, lesions harboring parasites, and spontaneous healing of the lesions. Our data and other findings in the literature allow us to speculate that domestic cats have all the properties needed to serve as potential reservoirs of Leishmania. Thus, further epidemiological studies are necessary to determine the real role of cats in the transmission of leishmaniasis in endemic areas. In addition, experimental studies on feline models infected with several Leishmania species may contribute to the knowledge of natural FL.

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