Canine Experimental Infection: Intradermal Inoculation of *Leishmania infantum* Promastigotes

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Five mixed breed dogs were inoculated intradermally (ID) with cultured virulent stationary phase promastigotes of Leishmania infantum *Nicole, 1908 stocks recently isolated. Parasite transformations in the skin of ID infected dogs were monitored from the moment of inoculation and for 48 h, by skin biopsies. Anti-*Leishmania *antibody levels were measured by indirect immunofluorescence assay, counterimmunoelectrophoresis and direct agglutination test, and clinical conditions were examined. Thirty minutes after ID inoculation the first amastigotes were visualised and 3 to 4 h after inoculation the promastigotes were phagocyted by neutrophils and by a few macrophages. These cells parasitised by amastigotes progressively disappeared from the skin and 24 h after inoculation parasites were no longer observed. Local granulomes were not observed, however, serological conversion for antibodies anti-*Leishmania *was achieved in all dogs. Direct agglutination test was the only technique positive in all inoculated dogs. Amastigotes were found in the popliteal lymph node in one dog three months after inoculation. This work demonstrates that, with this inoculum, the promastigotes were transformed into amastigotes and were up taken by neutrophils and macrophages. The surviving parasites may have been disseminated in the canine organism, eliciting a humoral response in all cases.*

Key words - *Leishmania infantum* - dogs - promastigotes - intradermal inoculation

Visceral leishmaniasis (VL) caused by *Leishmania infantum* Nicole, 1908, is a severe, often fatal disease common in the Mediterranean region where dogs and wild canids are the main reservoir (Abranches 1984, Peters & Killick-Kendrick 1987).

Many epidemiological studies have been carried out in the dog, the domestic reservoir of VL, especially in Italy where the highest prevalence of 23.9% was detected in Monte Argentario (Pozio et al. 1981). In France, Nice, the highest prevalence was cited as 17.1% (Jambou et al. 1986) and in Portugal the highest prevalence, 20.2%, was found in "Terra Quente" (hot land) of Alto Douro Valley (Abranches et al. 1993).

In recent years there has been an upsurge of interest in the study of leishmania animal models partly due to the promise of control of this disease but also reflecting the ability of such models to illustrate various aspects of the immune response and parasite transmission.

Dogs might be considered useful models for the study of human VL, as well as for the development of new prophylactic and therapeutic programmes since canine VL is similar to the human disease (Peters & Killick-Kendrick 1987). Leishmania infection is transmitted by the bite of a female phlebotomine sand fly, which inoculates metacyclic promastigote forms (Killick-Kendrick 1990).

This study describes an experimental model for infection of dogs by intradermic route (ID) using virulent cultured promastigotes of *L. infantum* zymodeme MON-1. Parasite transformation in the skin from the moment of inoculation and during a period of 48 h was monitored. It was also verified if the inoculation induced a humoral response in dogs.

MATERIALS AND METHODS

Parasites - Promastigotes of *L. infantum* zymodeme MON-1, subserotype B2, was used in the inoculations. This stock was isolated from a naturally infected Portuguese dog, in NNN medium and incubated to 24ºC. Virulent promastigotes were collected from the stationary phase of a subculture with less than five passages (Santos-Gomes & Abranches 1996).

Animals - Eight mixed-breed dogs, of both sexes, were housed in the Instituto de Higiene e Medicina Tropical animal facility under stable climatic and diet conditions throughout the experiments. Prior to inclusion in this study, dogs were treated with antihelminthics, vaccinated against leptospirosis, parvovirosis, hepatitis, distemper and rabies. The animals were observed for clinical signs of leishmaniasis and absence of specific antibody, anti-*Leishmania,* was determined by indirect immunofluorescence assay. The dogs were inoculated between the ages of 2 and 3 years.

Infection - Five dogs (D3, D4, D5, D6, D7) were inoculated intradermally (ID) in several places of the abdomen, with a total of 10^8 promastigotes/ kg of body weight. Three healthy dogs, not inoculated, were used as controls (D8, D9 and D10).

Parasitological studies - Skin biopsies from dogs inoculated ID were carried out at 5 min, 30 min, 1.5, 3.5, 6.5, 24 and 48 h after inoculation. The biopsies were taken from the exact site of the inoculations. Each biopsy was cultured in NNN medium. Impression smears were also done for microscopic observation.

The dogs were observed over a period of five months after inoculation. Animals were examined monthly for clinical signs of leishmaniasis (Pozio et al. 1981). Popliteal lymph nodes were enlarged in dogs D5 and D7. Parasitological examinations of these dogs were carried out by popliteal lymph node tissue biopsy and preparations stained with Giemsa were examined by microscopy. This material was also cultivated in NNN medium. Cultures were incubated at 24ºC, passaged and examined weekly over a five-week period.

Immunological surveillance - Peripheral blood samples were taken each month for immunological analysis. Parasite specific antibodies were determinated by indirect fluorescent immunoassay (IFI), counterimmunoelectrophoresis (CIE) and direct agglutination test (DAT). The IFI was carried out as described by Abranches (1984). The antigen was prepared from *L. infantum* zymodeme MON-1, maintained by weekly passages in NNN medium. The dilution of 1:128 was considered the limiting titre (Abranches 1984). CIE was performed according to Campino et al. (1995). The antigen was prepared from *L. infantum* zymodeme MON-1 according to Mansueto et al. (1978). Sera samples were used undiluted. All the reactions with at least one precipitation arc were considered positive (Campino et al. 1995). DAT was performed according to Harith et al. (1989). The antigen was kindly provided by A Harith and its preparation is described in Harith et al. (1986). The dilution of 1:320 was considered the limiting titre (Harith et al. 1989).

RESULTS

Skin biopsies - Five minutes after the ID inoculation of stationary phase promastigotes, the microscopic observation of skin biopsies impression smears showed the presence of promastigotes identical to the inoculated ones (Fig. 1A). After 30 min, a mixture of large promastigotes, short promastigotes, enlarged amastigotes (Fig. 1B), metacyclic-like promastigotes, and extracellular typical amastigotes was observed. At 1.5 h post inoculation, metacyclic-like promastigotes and extracellular amastigotes were visible in the skin biopsies impressions. By 3.5 h post inoculation, numerous neutrophils and some macrophages parasitised with amastigotes, extracellular enlarged amastigotes (Fig. 1C), extracellular amastigotes, promastigotes, and metacyclic-like promastigotes were observed. Other intermediate forms under dividing process were also noted. At 6.5 h after inoculation, metacyclic-like promastigotes, extracellular amastigotes (Fig. 1D), and promastigotes with short flagellum remained in the skin. Parasitised cells were not observed. Twenty-four h after inoculation, the presence of parasites in the skin was no longer observed. Epidermal Langerhans cells were not observed and the skin cultures were negative for all the dogs.

Clinical and parasitological observations - At the site of inoculations of all the dogs ID inoculated (D3, D4, D5, D6 and D7), nodules or lesions were not observed. All the animals maintained asymptomatic except for dogs D5 and D7, which exhibited moderate enlargement of popliteal lymph node 2-4 months after inoculation.

Only in dog D5 were parasites observed in lymph node biopsies two months after infection.

Immunological observations - All five inoculated dogs showed humoral responses between the first and the third month after the infection. DAT was positive with significant titre in all dogs. Only dogs D5 and D7 showed positive serological conversion by the three techniques, with the IFI (1:512) achieving significant titres. CIE was also positive in the dog D6. The reactions were negative in the three non-inoculated dogs (Table).

DISCUSSION

Leishmania transmission from sand flies to the vertebrate host is achieved by inoculation of infective metacyclic promastigotes. The developmental sequence of the parasite after being inoculated in the skin is not well known. In this study, dogs were experimentally infected by intradermal inoculation of promastigotes obtained from stationary phase of culture, when virulent metacyclic-like forms are more abundant (Mallison & Coombs 1989, Bandyopadhyay et al. 1991). During the first
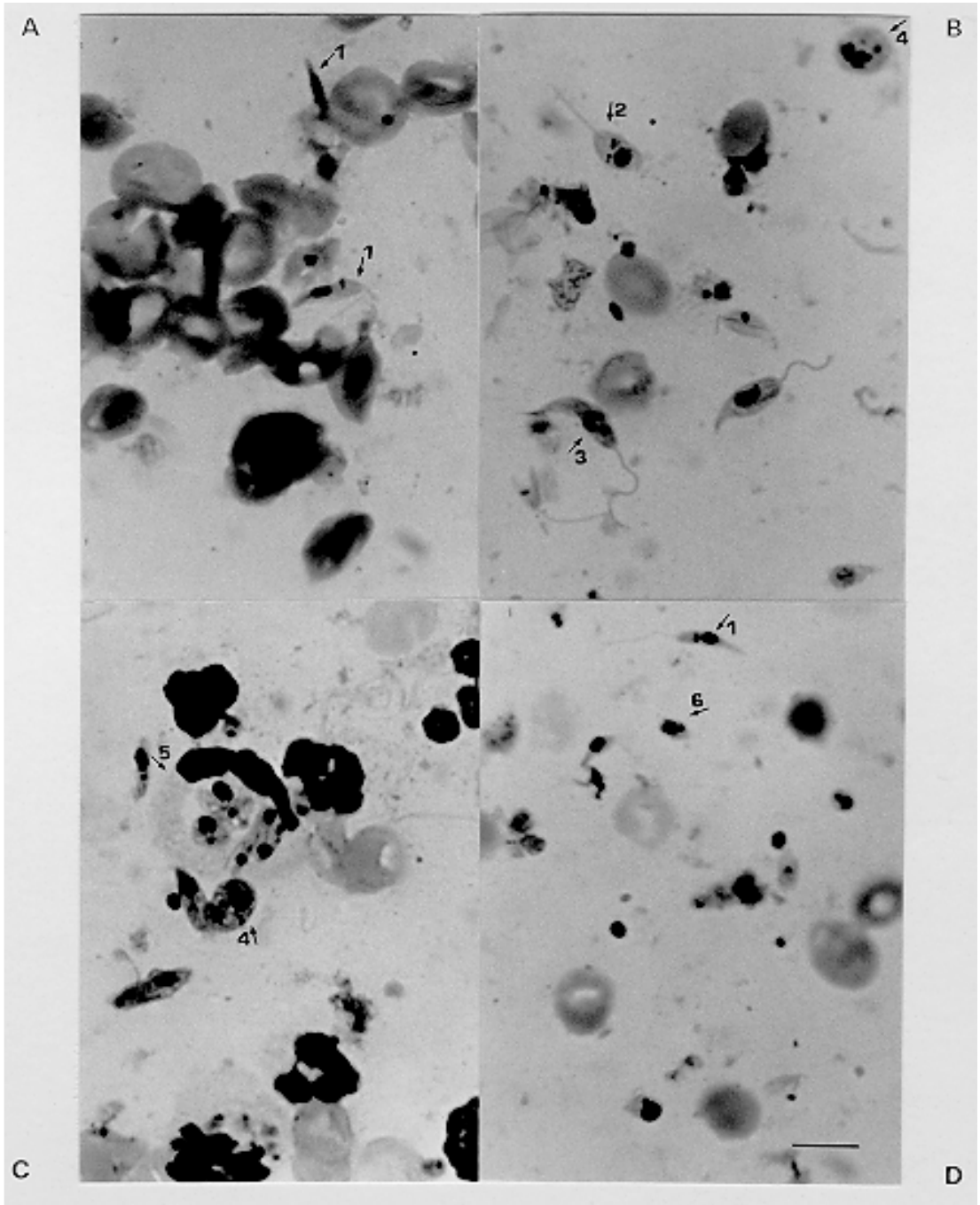
Morphology of parasites in Giemsa-stained skin biopsies impression from dogs intradermally inoculated with stationary phase promastigotes 5 min (A), 30 min (B), 3.5 h (C) and 6.5 h (D) after inoculation. All pictures are shown at the same magnification (X1000, Bar = 10 µm). 1: metacyclic-like promastigote; 2: short promastigote; 3: large promastigote; 4: enlarged amastigote; 5: neutrophil parasitised with amastigotes; and 6: amastigote.

TABLE

Anti-*Leishmania* antibodies detected by indirect
fluorescent immunoassay (IFI),
counterimmunoelectrophoresis (CIE) and direct
agglutination test (DAT) and the results of
parasitological examinations (Paras Exam) carried out
in the dogs

Dog no.	IFI	CIE	DAT	Paras Exam
D3	<1:128	-	1:1280	ND
D4	<1:128	-	1:2560	ND
D5	1:512	+	1:1280	+
D6	<1:128	+	1:5120	ND
D7	1:512	+	1:320	-

ND: not done

3 h following inoculation, promastigotes went through several modifications from promastigote to amastigote form. This last form was detected early, at 30 min after inoculation, but only at 3.5 h amastigotes were observed inside neutrophils and macrophages. These cells disappeared quickly and at 6.5 h post infection no more parasitised cells were found in the sites of inoculation. Promastigotes were more persistent; 6.5 h after inoculation, some of these forms still were observed in the collected material. The phenomenon described here, in experimental infected dogs, has never been described in natural infection, as far as we know.

The leishmaniome, a small local nodule in the place of inoculation, was never observed, and the parasites were no longer in the skin 24 h after being inoculated. The leishmaniome was described in human visceral leishmaniasis caused by *L. donovani* (Manson-Bahr 1959, 1961), in natural infected dogs by *L. infantum* (Vidor et al. 1991) and in experimentally infected dogs by *L. infantum*, 6-14 weeks after being inoculated with promastigotes collected from sand flies (Killick-Kendrick et al. 1994). The study described here is also of canine experimental infection, but differs in the following aspects. The experiment was carried out with a high inoculum of cultured promastigotes from the stationary phase, which refers the total number of promastigotes forms and not only virulent metacycle-like promastigotes. The inoculum used here does not include saliva of the insect vector. Indeed, a possible role of sand fly saliva in the formation of inoculation chancres could explain the absence of leishmaniome in the present study. However this role has not been proved, as verified by Killick-Kendrick et al. (1994). Although, the presence of sand flies salivary glands in the experimental inoculation of dif-

ferent species of *Leishmania* in the rodent model seems to increase the severity of infection (Titus & Ribeiro 1988, Samuelson et al. 1991, Theodos et al. 1991, Lima & Titus 1996, Belkaid et al. 1998).

The present study describes the appearance of neutrophils and macrophages parasitised with amastigotes, which were found 3.5 h after inoculation. Apparently they do not remain a long time at the site of inoculation, since parasitised cells are not observed at 6.5 h PI. The presence of neutrophils has been reported in several leishmaniasis infections, both in murine experimental models (Laison & Shaw 1979, Grimaldi et al. 1984, Pompeu et al. 1991, Belkaid et al. 1998) and in human ulcers of human natural *L. infantum* infection (Rab et al. 1992). Existence of neutrophils at the site of infection seems to be elicited by leishmania and serum components, these last generated by the presence of leishmania (Sorense et al. 1989). Most leishmanias taken up by neutrophils were probably destroyed, as was observed by Pearson and Steigbigel (1981) with human polymorphonuclear leukocytes infected with *L. donovani* and by Pompeu et al. (1991) in Balb/c mice infected with *L. amazonensis*, while the preservation of amastigotes inside macrophages is usual (Cook 1996).

The increase of Langerhans cells in the dermis of mice infected with *L. major* was reported by Moll et al. (1993). Based on this model, the authors proposed that Langerhans cells take up parasites in the skin and transport them to the draining lymph node for presentation to T cells and initiation of the immune response. Although, in the present study Langerhans cells were not observed in the skin.

The work described here demonstrates that the inoculum of virulent cultured promastigotes by ID route in canine model is successful in transformation of promastigotes into amastigotes which are phagocyted by neutrophils and macrophage cells, and that these transformations occur between 30 min and 24 h after infection. The fact that no parasites were found after 24 h indicates that this phenomenon is different from the one observed after inoculation of promastigotes collected from the vector in which all dogs developed skin lesions (Killick-Kendrick et al. 1994). The existence of immunological response in all inoculated dogs and the presence of parasites in the popliteal lymph node (confirmed in one case), may suggest that macrophages cells could play a role as carriers for the dissemination of parasites.

In different degrees, all the inoculated dogs showed significant humoral responses shown by one or more techniques which persisted during the observation period. DAT was the most sensitive

technique and the only one positive in all inoculated dogs.

The fact that such high inoculum of stationary phase promastigotes did not induced signs of disease could be due to the constitution of the inoculum, which did not have only virulent promastigotes and no sand fly saliva. Furthermore the period of incubation in dogs is usually larger than the period of observation in this study. In experimental infections by *L. infantum* either through the bite of a sand fly (Rioux et al. 1979) or by intradermal injections of metacyclic promastigotes from sand flies (Killick-Kendrick et al. 1994) the incubation period was from 16 to 24 months. Studies carried out in naturally infected dogs indicate that canine leishmaniasis has a prolonged asymptomatic period (Adler & Theodor 1932, Abranches et al. 1991). It should also be mentioned that in this study the evaluation of the consequence of the infection (self-cured or evolved) was not carried out.

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