

Experimental Infection of Canine Peritoneal Macrophages with Visceral and Dermotropic *Leishmania* Strains

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A study was carried out using macrophages cultured from the peritoneal exudate of dogs infected in vitro with three species of Leishmania: L. (L.) chagasi, L. (Viannia) braziliensis and L. (L.) amazonensis with the aim of investigating the growth kinetics and infectivity of these species in the host cell.

Results were expressed as the percentage of macrophages infected measured at 24 hr intervals over six days in RPMI - 1640 culture medium at a temperature of 34-35°C.

The findings open the possibility of using canine peritoneal cells as a model for the screening of leishmanicide drugs and to study the pathogenesis of these species.

Key words: leishmaniasis - macrophages - dogs

Leishmanias are obligate intracellular parasites that multiply inside the cells of the mononuclear phagocytic system of vertebrate hosts. The multiplicity of species associated with transmission conditions by different vector species and host immune conditions generate variable clinical manifestations (Grimaldi Jr 1982, Marzochi & Marzochi 1994).

Changes introduced by humans to natural environments favor the installation of non-sylvan foci, thereby leading to the involvement of various species of domestic animals, mainly canids and equids (Marzochi 1992, Marzochi & Marzochi 1994), thus fostering the maintenance and expansion of peridomestic cycles.

The frequent presence of household dogs with high infection rates associated with the human disease in endemic areas for American tegumentary and visceral leishmaniasis, as well as the presence of the same genotypic pattern of the parasite circulating in human and canine populations prove the involvement of dogs in the peri and intradomestic transmission cycle of both diseases (Lopes et al. 1984, Pacheco et al. 1986). The same has been observed with regard to equine species in American tegumentary leishmaniasis (ATL) areas (Barbosa-Santos et al. 1994).

Thus, domestic dogs have been the target of several studies and are considered an excellent model for experimental infections (Chapman et al. 1979, Pirmez et al. 1988). However data concerning the treatment of disease in dogs are limited.

In attempts to evaluate experimental treatments various models have been used in studies with these protozoa. However, *in vitro* cellular models offer the greatest advantages for the objective at hand concerning the *in vivo* system, including both simplicity and speed in obtaining results (Berman 1985).

Macrophages, described as pioneering cells surviving in axenic conditions (Carrel & Ebeling 1922) have been used under different experimental conditions and obtained from different anatomical sites from various animal species. Such cells represent an ideal target cell population, mainly for studies of parasites from the *Leishmania* genus.

We have performed an *in vitro* investigation employing canine macrophages infected separately with three species of *Leishmania* in order to monitor the multiplication kinetics and possible standardization for trials using drugs with leishmanicide properties.

MATERIALS AND METHODS

Parasites - We used stationary-phase cultured promastigotes of *L. (Viannia) braziliensis* (MHOM/BR/95DCB-22), amastigotes of *L. (L.) amazonensis* (MCAN/BR/94DCB-16) and *L. (L.) chagasi* (MHOM/BR/95/DCB-27).

Macrophages - Macrophages were obtained from healthy adult male and female mongrel dogs kept at the Jorge Waitsman Institute of Veterinary Medicine, under the Rio de Janeiro Health Secretariat. The health of the animals was established on

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the basis of results of a clinical examination and the evaluation for antibodies to *Leishmania* using the indirect immunofluorescence reaction. The dogs had their peritoneal cavity washed four days after stimulation with Sephadex G50 (Pharmacia) at a concentration of 70mg/kg body weight.

Dogs were anesthetized with an intravenous injection of sodium pentobarbital (Ceme, Brazil) at 25 mg/kg body weight. The peritoneal cavity was washed with a 500 ml volume of 0.85% physiological saline solution containing 0.3% sodium citrate, 100 U penicillin, and 100 µg streptomycin/ml. Approximately 80% of the lavage fluid was recovered, packed at 4°C, and immediately processed.

Isolation and infection of macrophages - The lavage fluid was concentrated by centrifugation at 800 g for 10 min at 4°C and resuspended in RPMI-1640 containing 100 U penicillin and 50 µg streptomycin/ml. Cell viability was determined using 0.2% trypan blue exclusion dye (Phillips 1973) and adjusted to give a cell density of 2×10^6 per ml, using a Neubauer hemocytometer in RPMI-1640 medium without serum.

Each milliliter of cell suspension was placed in a test tube (Vidrolabor 15cmx10cm) containing a coverslip. The tubes were incubated at 35°C for 2-3 hr. There after, the medium was removed and each tube was washed with RPMI-1640 medium to remove the non-adherent cells. After washing, RPMI-1640 was added, containing antibiotics and 10% sterile inactivated homologous canine serum.

After 24 hr in dishes, the cell monolayers were infected at a ratio of 5-10 parasites per host cell with incubation at 35°C when the infection was with *L. (L.) amazonensis* and *L. (L.) chagasi* and 34°C when the infection was by *L. (V.) braziliensis* for a period of 2-3 hr. After this period, the cell monolayers were washed, complete RPMI was added, and the respective temperatures were maintained. At 24 hr intervals the coverslips were washed in PBS, fixed in methanol, and stained with Giemsa. The percentage of infected cells was determined by individual counts on the slide covers under light microscopy, making a total of 100 cells.

RESULTS

The results of this investigation, as shown in Fig. 1, indicate that 24 hr after infection the percentage of infected cells were 81% for *L. (V.) braziliensis*, 67% for *L. (L.) amazonensis*, and 90% for *L. (L.) chagasi*. These values increase over the course of the study and by the sixth day post-infection they stood at 92%, 95%, and 98% of infected cells, respectively.

Fig. 2 shows peritoneal macrophages infected with *L. (V.) braziliensis*, *L. (L.) amazonensis* and *L. (L.) chagasi* at 3, 5 and 6 day post-infection re-

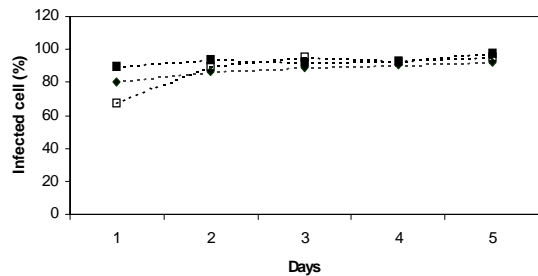


Fig. 1: the percentage of cells infected with *Leishmania* (*Viannia*) *braziliensis* (····), *L. (L.) amazonensis* (- - -) and *L. (L.) chagasi* (- · - ·) with mean of two experiments during six days of observation.

spectively. At least two different patterns of infection were observed during the experiment on macrophages infected by each *Leishmania* specie. The aspect of cells parasited with *L. (L.) amazonensis* is typical providing the appearance of many vacuoles. By the way on the two others epecies the parasite multiplication is closer.

DISCUSSION

The use of canine peritoneal macrophages for *in vitro* studies has been sparsely documented, although different cell lines have been used experimentally in various investigations, displaying advantages and disadvantages (Mattock & Peters 1975, Chang 1980, Berman & Wyler 1980).

To our knowledge this is the first report concerning the utilization of canine peritoneal macrophages experimentally infected with three species of *Leishmania*, since the literature only includes utilization of these cells in association with infection by *Ehrlichia canis* (Nyindo et al. 1971, Stephenson & Osterman 1977). Lack of utilization of this *in vitro* cellular model may be resultant from the inherent difficulties associated with keeping larger animals as compared to smaller rodents.

The specific purposes of this investigation led us to develop a relatively simple technique for washing the peritoneal cavity of dogs, providing up to 10^9 cells on total volume, while utilization of special cannulae in this procedure (Zeman & Neri 1970) eliminates the need to sacrifice the animals.

In our study, the characteristics displayed by the cells cultured *in vitro* were similar to those described elsewhere, presenting single spherical and kidney-shaped nuclei located excentrically in the cell (Stephenson & Osterman 1977). However, in some cases there was not a good spreading of the cells, which may be linked to biological factors including state of cell maturation and age and sex of the donor animals, factors which cannot be controlled by our methodology.

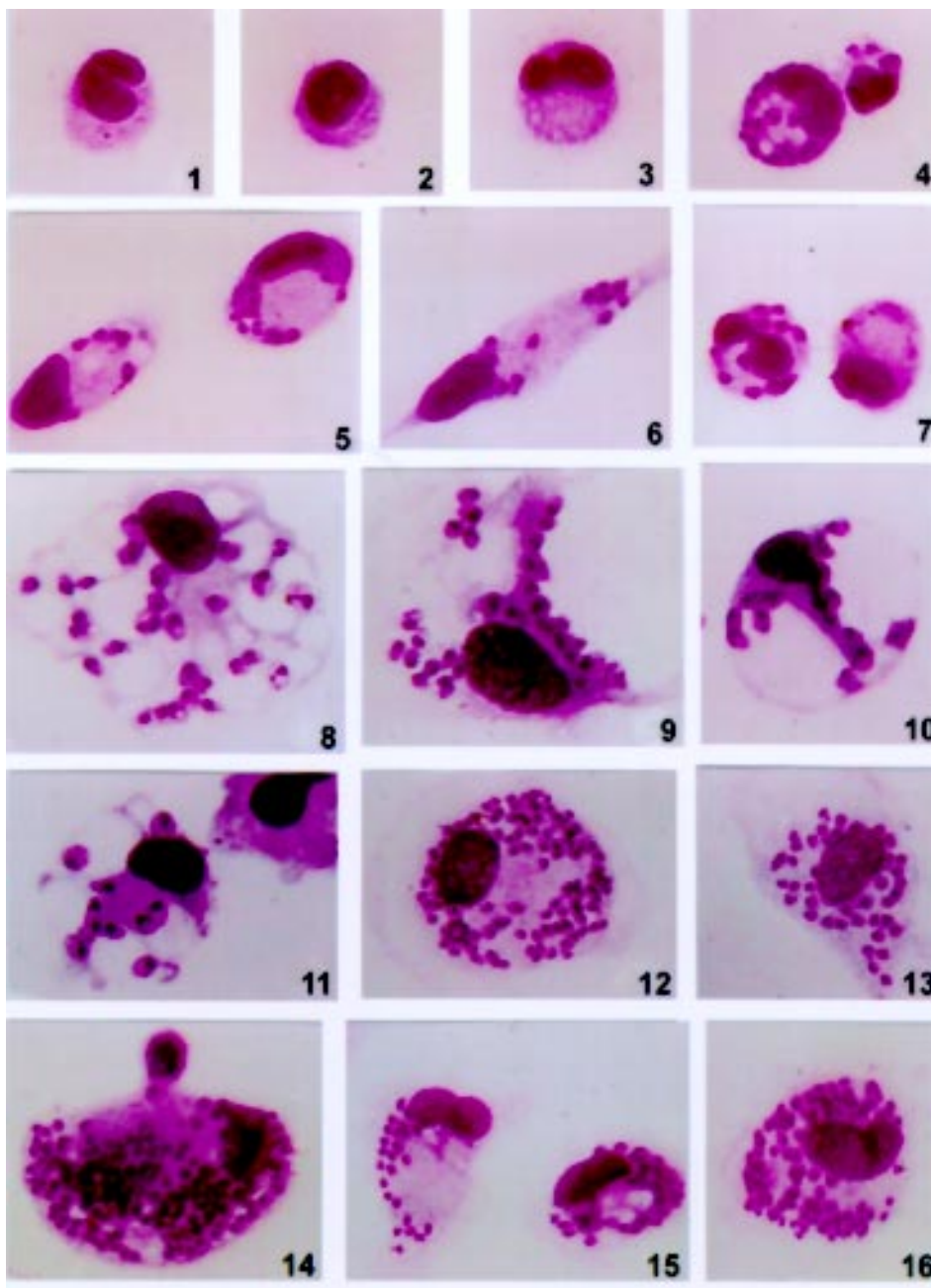


Fig. 2: photomicrograph of canine peritoneal macrophages cultured *in vitro* (Giemsa stain; x 1000). Figs 1-3: normal (non-infected) macrophages. Figs 4-7: macrophages infected with *Leishmania (Viannia) braziliensis*. Figs 8-11: macrophages infected with *L. (L.) amazonensis*. Figs 12-16: macrophages infected with *L. (L.) chagasi*.

Numerous reports on the evaluation of the *in vitro* multiplication of different *Leishmania* species suggest a correlation with the *in vivo* system, e.g., where it is possible to observe the limited capacity of *L. (V.) braziliensis* to grow in murine macrophages, while *L. amazonensis* displays exuberant multiplication in this cell type (Zeledón et al. 1969, Grimaldi Jr et al. 1983).

Actually the utilization of the two forms, amastigotes and viable promastigotes, could be a start and to follow up the macrophage infection by *Leishmania* (Berman & Lee 1984). In this study it was used to infect the canine cells a pool of metacyclic and non-metacyclic promastigotes harvested from stationary phase culture of *L. (V.) braziliensis* and amastigotes of *L. (L.) amazonensis* and *L. (L.) chagasi* and the three species clearly behaved differently with regard to their multiplication rate. The *L. (L.) chagasi* displayed greater capacity for multiplication than the two others (data not show).

So in order to make sure if the macrophage infection by *L. (L.) chagasi* really display greater affinity for canine cells as seen in this study a specific experiment is being performed with only *Leishmania* metacyclic promastigotes forms.

Our results support the hypothesis that these three organisms behave differently, both in their morphology and location in the host cell, showing characteristic patterns and the formation of large vacuoles as observed in the murine cells infected by *L. (L.) amazonensis* (Grimaldi Jr et al. 1983).

In addition the standardization of this efficacious system open perspectives to study the role of this animal as an important reservoir for both visceral and mucocutaneous leishmaniasis by *L. (V.) braziliensis*, besides the evaluation of new efficient drugs and/or natural products with potential leishmanicide activity.

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