

THE SUBCLINICAL FORM OF EXPERIMENTAL VISCERAL LEISHMANIASIS IN DOGS

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Pathological aspects of a subclinical form of experimental canine leishmaniasis is reported here for the first time.

Fifteen mongrel dogs were used in the present study. Eight dogs were infected and seven were used as control. Four of the control dogs were inoculated with spleen cells from non-infected hamsters. The eight mongrel dogs inoculated intravenously with amastigotes forms of Leishmania chagasi evolved for periods as long as 25 months without any clinical characteristic sign of classical Visceral Leishmaniasis (VL). Most of the laboratory test results were compatible to those of the seven control animals but culture of bone marrow aspirated material and serologic testing (IIF) demonstrated or provided evidence that the animals were infected.

The most important and predominant histopathological lesion in infected animals were epitheloid granulomas presented in the liver, spleen, adrenal gland and lung of some animals. Channels containing erythrocytes in some granulomas of the liver suggest that these granulomas are formed inside sinusoidal capillaries.

Despite the animals were proved to be infected and presented characteristic histologic lesions, they did not present external signs of disease. The granulomatous aspect of the lesions indicates a good immunologic reactivity and suggest that a host-parasite equilibrium does exist in the dog experimental model.

Key words: Leishmania chagasi – canine leishmaniasis – subclinical form – histopathology

Visceral Leishmaniasis (VL) caused by the *Leishmania donovani* complex is spread widely in tropical and sub-tropical areas. The disease is caused by intracellular heteroxenous protozoa which infect vertebrate host. Several mammalian species have been pointed out as natural reservoirs of the parasite (Hommel, 1978). In the American Continent and the Mediterranean, the dog seems to be the most important domiciliary reservoir (Alencar, 1959a; Lainson & Shaw, 1971).

Clinical features of human VL in its clas-

sical presentation are characterized by: fever, anemia, weight loss, spleen and liver enlargement and hemorrhagic diathesis. The clinical-pathological findings are hypoalbuminemia, hypergamaglobulinemia and pancytopenia (Napier, 1946; Most & Laviertes, 1947; Prata, 1957; Alencar, 1959b).

Many retrospective studies suggest that the majority of patients infected with *L. donovani* develop classical disease. However, other authors have mentioned the occurrence of cases with positive serology or even the presence of parasites within bone-marrow tissues, without clinical symptoms (Napier, 1946; Pampiglione et al., 1974). Badaro et al. (1986a) have demonstrated that under certain circumstances the parasite seems to behave as an opportunistic agent. The same authors (Badaro et al., 1986b), have performed a prospective study in an en-

This work was supported by FINEP, Brasil and INSERM, France.

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Received 6 August 1992.

Accepted 28 December 1992.

demic area of VL with 86 children presenting anti-*Leishmania* antibodies. Among them, 44.2% developed oligo-symptomatic disease which evolved to a complete recovery and 23.3% remained absolutely asymptomatic during the study. These data demonstrated that some patients develop sub-clinical disease.

Clinical features of exuberant canine VL are considered as being the classical presentation of disease and consist of: weight loss, anemia, ulcerated lesions, skin desquamation, loss of skin hair and an increase in nail size and length (Adler & Theodor, 1932; Donatien & Lestoquart, 1935; Souza Lopes & Sarno, 1956; Brener, 1957; Alencar, 1959a). Like man, most of the dogs naturally infected in endemic areas seem to present an asymptomatic evolutionary disease, since they do not present any symptom related to VL (Adler & Theodor, 1932; Balozet, 1932; Giraud & Cabassu, 1933; Donatien & Lestoquard, 1935, 1938; Alencar & Cunha, 1963).

Although it is important to study the anatomo-pathological and laboratory aspects of sub-clinical disease in canine VL, either to compare with the disease in human-beings or to evaluate the development of vaccines against canine infection, at the moment there is little information concerning these aspects in the literature. In the present study, the authors have described for the first time some of the

anatomo-pathological and laboratory aspects of VL subclinical disease in dogs.

MATERIALS AND METHODS

Animals – Fifteen mongrel dogs of both sex and age varying from three to six months were used.

Inoculation of eight dogs with promastigotes and/or amastigotes was performed, according to the Table. Four dogs injected with spleen cells from non-infected hamsters (1×10^7 cells/kg), and three healthy dogs which did not receive any injection formed the control groups.

Parasites – Infection was carried out with *Leishmania chagasi* (MHOM/BR/79L101 – “Imperatriz”), isolated by culture in NNN medium from a patient’s bonemarrow (Philip Marsden – University of Brasilia) and maintained in hamsters and in medium LIT-R9 (Sadigursky & Brodskin, 1986) for no more than ten passages.

The promastigotes were obtained from culture in LIT-R9 medium, washed three times in buffered saline-phosphate and counted in Neubauer’s hemocytometer. To obtain amastigotes, spleens from infected hamsters were aseptically removed, sectioned, imprinted in glass slides and stained by Giemsa. Imprints were examined by microscopy in order to detect parasitemia. The spleens presenting high lev-

TABLE

Protocol of experimental infection in eight dogs

Dog	Week (w) and or months after first injection																
	1st month					11th month					15th month	28th month					
	0w	1w	2w	3w	4w	0w	1w	2w	3w	4w		0w	1w	2w	3w	4w	5w
1		0.1				0.5						1000	10	0,5	0,6	100	
2		0.1				0.5							10	0,5	0,6	100	10
3		10				0.5	6.0	100	10								
4		16				5.0	6.0	100									
5		16				5.0	6.0	100									
6		5.0	6.0	100	10												
7		6.0	100	10													
8		6.0	100	10													

No. of promastigotes/kg $\times 10^7$ inoculated ip. per dog (first injection in dogs No. 1 and 2) or No. of amastigotes/kg $\times 10^7$ inoculated iv. per dog (all other injections).

els of parasites were homogenized using RPMI 1640. A cellular concentrate was obtained from the homogenate, centrifuged (Cytospin Shandon Souther, USA), applied to glass slides and stained by Giemsa. A relationship between the proportion of splenocytes and amastigotes was determined. The quantification of parasites was estimated by the multiplication of the relation parasites/cells and the number of cells per ml.

Follow-up of animals – The animals were clinically examined weekly. They were also anesthetized by peritoneal injections of sodium Pentobarbital (30 to 50 mg/kg), and skin biopsies were obtained from the ear. Liver needle-biopsy and bone/marrow aspiration were also performed at one to two months intervals. Finally, the routine laboratory exams such as urine analysis and hemogram were also carried out.

Indirect immunofluorescence – *L. chagasi* promastigote forms were used as antigen. They were prepared in glass slides and fixed with acetone. Dog sera were serially diluted from 1:10 to 1:5120 and distributed in different wells. After 30 min of incubation at 37 °C, the slides were washed in PBS and incubated with fluorescein conjugated anti dog IgG antibodies (Cappel Laboratories, USA). After 30 min of incubation the slides were washed in PBS and examined in an immunofluorescence microscope.

Sacrifice and necropsy – Dogs were sacrificed after 3, 12 and 22 months of infection (45 days after the last inoculation). They were submitted to necropsy and the organs were fixed with Bouin liquid and/or a neutral phosphate buffered formalin. Tissue specimens were prepared in paraffin-waxed, sectioned (5 µm of width) and stained by six different techniques: Hematoxylin-Eosin (HE), Gomori's reticulin, Schiff-PAS, Red Sirius Picro, Perls methanamine Silver and Azan).

RESULTS

Clinical aspects – During the period of study the dogs put on weight until the adult phase. All of them remained healthy throughout the experiment until the day of sacrifice.

The hemogram revealed that the concentration of hemoglobin in the prephase group varied from 8.2 to 14.2 g/dl. The white blood cell count varied from 5,000 to 14,600 in in-

fecting animals and from 4,000 to 7,200 in the controls. These results did not show a statistically significant difference between the studied group and the controls (Student's test). However, the differential count of neutrophils presented a significant difference when control and study groups were compared (X 2545 and X 5575, respectively). The urine analysis were similar in both groups and granular cylinders were rarely seen. Moreover, urine culture for bacteria were all negative. The culture of bone marrow aspirate samples was positive for *Leishmania* in six out of eight infected dogs, 40 days after the inoculation of amastigote forms.

By using indirect immunofluorescence, the infected animals presented high levels of anti-*Leishmania* antibodies, varying from 1:320 to 1:1280, except for one dog (1:40). In the control group, 2 dogs presented a positive test at 1:10 dilution. The other control animals remained negative.



Fig. 1: epithelioid granuloma in the liver of an infected dog. H.E. x 200.

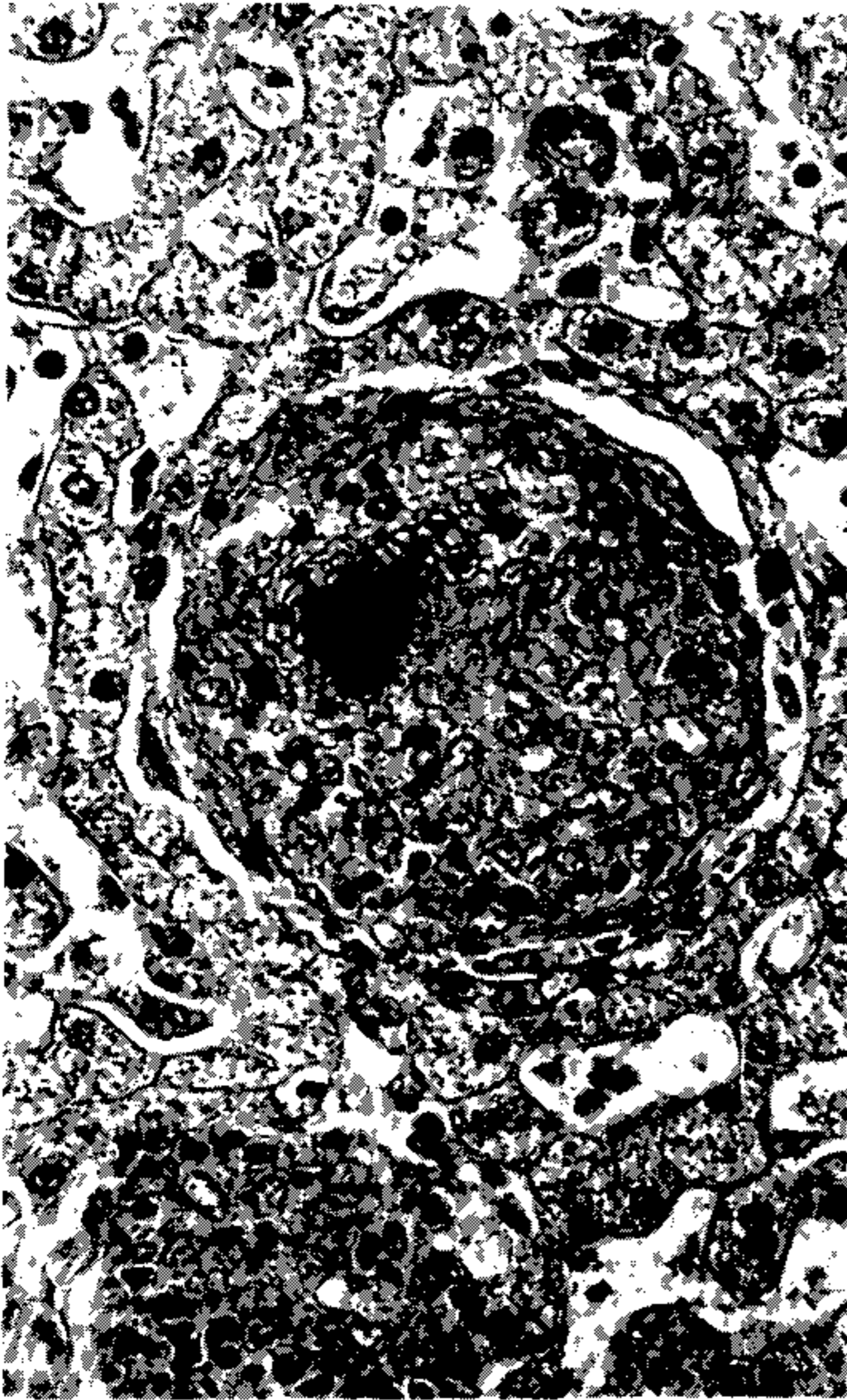


Fig. 2: liver of an infected animal with epithelioid granuloma showing a small channel containing erythrocytes. H.E. x 200.

Only non specific inflammatory alterations were seen in the skin biopsies performed periodically. In the liver samples obtained two months before sacrifice, we have observed epithelioid granuloma and macrophages containing amastigote forms in two infected animals.

Necropsy gross aspects – The most important macroscopic change observed in the necropsy was the absolute increase of spleen weight among the infected animals (70 to 129 g; average 90,0 g), when compared to the control group (16 to 33 g; average 24 g). Liver weight varied from 224 to 650 g (average 406 g) in the study group against 128 to 251 g in the controls (average 238 g).

Histopathological aspects – The characteristic lesion found in infected animals was the epithelioid granuloma. Among the eight infected dogs, four presented this type of lesion in more than one organ.

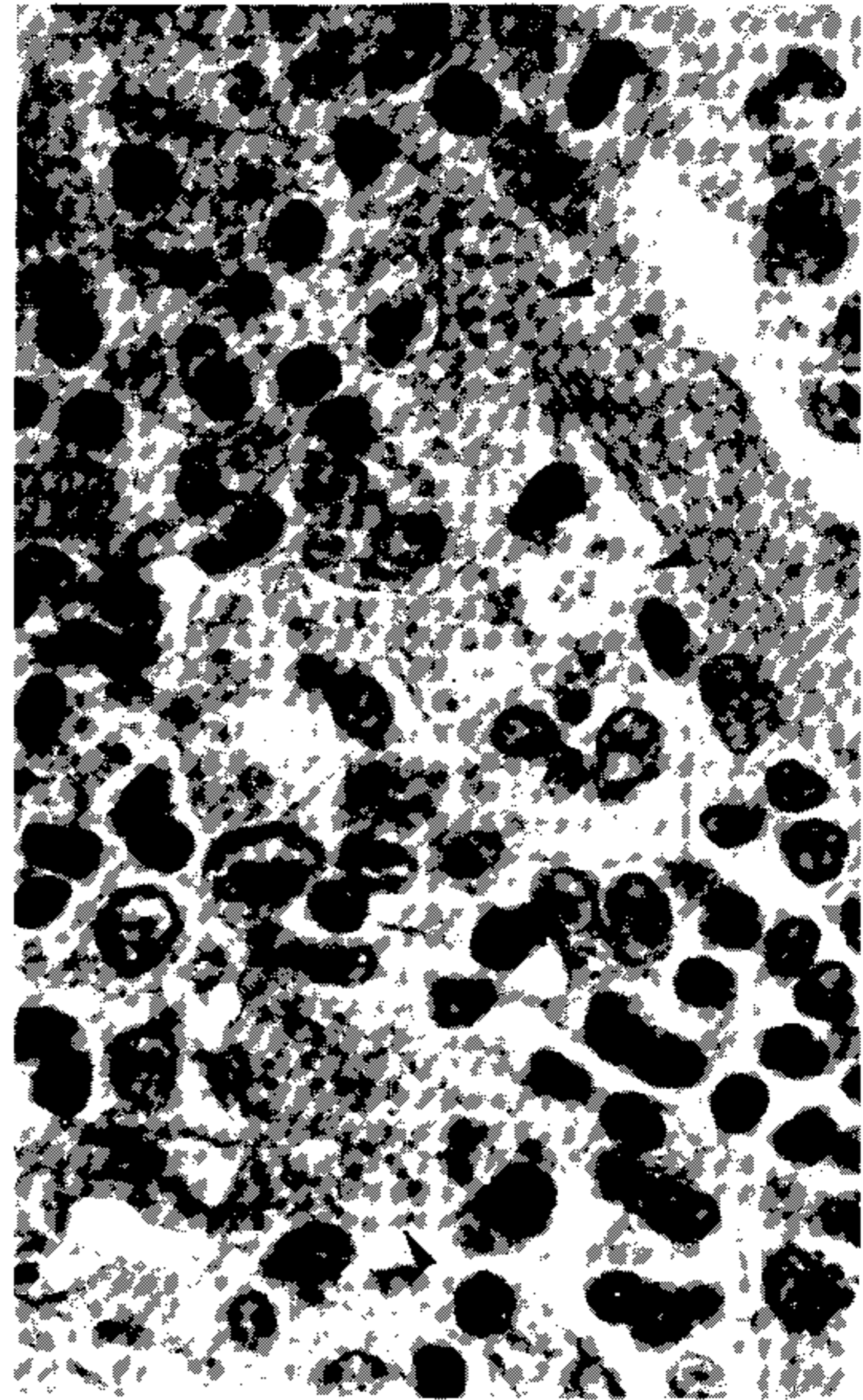


Fig. 3: spleen of an infected dog with large number of amastigotes forms of *Leishmania* inside macrophages.

In the liver, the granuloma lesions were observed in parenchyma as well as in the portal spaces. They were quite organized and presented several cellular components. The lesions varied from small irregular groups of macrophages and lymphocytes without evident organization to very complex oval or round granulomas, with precise limits showing sometimes a concentric cellular disposition and containing epithelioid macrophages, lymphocytes and plasmocytes in peripheral areas (Fig. 1). Although many intermediate stages were seen, there was always one predominant type of granuloma in each animal. An interesting histological lesion, characterized by the existence of granuloma with a channel containing red cells but no endothelial exterior limits was observed (Fig. 2). Intact amastigote forms of *Leishmania* were found into the macrophages presents in the hepatic samples of one animal. There was also hypertrophy and hyperplasia of the Kupffer cells.

The granuloma observed in spleens presented lymphocytes and PMN neutrophils. Besides these granuloma, there were groups of macrophages in sub-capsular areas and hyperplastic lymphoid follicles. In one of the cases we have noticed amastigote forms into the macrophages (Fig. 3).

In lungs and adrenal glands there was a predominance of epithelioid cells in the granuloma. Other organs presented only non specific inflammatory processes. In only one case it was possible to observe a mild parasitemia in macrophages from a bone-marrow sample.

Neither the control animals inoculated with spleen cells from uninfected hamster nor the other control group presented specific histopathological lesions.

DISCUSSION

Major signs observed in dogs affected by VL are mainly represented by weight loss, of hair and skin ulcers (Souza Lopes & Sarno, 1956; Brener, 1957; Alencar, 1959a). In our study animals did not present any symptomatology that could be attributed to VL.

Six of eight infected dogs presented amastigotes in bone-marrow aspiration samples. In the histopathological study, amastigotes were also seen into liver and spleen macrophages of one animal and in the bone-marrow of another. The presence of round corpuscles in macrophages of hepatic granulomas might represent the occurrence of partially digested *Leishmania*.

Pampiglione et al. (1974) followed six cases of asymptomatic human VL using serological test. They observed high levels of anti-*Leishmania* antibodies during the subclinical phases, but with the evolution and cure of the disease, the levels of antibodies decreased. In the infected dogs, high titres of anti-*Leishmania* antibodies were detected during all the experiment. These results are probably associated with the persistence of the infection.

All these data strongly suggest that our animal inoculated with *Leishmania* developed a subclinical form of VL.

The enlargement of spleens among infected dogs reached average values of 90.9 g is in accordance with those obtained by Alencar & Cunha (1963), studying dogs with VL disease (average value 81g).

The fundamental histopathological finding was the presence of epithelioid granuloma. They were localized in liver, spleen, adrenal glands and lungs. Detection of amastigote forms was rare and may represent a steady-state in the host-parasite relationship. Among the dogs with subclinical VL, granulomatous hepatitis was a common finding. Similar data were reported by Pampiglione et al. (1974), dealing with human asymptomatic VL.

An unusual appearance was the detection of an inner space filled with red cells inside the granulomas. As no other vascular structure surrounding red blood cells was identified, this finding suggests that the macrophages themselves may form the granuloma structure around the sinusoidal lumen. Since there is no previous references in literature concerning this description, we think that it is the first time it has been suggested.

In the present study, a group of mongrel dogs was inoculated with a high number of parasites. Although long follow-up of several animal was possible there was no clinical evidence of VL. This data corroborates the findings of Alencar (1959a), that in endemic areas, most of naturally infected dogs are asymptomatic. According to Lanotte et al. (1979), about 90% of dogs with inapparent infection will develop a symptomatic disease. Nevertheless, the period between infection and the appearance of symptoms is often long. It is possible that subclinical form of VL occurs when a good host-parasite equilibrium exists.

All this data strongly indicate the importance of dogs in the epidemiological network involving leishmaniasis. Although, as in human, it is a victim of the disease, the dog with its subclinical form become more dangerous as a domiciliary reservoir of *Leishmania* in the presence of the insect vector (phlebotomines).

ACKNOWLEDGEMENTS

To Miss Odile Brasier and Miss Sônia Guimarães for secretarial assistance.

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