

HISTOPATHOLOGY AND IMMUNOCYTOCHEMICAL STUDY OF TYPE 3 AND TYPE 4 COMPLEMENT RECEPTORS IN THE LIVER AND SPLEEN OF DOGS NATURALLY AND EXPERIMENTALLY INFECTED WITH *LEISHMANIA (LEISHMANIA) CHAGASI*

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SUMMARY

The objective of this study was to compare the histopathological changes and expression of CR3 and CR4 in the liver and spleen of dogs naturally and experimentally infected with *L. chagasi*. The basic histopathological lesions observed mainly in naturally infected dogs were: epithelioid hepatic granulomas, hyperplasia and hypertrophy of Kupffer cells, Malpighi follicles and mononucleated cells of the red pulp of the spleen. Sections from the liver and spleen by immunocytochemistry technique showed the presence of CD11b,c\CD 18 antigens in the control and infected animals and no qualitative or quantitative differences in the liver. Nevertheless, CD18 was always increased in the spleen of naturally and experimentally infected dogs. These results indicate that there is a difference in the activation of CD18 in both experimental and natural cases of canine visceral leishmaniasis that should play an important role in the immunological response to *Leishmania chagasi* infection.

KEYWORDS: Dogs; *Leishmania chagasi*; Histopathology; Immunocytology; Complement receptors.

INTRODUCTION

American visceral leishmaniasis is a zoonosis in which dogs represent the principal domestic reservoir^{1,12}. In the New World the etiological agent of the disease is *Leishmania (Leishmania) chagasi* which is transmitted by the phlebotomine *Lutzomyia (Lutzomyia) longipalpis*^{15,27}. The mammalian host is infected with the promastigote forms through the bite of insect vectors. Once inoculated, the promastigotes infect cells from the mononuclear phagocyte system (MFS) and later transform into amastigotes²⁸. The *Leishmania*-macrophage interaction involves complex mechanisms between the specific receptors and ligands present on the surface of their membranes. Type 3 and 4 complement receptors (CR3 and CR4) present on the surfaces of the macrophages participate in this interaction with other ligands^{31,41}.

CR3 and CR4 are members of the family of leukocyte integrins, transmembrane dimeric glycoproteins constituted by a single variable chain designated α (120-180 kD) non-covalently linked to another single constant chain, designated β (90-110 kD)¹⁸. The integrins can be classified into various "subfamilies" according to β chain molecule. Eight types are currently known (β 1 through β 8) where CR3 and CR4 are classified into subfamily β 2. These molecules, principally present on the monocytes and macrophages, act basically in cell adherence^{19,20}. The Third International Workshop on Antigens of Human Leukocyte Differentiation identified the α subunit of CR3 and CR4 as CD11b, and CD11c, respectively, and the common β subunit as CD18¹⁹. Therefore, CR3 is also known as CD11b\CD18 and CR4 as CD11c\CD18.

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The tissue distribution or expression of CR3 and CR4 can be outlined as follows: CR3 is mainly found on cells of the MFS, but it can be expressed on polymorphonuclear neutrophils, large granular lymphocytes, CD5 positive B lymphocytes and NK cells^{18,22}. In contrast, CR4 glycoproteins possess a distribution similar to that of CR3 although they can be expressed on cytotoxic T-cells and some activated lymphocytes¹¹. Various authors have demonstrated the participation of these glycoproteins in the *Leishmania*-macrophage interactions in systems that contain complement proteins^{30,32} as well as those that do not^{10,38,41}.

The objective of this study was to investigate the histopathology and expression of CR3 and CR4 in the liver and spleen of 8 dogs experimentally infected with *L. chagasi*, and 5 naturally infected dogs, in order to relate the histopathological to the immunocytochemical findings. This study in both groups of animals is important because the naturally infected dogs could be infected with other pathogens that may interfere with the pathogenesis of the disease.

MATERIAL AND METHODS

Control dogs

Five animals, 4 females and 1 male, 36 months-old, were obtained from the Federal University of Ouro Preto, Minas Gerais. The animals were treated previously with antihelminthic drugs (Mebendazole-Univet) and vaccinated against cynomosis, parvovirus, leptospirosis, parainfluenza, and infectious hepatitis (six-dose vaccine from Masterguard-Plus-Salsbury Laboratórios Ltda.). The dogs were provided with water and ration (Kinus) *ad libitum*.

Naturally infected dogs

Five mongrel dogs, 4 females and 1 male of undefined age, previously used in an epidemiological survey of visceral leishmaniasis held by the City Hall of Belo Horizonte, were studied. This inquiry was based on a serological survey of the canine population by anti-leishmania indirect immunofluorescence (IIFT) and complement fixation reaction (CFT) tests that were confirmed by the Serology Laboratory of the Department of Parasitology, Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG).

Experimentally infected dogs

Eight dogs, 6 females and 2 males, 36 months old raised in the kennel of the Leishmaniasis Laboratory, ICB, UFMG, were used. These animals received the same treatment as those of the control group.

Experimental infection

The protocol for experimental infection was similar to that described by GENARO¹³. The inocula was done with 2.3×10^6 stationary-phase promastigotes of the *Leishmania (L.) chagasi* strain (MHOM/BR/72/BH/46) that were grown in LIT medium¹³. The promastigotes were injected intravenously into the dogs through the radial vein of the front right leg. Blood samples were collected from each dog every 30 days until the end of the experiment and serum was obtained for anti-leishmania IIFT and CFT immunological studies³⁵. The parasitological diagnosis was done by determination of parasites in the bone marrow aspirate.

Collection and processing of material

The infected and control dogs anesthetized with Thionembutal® (33%, 5 ml/kg dose, by the intravenous route) were sacrificed by induction of pneumothorax. Liver and spleen fragments were fixed in Bouin solution (18 hours) and later dehydrated, cleared, embedded in paraffin, cut (3-4 µm thick) and stained with Hematoxylin and Eosin (HE). Special staining with Gomori and ammoniacal silver was used for the observation of collagen and reticulum, respectively.

For the immunocytochemical study of the same sample (0.5 x 0.5 cm) was placed in saline (pH 7.4). Two to three sections (4-5 µm thick) were obtained with a cryostat and mounted on slides. Immunoperoxidase anti-peroxidase (PAP)⁴⁰ technique was employed. Antic canine CD18, CD11b, CD11c IgG1 mouse monoclonal antibodies (MAbs), were a kindly gift from Professor Peter F. Moore, School of Veterinary Medicine, University of California, Davis, USA. The MAbs were used at the following dilutions: 1:100 for anti-CD18 and anti-CD11c (CR4) MAbs, and 1:300 for anti-CD11b MAbs.

Microscopic and statistical analysis

The slides were examined blind with a common light microscope using an 8x eyepiece and a 10x objective. The slides were classified in increasing order of intensity according to specific immunocytochemical labelling and subjected to non-parametric Wilcoxon test modified by Mann-Whitney³⁹. The difference was considered significant if $p \leq 0.05$.

RESULTS

Liver: Microscopically intralobular granulomas were observed in all of the naturally infected dogs. The granulomas, rarely confluent, were mainly localized in the sinusoid lumen, partially occluding it or not. At times they were present in the porto-biliary spaces in the lumen of the centrilobular veins. They consisted predomi-

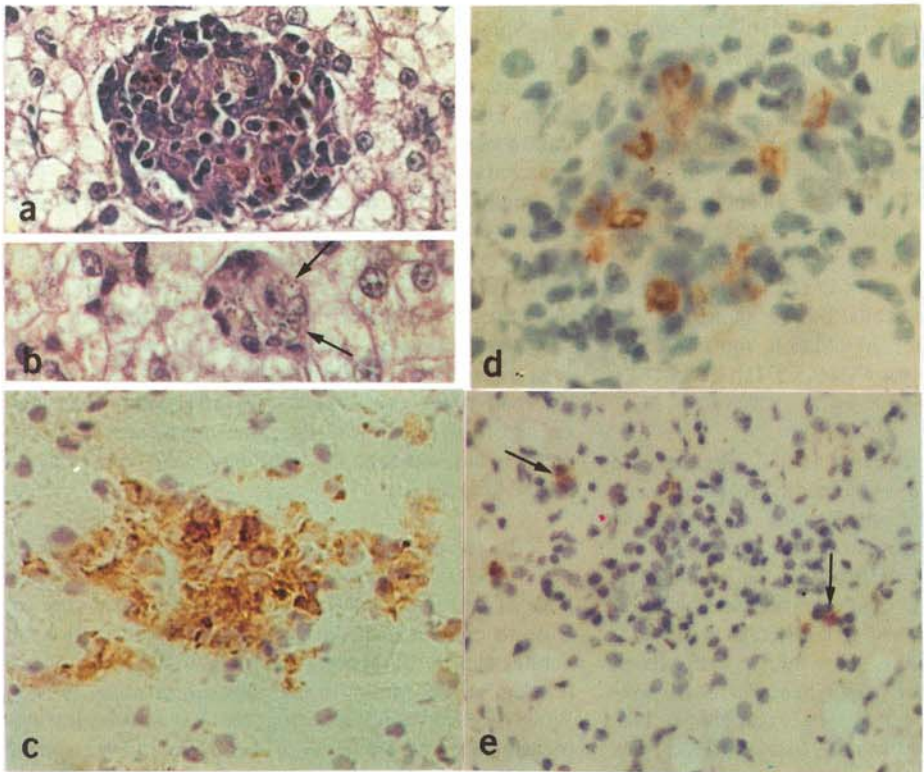


Fig. 1: Liver of naturally infected dog; (a,b) Two granulomas consisting of macrophages, epithelioid cells and lymphocytes. Presence of parasites in macrophages (arrows) HE. x200 (a), x400 (b); (c) positive PAP staining for CD18 and (d) CD11b directly related to the cells of the granuloma. x200 (c), x400 (d); (e) positive PAP for CD11c in some cells of the periphery of the granuloma (arrows) x100.

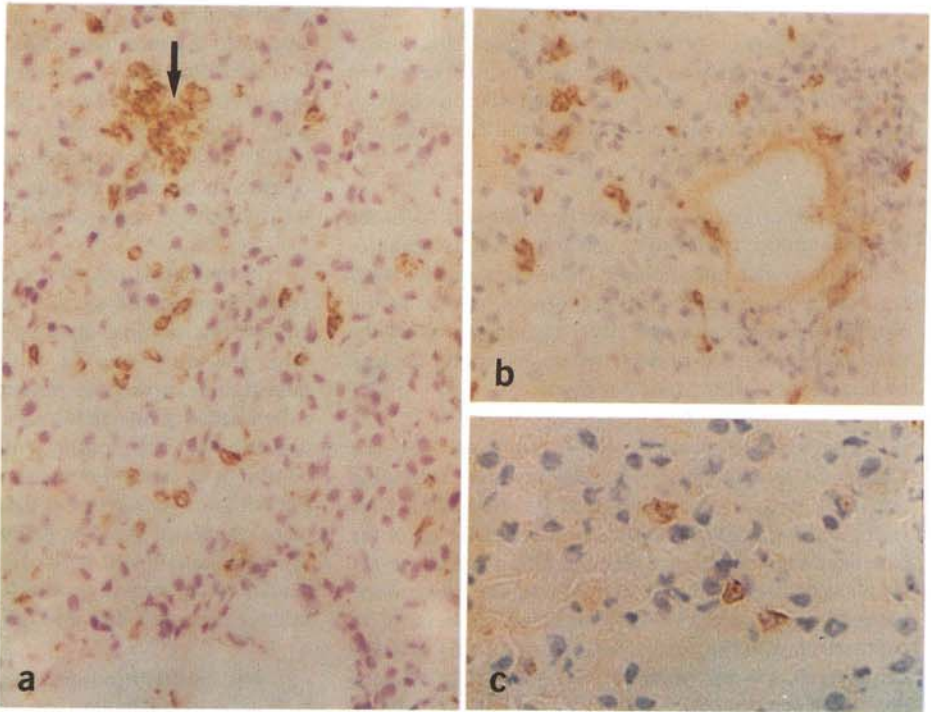


Fig. 2: Liver of naturally infected dog; (a) positive PAP for CD18; (b) CD11b and (c) CD11c. Note the reactive cells in the periportal and sinusoidal spaces and at times clustered (a) (arrows). x200; in (c) only some labelled cells x400.

nantly of macrophages, parasitized or not, some epithelioid cells, small number of lymphocytes and rare polymorphonuclear neutrophils (Figure 1a, b).

The Gomori trichrome stain and ammoniacal silver did neither reveal fibrosis nor evident changes in reticulum in the granulomas. The immunocytochemical reaction (PAP) for the CD 18 and CD 11b (CR3) antigens was positive in the cells in the granuloma of all animals (Figure 1, c,d). The labelling of CD 11c (CR4) was positive in only some isolated cells, but not those forming the granulomas (Figure 1e). In addition, sinusoidal and portal cells were positive for CD18, CD11b and CD11c (Figure 2a, b, c).

In two naturally infected dogs, besides the granulomatous lesion, there was a marked thickening and multiplication of reticular fibers forming a different pattern. In fact, the reticulum was thickened mainly in the portobiliary space and on the walls of the sinusoids of the peripheral region of the lobules of one animal, and it was diffuse in another. In the latter, reticular fibers were aligned in various directions, some thicker than others, that ran parallel, oblique or perpendicularly to the sinusoids, forming a compact network in certain points. Frequently the fibers encircled small groups of hepatocytes or a single cell acquiring the aspect of the NATAN-LARRIER³³ monocellular cirrhosis and in a diffuse manner mainly in certain areas similar to ROGERS³⁷ hepatic fibrosis (Figure3).

The presence of amastigotes inside the hypertrophic and hyperplastic Kupffer cells was observed in all of the dogs. Yet, there was no correlation between parasitism and the number of cells, and consequently, the size of the granuloma.

The experimentally infected dogs showed, as in the previous group, intralobular and intravascular granulomas with similar morphology. However, parasites were rare or even absent in the granuloma, or inside the Kupffer cells. Gomori and silver staining revealed the same pattern as in the previous group, except for the lack of derangement of the reticular fibers.

The immunocytochemical reactions for the CD11b,c/CD18 integrins was also positive, following the same pattern as that described for the naturally infected dogs. Statistical analysis of the data for all of the examined animals did not reveal a difference in the expression of the CD18 and CD11b antigens between the naturally and experimentally infected animals, or in comparison with the control animals ($p \geq 0.05$). Statistical analysis was not possible with the CD11c antigens due to the small number of labelled cells in all cases.

Spleen: Microscopically the spleen of all of naturally infected animals showed similar histological pattern, although more relevant data were observed only in some. In one animal the capsule was thicker as also was the trabecular system than the controls (Figure 4a). The trabecular vessels were dilated and congested. The Malpighi follicles, especially in three dogs, had bigger volume, sometimes confluent and consisting of cells arranged in different layers according to their staining affinity (Figure 4b). The central cells were lighter with more vesicular nuclei and acidophilic cytoplasm, with or without parasites, small lymphocytes and cells with fusiform nuclei, with cytoplasmic prolongations. More externally there were lymphocytes arranged in finer layers and finally cells with the same characteristics as the central ones, although the light ones were heavily parasitized (Figure 4c).

The red pulp suffered profound modifications due to the considerable increase in the cellular proliferation both of marginal macrophages of the sinuses and of Billroth cords and of the pronounced neof ormation of connective tissue (young fibroblasts and fibrocytes) leading, on the whole, to the reduction in sinus lumen. With the exception of one dog, the rest showed intense parasitism in the thickness of the capsule, and subcapsular and perifollicular region (marginal zone) (Figure 4d).

The specific reactions for CD 18 and CD 11b were positive in a large number of cells in two animals that were also the most parasitized. The distribution pattern was the following: (1) the expression of the CD 18 antigens mainly occurred near the marginal zone and the red pulp (figure 5a); (2) the specific characterization of CD11b (CR3) showed the same distribution of CD18 (Figure 5b, c), while the glycoprotein CD11c (CR4) was only positive in some cells (Figure 5d).

In the experimentally infected dogs, the examined spleen samples of all the animals generally showed similar microscopic profiles with some nuances. One dog, the only one in which liver we observed amastigotes, presented bigger changes than the other experimentally infected dogs, i.e., moderately thickened capsule and trabeculae, follicles that were either confluent or not, with predominance of lymphocytes. Parasitism was rare with presence of amastigotes in macrophages of the marginal zone and in the red pulp. The remaining of the dogs showed some changes, quite similar to the control group. Parasites were always few in three dogs and absent in the others.

The immunohistological pattern of the CD11 b,c/CD18 integrins was the same as described for the

previous group. The statistical analysis revealed an increased expression of CD18 antigens among the infected animals compared with control group ($p \leq 0.05$). However, such a correlation did not occur between the two groups of sick animals ($p \geq 0.05$). In relation to the CD 11c antigens, statistical analysis was not possible due to the small number of labelled cells.

DISCUSSION

In all of the livers of the naturally infected group, and in 5 experimentally infected animals, the basic damage was the presence of intralobular and intravascular granulomas with the presence or absence of parasitized macrophages, epithelioid cells and lymphocytes. Most noteworthy was the ever present parasitism of macrophages in the naturally infected animals. In contrast, in the experimentally infected animals only one dog had parasites, even though the granulomas had the same morphology and similar number of cells per granuloma. The hypertrophy and hyperplasia of Kupffer cells was a common finding in all of the animals examined in both groups. Again, the basic difference between the two groups was the ever present parasitism of the cells in the naturally infected dogs. Nevertheless, we did not observe parasites in the hepatocytes, as it occurs in some cases of the human form of the disease⁵. Only one naturally infected dog exhibited changes in the reticular pattern that resembled what is described in human visceral leishmaniasis^{6,33,36,37}. The occurrence of the so called "Rogers cirrhosis" is rare in humans^{3,6} as appears to be the case in dogs as well^{13,21,34}.

The immunocytochemical labelling for the CD11b,c/CD 18 integrins maintained a similar pattern among all of the animals, i.e., only sinusoidal cells were positive, those related to the granulomas and to the periportal inflammatory infiltrates. The integrins of the CD11b,c/CD18 complex are expressed by all leukocytes (leukocyte integrins – $\beta 2$ subfamily), with CD11b and CD11c mainly expressed by macrophages. We can see, then, that all cited cell types can be present in the granulomas as well as in the sinusoidal and periportal and hepatic spaces. The cellular positivity in the periportal inflammatory focal points and in the granulomas was expected since the integrins play an important role in the inflammatory reactions^{20,25}.

The statistical analysis of the immunocytochemical characterization of CD 11b,c/CD 18 in the liver did not reveal any quantitative differences among animals. The qualitative aspect of the reactions also did not differ in any of the animals examined. This indicated to us that *L. chagasi* infection, at least in the 30-month infection period for the experimentally infected dogs, did not pro-

voke marked changes in the expression of these glycoproteins in the liver. It should also be mentioned that the infection of the naturally infected group from metropolitan Belo Horizonte also has *L. chagasi* as an etiological agent⁴⁴.

The dynamics or kinetics of the formation of the hepatic granulomas in experimental murine visceral leishmaniasis have been described by various authors^{9,16,25}. Balb/c mice infected with *L. donovani* developed hepatic granulomas within the first four weeks of infection. In the first week, the granulomas consisted of some parasitized macrophages, some granulocytes (neutrophils and eosinophils) and interspersed mononucleate cells (monocytes and lymphocytes). Staining for the reticulum was negative. In the second week the fusion of macrophages (epithelial cells) and a rise in the afflux of mononucleate cells were observed, leading to the formation of nodules. Staining for the reticulum was positive in some granulomas indicating the presence of collagen. From the fourth week on, the granulomas became mature as the parasitism and the number of cells diminished, and as the deposition of collagen increased. CERVIA et al.⁹, working with Balb/c mice infected with *L. donovani* treated with anti-CR3 (CD11b) MAbs, demonstrated that the afflux of mononuclear cells that occurs after macrophage fusion is dependent on the presence of positive CD11b monocytes. In addition, the action of the anti-CR3 MAbs did not interrupt the fusion of Kupffer cells or the organization of the lymphocyte mantle. Studies related to the pathogenesis of hepatic granulomas in dogs, as far as we know, do not exist, but in accordance with the above studies we may consider that the granulomas found in all of the animals are compatible, although we cannot extrapolate from one animal to another, with the second phase described by GUTIERREZ¹⁶ or McELRATH²⁵ in mice. The positive cells for CD 11b in the histochemical analysis seem to be related to monocytes-macrophages (Figure 1d).

Microscopically, the spleen fragments of all dogs showed hypertrophy and hyperplasia of the cells of the Malpighi follicles of greater or lesser intensity. The naturally infected dogs were the most reactive with formation of clearly visible germinative centers showing many lymphoblasts, macrophages (containing or not parasites), lymphocytes and some myototic cells and others in apoptosis. There was an intense proliferation of the marginal zone consisting of small cells with dense nuclei and sparse cytoplasm (lymphocytes) and larger cells with a weak nucleus and ample cytoplasm (macrophages). Generally the macrophages were heavily parasitized. The red pulp also showed hypertrophy and hyperplasia, consisting primarily of mononucleate cells with heavy parasitism. These histopathological findings agree with some

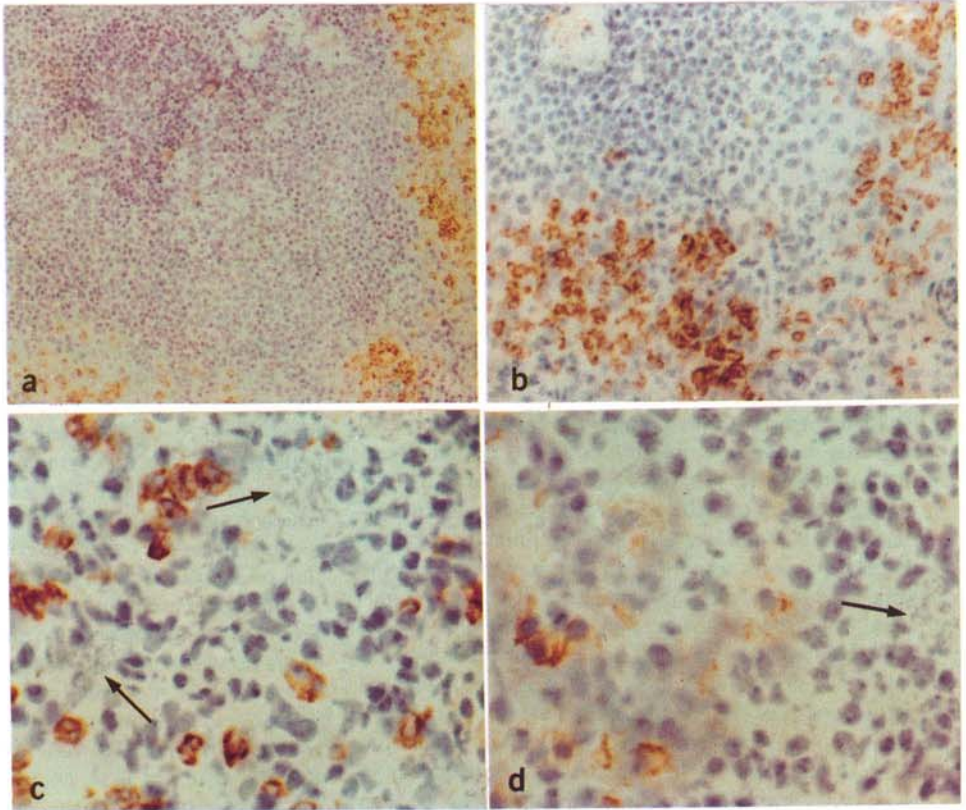


Fig. 3: Liver of naturally infected dog: Thickened reticulum, showing thick and thin fibrils, enclosing group of hepatocytes or isolated hepatocytes, with more localized or diffuse distribution. Silver. x200.

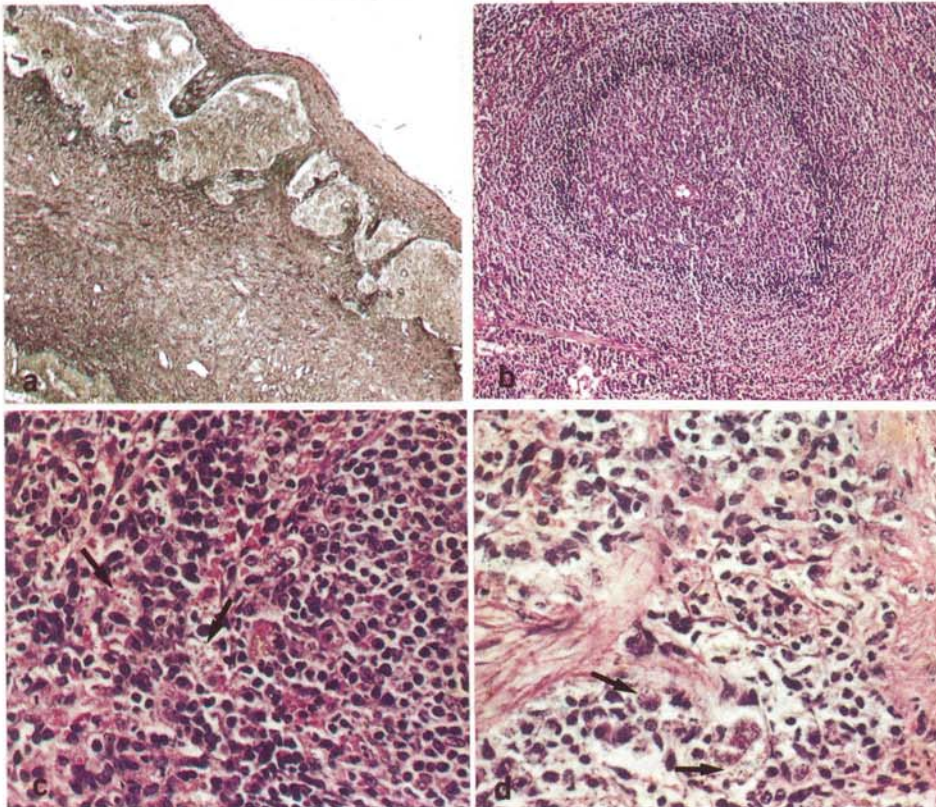


Fig. 4: Spleen of naturally infected dog: (a) Thickened capsule and trabeculae. Silver. x200.; (b) Malpighi follicles showing three distinct layers of cells. HE. x400.; (c) Macrophages from the marginal zone heavily parasitized (arrows). x400.; (d) Heavy parasitism of macrophages of the subcapsular region (arrows). HE. x400.

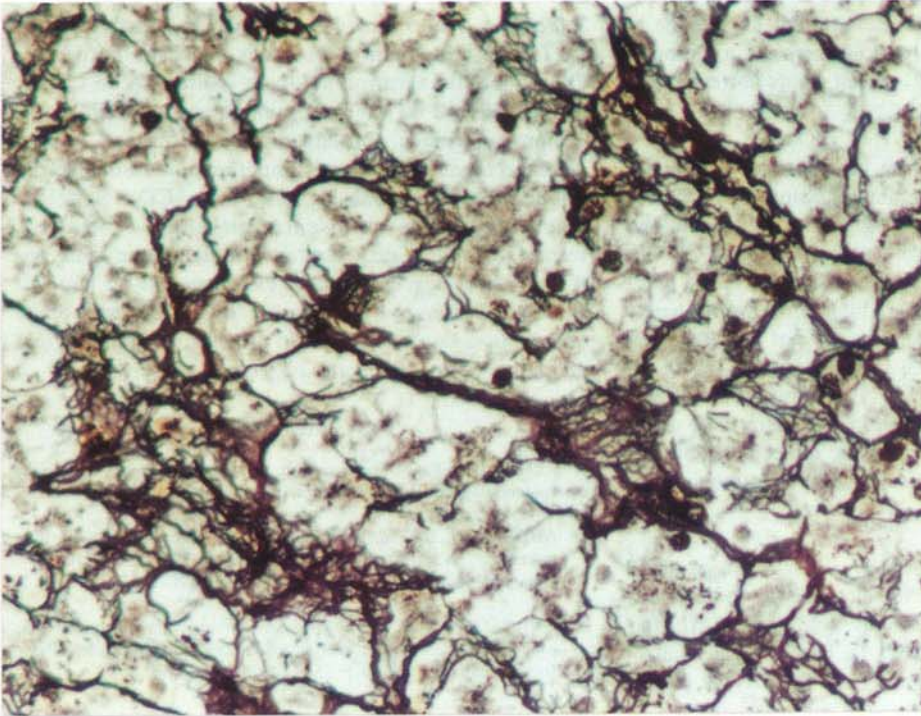


Fig. 5: Spleen of naturally infected dog: (a) positive PAP for CD18 and (b) for CD11b in the cells of the marginal zone x40 (a); x200 (b); (c) positive PAP for CD11b and (d) for CD11c in cells of the red pulp. Presence of numerous parasites (arrows). x400.

studies on spleen of dogs naturally infected with *L. donovani*² and *L. infantum*²⁴.

In one case, the same animal that showed the fibrous hepatic lesions, the connective proliferation in the red pulp was evident, approximating the profile of a sclero-congestive spleen. Spleen fibrosis is a pathological process that can be found in human visceral leishmaniasis⁴³.

We did not observe the deposition of amyloid material in the white pulp (in the Malpighi follicle and around the central arteriole) as described by GEORGE et al.¹⁴ in a two year-old, naturally infected dog from Greece. This fact would also be relevant for an immunocytochemical study of the integrins of the CD11b,c/CD18 complex since the amyloid substance is capable of provoking a greater expression of these integrins^{42,45}.

As to the experimentally infected dogs, the changes were the same as described for the earlier group, but less obvious or intense. In fact, the reactivity of the Malpighi follicles with quite evident formation of ger-

minative centers occurred in a much less intense fashion than in the naturally infected dogs. The red pulp showed slight changes, but always with more cellularity than in the control group. Another marked difference compared to naturally infected animals was the scarcity of parasitism in three dogs and the absence of parasitism in the others. These differences may be due to longer duration of the infection or with the presence of pathogens in naturally infected animals. However our data does not allow definitive conclusions about that. Fibrosis and spleen amyloidosis were not observed in agreement with GENARO¹³ and OLIVEIRA³⁴.

The immunocytochemical reactions for the CD11b,c/CD18 integrins showed a similar labelling and distribution pattern in all groups, i.e. the cells of the marginal zone and of the red pulp, and at times occasional ones in the white pulp, were always positive. The labelling for CD18 was in agreement with the studies of MOORE et al.²⁹ on dogs. However, as far as we know the distribution of CD11b in dog spleens has not been described. In mice, HO & SPRINGER¹⁷, using immunofluorescence, found positive cells for CD11b in the same regions that we described.

The larger number of cells labelled for CD18 in the naturally and experimentally infected animals compared to the control group, agrees with the histopathology that clearly showed a greater cellularity in the spleens of sick dogs. However, there was no increase in the number of cells labelled for CD11b that would correspond to a macrophage proliferation.

The possible correlation between the parasitism and expression of all of the antigens studied, especially CD11b (CR3) and CD11c (CR4), leads us to the following considerations: although the expression of CD 11b was subjectively more intense and defined in the naturally infected dogs, in at least two animals with heavy parasitism, the statistical analysis did not reveal significant differences among the animals studied. With respect to the expression of CD11c, as the staining in all cases was very slight, we believe that there is no direct correlation of the expression of the molecules with that of the level of parasitism or infection by protozoans of the genus *Leishmania*.

The immunological cellular response is considered to be the most important in the outcome of the disease^{7,8}. The important fact revealed in this study was the difference in the activation of CD 18 between the control group and the infected group. This fact may be important as far as the immunological response to visceral leishmaniasis is concerned.

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RESUMO

Histopatologia e estudo imunocitoquímico dos receptores do tipo 3 e 4 do complemento no fígado e baço de cães natural e experimentalmente infectados com *Leishmaniose Visceral*

Os objetivos deste trabalho visaram uma análise comparativa das alterações histopatológicas e da expressão de CR3 e CR4 no fígado e baço de cães natural e experimentalmente infectados com *L. chagasi*. As lesões histopatológicas fundamentais observadas principalmente nos cães naturalmente infectados foram: os granulomas epitelióides hepáticos, a hiperplasia e a hipertrofia das células de Kupffer, dos folículos de Malpighi e das células mononucleadas da polpa

vermelha do baço. Os cortes de fígado e baço corados pela técnica de imunocitoquímica mostraram a presença dos antígenos CD11b,c\CD18 nos animais controles e infectados, sem diferenças qualitativas e quantitativas no fígado. Entretanto, no baço dos cães natural e experimentalmente infectados a expressão de CD18 (subunidade $\beta 2$ da molécula comum aos leucócitos) foi sempre aumentada. Em leishmaniose a resposta imune celular é considerada a mais importante na resolução da doença. A expressão de CD11 b,c\CD 18 pelos macrófagos deve representar um papel central na resposta celular. Estes resultados indicam que tanto na leishmaniose visceral canina experimental ou natural existe uma diferença na ativação de CD18, o qual deve exercer uma importante função na resposta imunológica na infecção por *Leishmania chagasi*.

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