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Presence of antibodies in the aqueous humour and cerebrospinal fluid during *Leishmania* infections in dogs. Pathological features at the central nervous system

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SUMMARY

In the present paper we show that in dogs, naturally infected with Leishmania infantum, *the aqueous humour and the cerebrospinal fluid contain anti-*Leishmania *IgGs and that the specificity of antigen recognition of these fluids is similar to that of the sera. We also show that in the encephalon and cerebellum of these dogs there is a pathological sponge-like reaction accompanied by neuronal degeneration, mobilization of glial cells together with accumulation of amyloid deposits. The interstitial and intravascular deposition of IgGs and* Leishmania *antigens in choroid plexus suggest that in these animals there is a failure of the blood-cerebrospinal and ciliary bodies filtration barriers which may allow the transfer of anti-*Leishmania *IgGs from the blood stream to these fluids. We suggest that the failure of the blood-cerebrospinal barrier and the in situ concentration of anti-*Leishmania *IgGs and antigens in brain tissues may predispose to the pathological features detected in this compartment.*

Keywords *aqueous humour, cerebrospinal fluid,* Leishmania, *central nervous system*

INTRODUCTION

Most of the multiple and dramatic organic alterations which are produced during visceral leishmaniasis infections both in humans and dogs are being unveiled in recent years by several biochemical and immunological studies. The studies have been mostly directed to determine the concentrations and the types of immunoglobulins existing in sera of infected animals and the modifications of the complement system together with the formation and accumulation of immune complexes in sera (Galvao-Castro *et al*. 1984, Oliveira *et al*. 1984, Carvalho *et al*. 1993) and in particular tissues (Oliveira *et al*. 1985, Castañho *et al*. 1990, Nieto *et al*. 1992). However, most of the studies concerning the leishmaniasis disease have been, recently, focused in the analysis of the mechanisms by which *Leishmania* eludes the lytic response in the host macrophages (Chang & Dwyer 1978, Channon & Blackwell 1988, Russel & Wilhelm 1989) and to determine the $CD4^+$ and $CD8^+$ Th cell subsets and cytokines that dominate during infection in susceptible as well as in resistant animal models or which develop after immunization (Muller *et al*. 1991, Romagnani 1991, Liew & O'Donnell 1993). However, the pathological damages occurring during visceral leishmaniasis at the spleen, liver, skin and kidney level associated with immune responses remain greatly unknown particularly those relative to the central nervous system (CNS).

In the present paper we show that anti-*Leishmania* antibodies are present in the sera, aqueous humour (AH) and cerebrospinal fluid (CSF) of dogs showing the acute and chronic forms of the leishmaniasis disease. We were interested in the analysis of the antigen-binding specificities of the AH and the CSF since it has been reported that ocular immune complex deposits are believed to contribute to the anterior segment inflammation of the eye in Mycoplasma arthritis in association with human arthritides (Moore *et al*. 1982) and that the pathogenesis of human cerebral malaria

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appears to involve immunological mediated mechanisms (Wright 1968, Grau *et al*.) some of which have been detected at the CSF level (Chapel *et al*. 1987, Mitra 1991). Our data indicate that in the infected animals the pattern of reactivity of the IgGs from the AH and CSF fluids is similar to that detected in sera and suggest that there is a break down of filtration barriers and a transfer of antibodies and antigens from the blood stream to the CSF compartment. We show, moreover, that lesions, similar to those reported in particular *Plasmodium falciparum* and *berghei* infections (Finley 1982, Wyler 1983) and amyloid deposits may be detected in the *choroid plexus* and the cerebral parenchyma, close to the third ventricle, in dogs with the acute and chronic forms of the leishmaniasis disease. We believe that this is the first report of the presence of anti *Leishmania* IgGs and antigens in immune privileged sites during natural VL and VCL and of the presence of pathological reactions in the central nervous system during these stages of the disease.

MATERIALS AND METHODS

Animals

Twenty-seven dogs naturally infected with *Leishmania infantum* and five dogs parasitologically and serological negative for *Leishmania* were used as controls. All of them came from the Caceres region of Spain. The clinical and analytical studies were carried out at the University Veterinary Hospital. The analysis indicated that the infected animals used in this study had the VCL (with visceral and cutaneous lesions) and the VL (visceral lesions) acute forms of the disease with rapid evolution.

Tissue and serum samples

After an intramuscular injection of 0·5 mg/kg of chlorpromazine and an intravenous injection of 1 ml of sodium thiobarbital at a dilution of 1/20 to each animal several samples were taken. (A): 20 ml of blood which were frozen to −80°C. (B): 10–15 ml of spinal fluid which were frozen at −80°C. The fluid was taken by centesis from the cerebellar-bone marrow cistern. (C): 2 ml of aqueous humour following centesis of the anterior chamber of the eye. (D): an encephalic tissue block containing *choroid plexus* from the di-encephalic cavity (third ventricle). The *choroid plexus* sample was taken after complete necrosis following euthanasia using 1 g of sodium barbital. The tissue sample was fixed for 72 h in 10% formalin pH7·2. and embedded in paraffin. Serial cuts in the tissue block were made parallel to the cerebral encephalic longitudinal fissure. The cuts were stained with haematoxylin-eosine and Congo red following standard methods.

Table 1 Reactivity of the sera (S) aqueous humour (AH) and cerebrospinal fluid (CSF) against *Leishmania* antigens. The ELISA values are expressed in OD

Dog no.	Disease form	IIF	ELISA S.[a]	ELISA AH.[b]	ELISA CSF.[c]
1	VCL	1/640	0·48	0·31	nd
2	VCL	1/320	0·42	0·18	—
3	VCL	1/160	0·33	0·32	—
4	VCL	1/640	0·30	0·15	—
5	VCL	1/640	0·45	0·25	—
6	VL	1/320	0·54	0·31	—
7	VCL	1/640	0·48	0·29	—
8	VCL	1/160	0·39	0·28	—
9	VL	1/320	0·30	0·07	—
10	VL	1/640	0·51	0·36	—
11	VCL	1/160	0·36	0·12	—
12	VCL	1/640	0·54	0·42	—
13	VCL	1/320	0·39	0·16	—
14	VCL	1/640	0·48	0·24	—
15	VCL	1/640	0·60	0·51	0·69
16	VCL	1/160	0·39	0·04	0·03
17	VCL	1/640	0·60	0·25	0·27
18	VCL	1/320	0·42	0·04	0·07
19	VCL	1/640	0·63	0·46	0·66
20	VL	1/640	0·60	0·29	0·42
21	VCL	1/640	0·48	0·22	0·30
22	VCL	1/160	0·36	0·19	0·08
23	VCL	1/640	0·57	0·33	0·07
24	VCL	1/160	0·42	0·15	0·08
25	VCL	1/640	0·51	0·28	0·57
26	VCL	1/640	0·63	0·28	0·54
27	VCL	1/640	0·60	0·36	0·48

Control (sera): $0{\cdot}08 \pm 0{\cdot}024$. Control (AH):$0{\cdot}02 \pm 0{\cdot}006$. Control (CSF): $0{\cdot}03 \pm 0{\cdot}005$

[a] serum dilution 1/400; [b] AH and [c] CSF were used at 1/2 dilution, nd = not determined.

Analytical determinations

The antigen was prepared by sonication of the MON 1 autochthonous strain of *Leishmania infantum* (LEM 2002, reference M/CAN/ES/88/CHUMI). The wells were coated with 100 μl of a solution of 4 μg/ml of the antigen dissolved in sodium carbonate (pH9·6). The sera, the aqueous humour and the cerebral spinal fluids were used at a dilution of 1/400, 1/2 and 1/2 respectively. As second antibody a peroxidase labelled anti-dog IgG was used (Sigma) at a dilution of 1/10 000. OPD dissolved in citrate buffer containing 0·5% Tween 20-H_2O_2 was used as substrate. The reaction was stopped by addition of 50 μl of 3 N H_2SO_4 to each well. The reading was done at A 450 in a Micro-ELISA autoreader. Four positive and four negative controls were included in each plate. The positive controls obtained from hyperimmune animals were included to check the ELISA reaction.

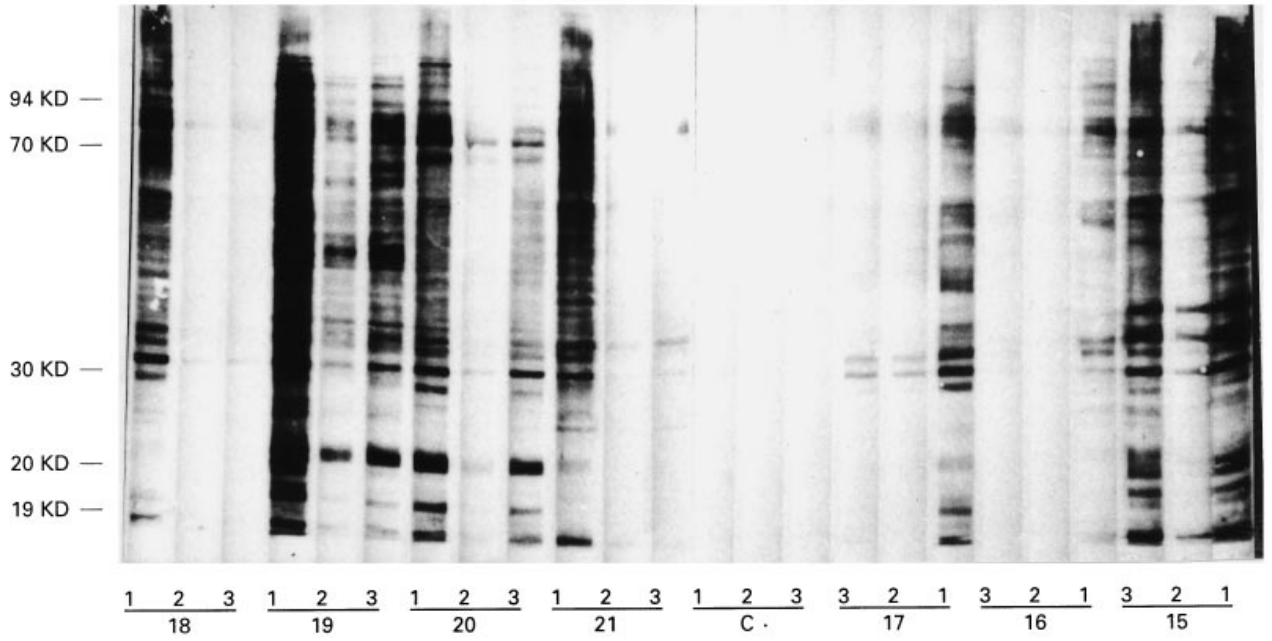


Figure 1 Immunoblot analysis of *Leishmania infantum* polypeptides reacting with sera (Lane 1), AH (Lane 2) and CSF (Lane 3), from VCL dogs (nos 15 to 21). C: control sera (1), AH (2) and CSF (3).

The mean absorbance value of the control sera was $0{\cdot}08 \pm 0{\cdot}024$. The mean value of the control AH was $0{\cdot}02 \pm 0{\cdot}006$ and of the CSF fluid was $0{\cdot}03 \pm 0{\cdot}005$. Values exceeding the mean value of the negative controls plus four standard deviations were considered positive.

Immunoblotting

The *Leishmania* proteins were obtained from a 20 ml promastigote culture of parasites grown to a density of 10^8/ml. After extensive washing in PBS the parasites were lysed at room temperature in 400 μl of Laemmli buffer (Laemmli 1970). After heating at 90°C for three min the solution was placed in ice. Afterwards the proteins were separated in 12% polyacrylamide gels using the Mini-Gel-System (Milipore). The amount of proteins added to each slot was equivalent to 10^6 parasites. Afterwards, the proteins were blotted into a nitrocellulose paper for 3 h at 600 mA using a miniblotter system (Pharmacia). Blocking was done in 2% de-fatted milk. The sera, the aqueous humour and the CSF, used at a dilution of 1/40, 1/2 and 1/2, respectively, were incubated for 1 h with the protein blots, washed and incubated in a second step with a peroxidase labelled anti-dog IgG for 1/2 h at a dilution of 1/1000 in TBS Tween 20-H_2O_2 and revealed with cloronaphtol. The reaction was stopped with distilled water when a background colour began to appear. Proteins of know molecular size were run in parallel as markers.

Determination of IgGs and antigens in tissue samples

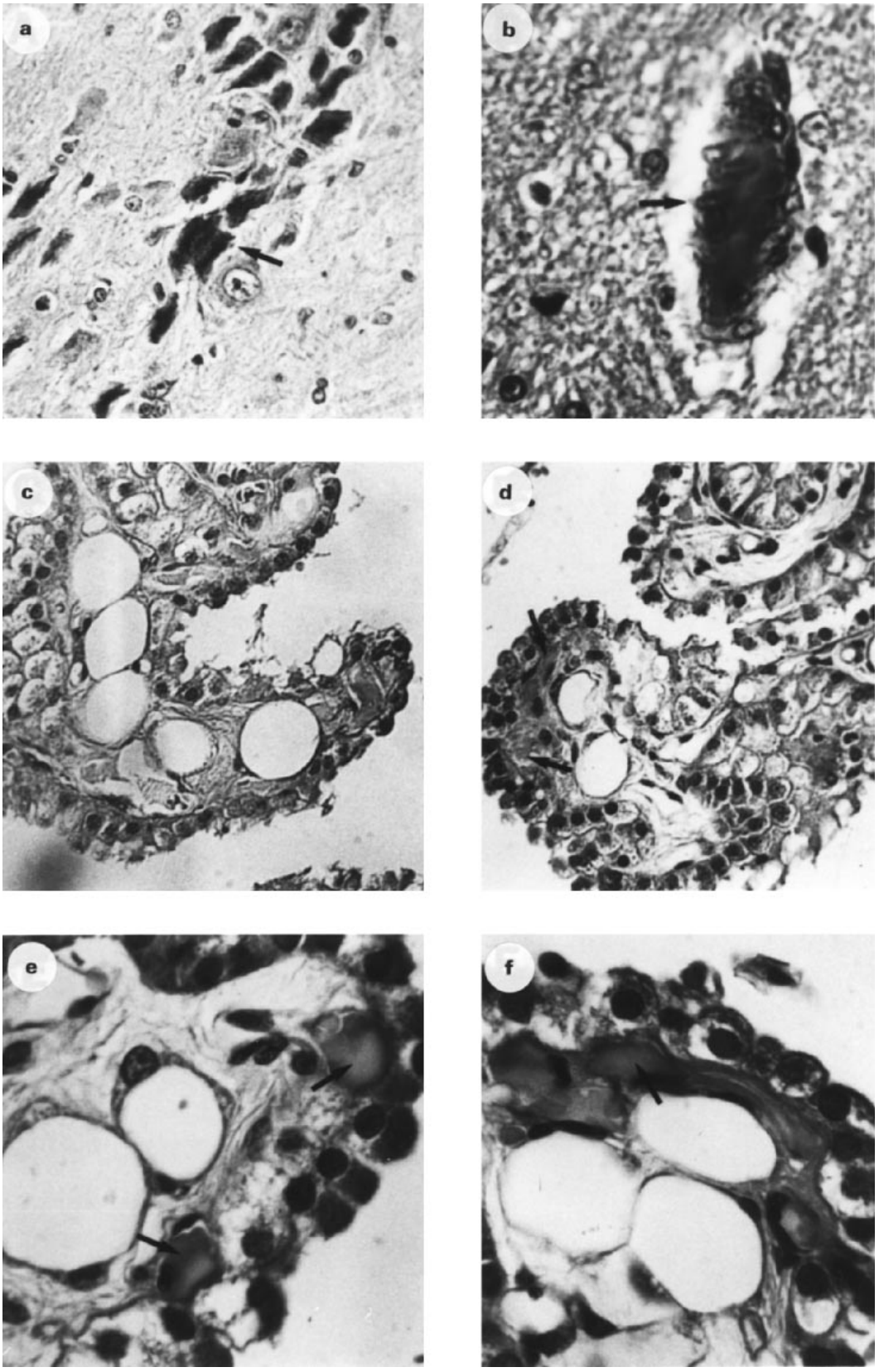
The hyperimmune anti-*Leishmania* serum was obtained by subcutaneous and intravenous immunization of two rabbits with a *L. infantum* promastigote lysate. Three doses of the antigen (500 μg per dose) were administered on day 0, 15 and 30 in Freund's incomplete adjuvant. Fifty days after the last immunization the serum of one of the rabbits which had been absorbed to rabbit red blood cells was probed against the promastigote lysate. The titre by IIF was 1/640.

The immunohistochemistry was done on deparaffined 4 μm thick sections of *choroid plexus.* The sections were probed with rabbit anti-dog IgG (RAD/IGG H+L, Nordic immunological Lab.) at a dilution of 1/160 to label immunoglobulins and with the rabbit hyperimmune anti-*Leishmania* serum described above (dilution 1/200) to detect *Leishmania* antigens. The sera were allowed to react with the tissue sections for 15 min at 25°C. The labelling was done by peroxidase using the biotin-streptavidin system (Dako corp., LSAB® 2 Kit, Peroxidase).

RESULTS

Clinical data of the animals and reactivity of the sera against *Leishmania* antigens

The clinical data and the microscopic observation of the macrophages from the popliteus and preescapular lymphoid



nodes of the control dogs revealed that the animals were free from the leishmaniasis disease and infection. Since the IIF test indicated, moreover, that the sera from these animals did not have anti-*Leishmania* IgGs those sera were taken as controls for the ELISA tests. Only four of the 27 dogs (dogs 6, 9, 10 and 20) under study presented the acute visceral form of the disease (VL) without signs of cutaneous lesions. All the rest of the dogs presented the visceral form together with cutaneous lesions (VCL) as shown by the presence of several syntomps such as general dermopathies, ulcers and conjunctivitis associated to blepharitis and alterations of the haematimetric and seric constants and renal and kidney disfunctions (Table 1[a]). The presence of amastigote forms of the parasite in macrophages from the popliteus and preescapular lymphoid nodes confirmed, moreover, the existence of the leishmaniasis infection in all these animals. None of these animals showed the presence of other infecting pathogens. All the animals were serologically positive when confronted with the *Leishmania* antigen as detected by IIF with titre values between 1/160 and 1/640 (Table 1[b]) and by ELISA (Table 1[c]) with a mean OD value of $0{\cdot}47 \pm 0{\cdot}03$ (mean OD value of controls $0{\cdot}078 \pm 0{\cdot}024$).

Immunological reactivity of the AH and CSF against *Leishmania* antigens

The ELISA technique was also able to detect the presence of anti-*Leishmania* antibodies in most of the AHs of the infected dogs (Table1, AH[b]). The reactivity of the AH is lower than the reactivity of the sera from the same animal since the values of ELISA shown were obtained at low dilutions of the fluid (1/2). The titre of the most reactive serum was 1/1600 while that of the most reactive AH was 1/16. Dogs 16 and 18 were positive for pathology and infection and by IIF and seric ELISA values but negative for AH ELISA values. We observed that, in general, the level of the reactivity of the AHs correlates well with the level of the seric reactivity. Dogs 15 and 17 have, however, identical ELISA values but different AH ELISA values.

The reactivity of the CSF from 13 of the infected dogs against the antigen is also lower than the reactivity of the sera but somewhat higher that the reactivity of the AHs (Table1 CSF[c]). The values of ELISA shown were obtained at a 1/2 dilution. The titre of the most reactive CSF was 1/32. The immunological response of the CSF from particular infected dogs with the VCL pathology (dogs 16, 18, 22, 23 and 24) was negative as an indication of the absence of anti-*Leishmania* IgGs in the CSF fluid of these animals. Thus, although in general elevated seric ELISA values have a counterpart in elevated CSF values there is no strict correlation between these values since particular dogs with elevated seric ELISA values are negative for CSF values (for example dog 23).

Immunoblot analysis of the reactivity of sera, AH and CSF

A detailed analysis of the specificity of the IgGs present in sera and of those present in AHs and CSFs was performed by Western blots (Figure 1). We selected samples from dogs 15, 17, 19, and 21 and dog 20 since they were VCL and VC positive, respectively, by IIF and seric AH and CSF ELISA values. Also samples from dogs 16 and 18 were selected because they were positive by IIF and seric ELISA but negative for AH and CSF. About 30 antigens with molecular masses ranging between $\pm$ 18 kD and $\pm$ 120 kD were recognized by the sera from the positive dogs. Although most of the bands were common to all sera we detected a certain variability in the antigen-binding pattern revealed by different samples, particularly in the 30–19 kD region. In agreement with previous reports for the humoral response to *Leishmania donovani* in human visceral leishmaniasis (Rolland-Burger *et al*. 1991) we observed that the most intensively labelled bands corresponded to molecular masses of 94, 75, 70, 66–60, 40–30, 20–17. We also observed that the sera from these dogs strongly react with the *Leishmania* purified Hsp70 (70Kd), LiP0 (32kd), H2A (14kd), LiP2a and LiP2b (13kd) proteins (Laboratory data). The CSF of dogs 15, 19, and 20 also reacted with a large number of *Leishmania* polypeptides having a specificity similar to that of the sera from the same animal. The AH of dogs 15 and 19, having the highest ELISA OD values of all the tested animals, reacted also with several protein bands. It was interesting to observe that the sera from dog 18 recognized a number of polypeptides larger than those recognized by the sera from dog 16 in spite of having similar

Figure 2 Pattern of the pathological reactions detected in the encephalon and cerebellum of dogs having the VCL pathology. The tissue was stained with haematoxiline-eosine. The amyloid deposits were revealed by Congo red and polarized light. The photographs correspond to tissue sections of dogs 17, 21 and 26. (a) The encephalitic reaction is revealed by mobilization of glia cells with condensation of peripheral glial micronodules towards degenerated pyramidal neurons with amyloid deposits ($\times$ 280). (b) Sponge-like reaction with intense neuronal necrosis. There is swelling of the Virchow Robbins spaces with amyloid deposits ($\times$ 320). (c) Plexus choroiditis with hypercellularity ($\times$ 180). (d) *Choroid plexus*: subependimal deposits of amyloid substance ($\times$ 280). (e) Interstitial localization of the amyloid deposits in the connective glial backbone of the *Choroid plexus* ($\times$ 320). (f) *Choroid plexus*: the amyloid deposits are located within sinusoidal capillaries ($\times$ 320).

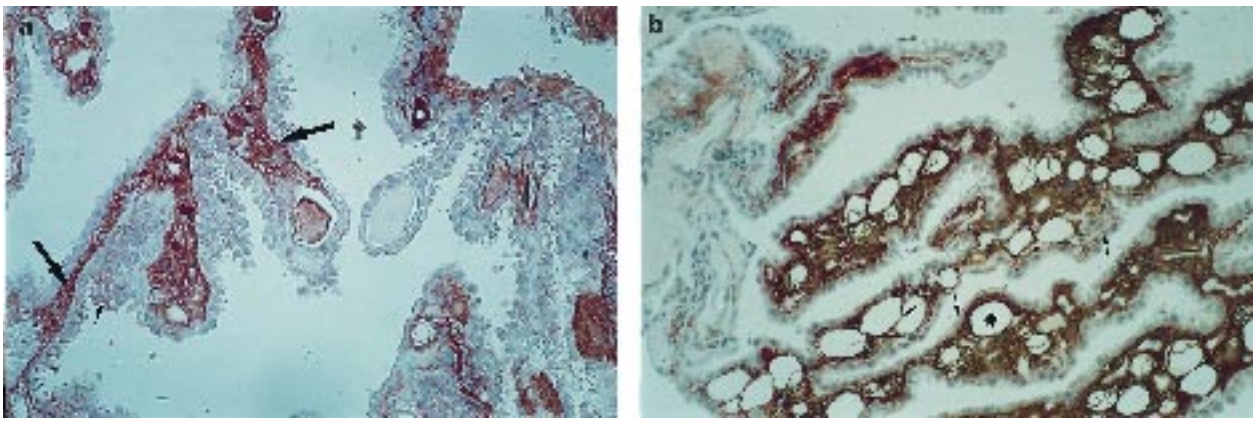


Figure 3 Immuno-staining for *Leishmania* antigens (b) and Immunoglobulins (a) in *Choroid plexus* of VCL dogs. The arrows indicate that the antigens are predominantly detected in interstitial, subepithelial and perivascular spaces. The immunoglobulins are detected in the interstitial and intravascular spaces. The picture also shows the existence of intense vascularity (*) and of some thrombi.

ELISA values. The pattern of labelled bands recognized by the sera from dog 18 was similar to that of dog 20 with the exception of the proteins in the 20 kD range. However, while dog 20 was clearly positive for seric and CSF ELISA dog 18 was negative for CFS ELISA. The CSF of dogs 19 and 20 also reacted with a large number of proteins with an specificity similar to that of the sera from the same animals. The AH reactivity pattern was poor and only that from dogs 15 and 19, having the highest ELISA values of all the animals tested, recognized a few protein bands.

Pathological data and immunohistochemistry

Since the presence of anti-*Leishmania* IgG in the CSF of particular infected animals suggested the existence in these animals of a pathological situation within the CNS we looked for possible alterations in the morphological pattern of particular CNS tissues. The histological analysis was performed on VCL dogs 17, 21 and 26 because they showed different but significative CSF ELISA values. Figure 2 shows the typical pathological features observed in the encephalon and cerebellum and *Choriod plexus* of these dogs. A clear pathological reaction was detected in the CNS as shown by the Walleriana degeneration of the Purkinge cells accompanied by satellitosis, neuronophagia, intense microglia mobilization, pyramidal neurons degeneration and amyloid deposits (Figure 2a, dog 17). An sponge-like reaction was also detected in the encephalon with neuronal degeneration and necrosis together with swelling of the Virchow-Robbin spaces containing intravascular deposits of amyloid substances (2b, dog 26). At the *choroid plexus* level we observed the presence of an inflammatory processes with hipercellularity (Figure 2c, dog 21) and a subependimal (Figure 2d, dog 26), interstitial (Figure 2e, dog 21) and intravascular localization of amyloid deposits within sinusoidal capillaries (Figure 2f, dog 17). None of these alterations was detected in the *choroid plexus* of the controls animals analysed (two dogs). We also performed an immunohistochemical analysis of these tissues looking for deposition of IgGs and *Leishmania* antigens. Extensive deposits of these antigens (Figure 3b) and of immunoglobulins (Figure 3a) were detected at the interstitial, perivascular and intravascular level of the *choroid plexus* (dogs 17 and 26). These deposits were not observed in the *choroid plexus* of control dogs.

DISCUSSION

The findings of this paper offer direct evidence of the presence of anti-*Leishmania* antibodies in the AH and CSF of animals with acute and chronic clinical forms of leishmaniasis and that the antigen binding specificity of the antibodies present in those fluids is similar to that found in the sera of the same individual animal. The level of the reactivity against the *Leishmania* antigens of the AH and CSF is, however, significative lower than that of the sera. The origin of the IgGs present in the AH and CSF is not clear although the good correlation existing between the level and specificity of reactivity of individual sera and that of the AH and CSF of the same animal would favour the hypothesis of a transfer of antibodies from the blood stream to the AH and CSF. In fact several authors have demonstrated increased blood-filtration barrier permeability and immune complex vasculitis in the kidneys of animals with the LVC and LV pathology (dogs 4, 21, 22) as well as abnormal filtration due to glomerulonephritis in experimental Kala-azar (Sartori *et al*. 1987). Increased blood-brain permeability and sequestration of parasitized erythrocytes

within the capillaries of the cerebral cortex occur also in some cases of cerebral malaria in which areas of marked endothelial swelling are observed in the brain vessels (Grau *et al*. 1987). Our data favour the hypothesis of a failure of the blood-CSF and ciliary bodies filtration barriers since extensive deposits of *Leishmania* antigens and immunoglobulins were observed in interstitial and intravascular spaces of *choroid plexus* of VCL dogs. We can not exclude, however, that the transferred antigens may also induce an intrathecal synthesis of immunoglobulins as it is the case in acute cerebral malaria (Chapel *et al*. 1987). Circulating *Leishmania* antigens have been also described in CL and MCL (Carvalho *et al*. 1993).

The factors contributing to the pathogenesis detected at the *choroid plexus* level and the encephalon and cerebellum in chronic VL and VCL leishmaniasis are difficult to be assigned. It may occur that the presence of *Leishmania* antigens and immunoglobulins in the CSF and *choroid plexus* of the infected animals induce the localized lesions and the inflammatory and immune reactions with intense microglia mobilization followed by pyramidal neurons degeneration. In fact, T cell deficient nu/nu mice do not exhibit cerebral malaria (Finley *et al*. 1982). However, while Howes & McKay (1975) demonstrated that in the experimental mycoplasma-induced arthritis model ocular lesions and intravascular deposits may result from circulating immune complexes which eventually might initiate the inflammatory reactions, Hylkema *et al.* (1983) and Thirkill, Tyler & Roth (1992) reported the failure of the circulating immune complexes to elicit ocular inflammatory reactions. Thus, in addition to the immune complexes deposition several other factors might contribute to the brain pathology. As indicated by Grau *et al*. (1987) the release of TNF-alfa by activated macrophages in *Plasmodium yoelii* infections may lead to the predominance of lesions in the central nervous system in the murine model or to predispose in *P. falciparum* infections to human cerebral malaria and to a fatal outcome (Kwiatkowski *et al*. 1990, Clark, Rockett & Cowden 1991). Thus, we believe that, as it occurs in malaria infections, particular cases of *L. infantum* infection in dogs leading to VCL may end up in canine cerebral lesishmaniosis (CCL) with a histopatholy similar to that observed in cerebral malaria.

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