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Immunopathology of the uveitis in canine leishmaniasis

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SUMMARY

Particular immunopathological features and their effects on the vascular permeability of different ocular structures were analysed in two dogs naturally infected by Leishmania infantum. *The existence of specific anti-*Leishmania *immunoglobulin G* (*IgG*) *in the aqueous humour was confirmed by the ELISA technique. There was no correlation between antibody levels in the aqueous humour and the related serum. The histopathological study of the eyes showed the existence of lesions in various ocular structures. The ciliary processes, ciliary body, sclerocorneal limbus, iris and lacrimal duct showed intense inflammatory zones with lymphocyte infiltrates, plasmatic cells and macrophages with amastigote forms of* Leishmania. *In addition vasculitis with dilation and thrombi were also detected in both cases, with consequent oedema and hyalinization. The immunohistochemistry analysis revealed the presence of granular and diffuse IgG deposits in the ciliary body, ciliary processes, sclerocorneal limbus and iris. Furthermore, numerous thrombosed vessels were observed in the sclerocorneal zone and iris. Complement 3* (*C3*) *fraction deposits were not present in the ocular structures. The present data suggest that the ocular lesions may have an immunopathological origin.*

Keywords *Leishmaniasis, dog, uveitis, aqueous humour, antibodies, antigens, immune complexes, complement 3*

INTRODUCTION

Leishmaniasis is a zoonosis with various manifestations, one of these being ocular lesions. The main characteristics of these ocular lesions are their high incidence (McConnell *et al*. 1970, Slappendel 1988), a predisposition for the anterior segment of the eye (Puchol & Gonzalez 1989) and their persistence despite specific treatments.

Some of the most important ophthalmological manifestations of the disease are blepharitis (Longstaffe *et al*. 1983, Boldy & Clerc 1989), conjunctivitis (Slappendel 1988), keratoconjunctivitis (Puchol & Gonzalez 1989) and keratouveitis (McConnell *et al*. 1970, Roze, 1986, Boldy & Clerc 1989).

There is accumulating evidence that circulating immune complexes (CICs) play an important role in many disease processes, in various forms of glomerulonephritis, arthritis (Thirkill, Tyler & Roth 1992), vasculitis (Cochrane & Koffler 1973) and uveitis (Dernouchamps *et al*. 1977, Rahi *et al*. 1979). Immune complex (IC) damage is likely to occur in areas with specialized vasculature such as the ciliary processes, the renal glomeruli and the choroid plexus, as well as in the choriocapillaries and the skin (Peress, Miller & Palu 1977).

Uveitis is a symptom frequently seen in a classical immune complex disease such as serum sickness (Woods 1961). In this disease, IC depositions have already been reported in sites such as the iris, the ciliary body (Wong, Anderson & McMaster 1971, Peress & Tompkins, 1978, Peress, 1980) and in the scleral capillary area (Hylkema, Rathman & Kijlstra 1983).

Due to the similarities of symptoms like uveitis between leishmaniasis and other immune complex diseases, and that in patients with leishmaniasis circulating immune complexes have been detected (Desjeux *et al*. 1980, Sehgal, Aikat & Pathania 1982, Carvalho *et al*. 1983, Galvao-Castro *et al*. 1984) with deposition at renal (Oliveira *et al*. 1985, Sartori *et al*. 1987) or skin level (Brenner *et al*. 1984, Ginel *et al*. 1993), in the present work we have made an analysis of the histological lesions and of particular immunopathological

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features of the anterior segment of the eye from dogs with leishmaniasis. We believe that this is the first time in which these lesions, presumably caused by the deposition of ICs, are demonstrated to be present in dogs naturally infected by *Leishmania infantum*.

MATERIALS AND METHODS

Animals

Two dogs naturally infected by *Leishmania infantum* were taken from the province of Caceres, Spain for the current study. Both animals were parasitologically confirmed as positive by observation of amastigote forms in samples obtained from biopsy of lymph nodes, stained by the Giemsa method.

The serological diagnosis was supported by the appearance of antibodies in serum by an indirect immunofluorescent antibody test (IFAT) and an enzyme-linked immunosorbent assay (ELISA). This technique was performed as described by Jaffe *et al*. (1988) using FTS-*Leishmania* antigen (Freeze-Thaw-Sonicated promastigotes) and anti-dog IgG peroxidase conjugate (Sigma, USA). A single dilution was used for each serum (1/400) and aqueous humour (1/2) and the limit was established at an index of 3 (ratio absorbance positive/negative). The animals had slowly evolving chronic viscerocutaneous leishmaniasis with a wide and varied symptomatology, including ophthalmological alterations. One parasitological and serological negative dog was used as control.

Procedure

The dogs were killed by an intravenous injection of 2 g of sodium pentothal (Abbot La., Spain). Whole eyes, from the test and control dogs, were removed immediately following death and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E).

Table 1 Level of anti-*Leishmania* antibodies determined by ELISA in the serum and aqueous humour

Dogs	Serum	Aqueous humour
1	0·254 (40·32%)	0·43 (73·89%)
2	0·567 (90·00%)	0·44 (74·57%)
Control	0·043 (6·90%)	0·015 (2·55%)

Immunohistochemistry

Sections (4 μm) of dogs' eyes from each group were deparaffined and placed in normal physiological saline. Sections were probed with the following antisera to detect intraocular deposits of immunoglobulins and C3 fraction: (i) rabbit anti-dog IgG conjugated with peroxidase (RAD/IgGH+L; Nordic Immunological Lab.) at a dilution of 1/400 and (ii) goat anti-dog C3 (Sigma Immunohistochemicals, USA) and rabbit biotinylated anti-goat IgG (H + L) (Vector Laboratories, USA).

The staining method used was the streptavidin-biotin system DAKO corp. (LAB® 2 Kit, Peroxidase).

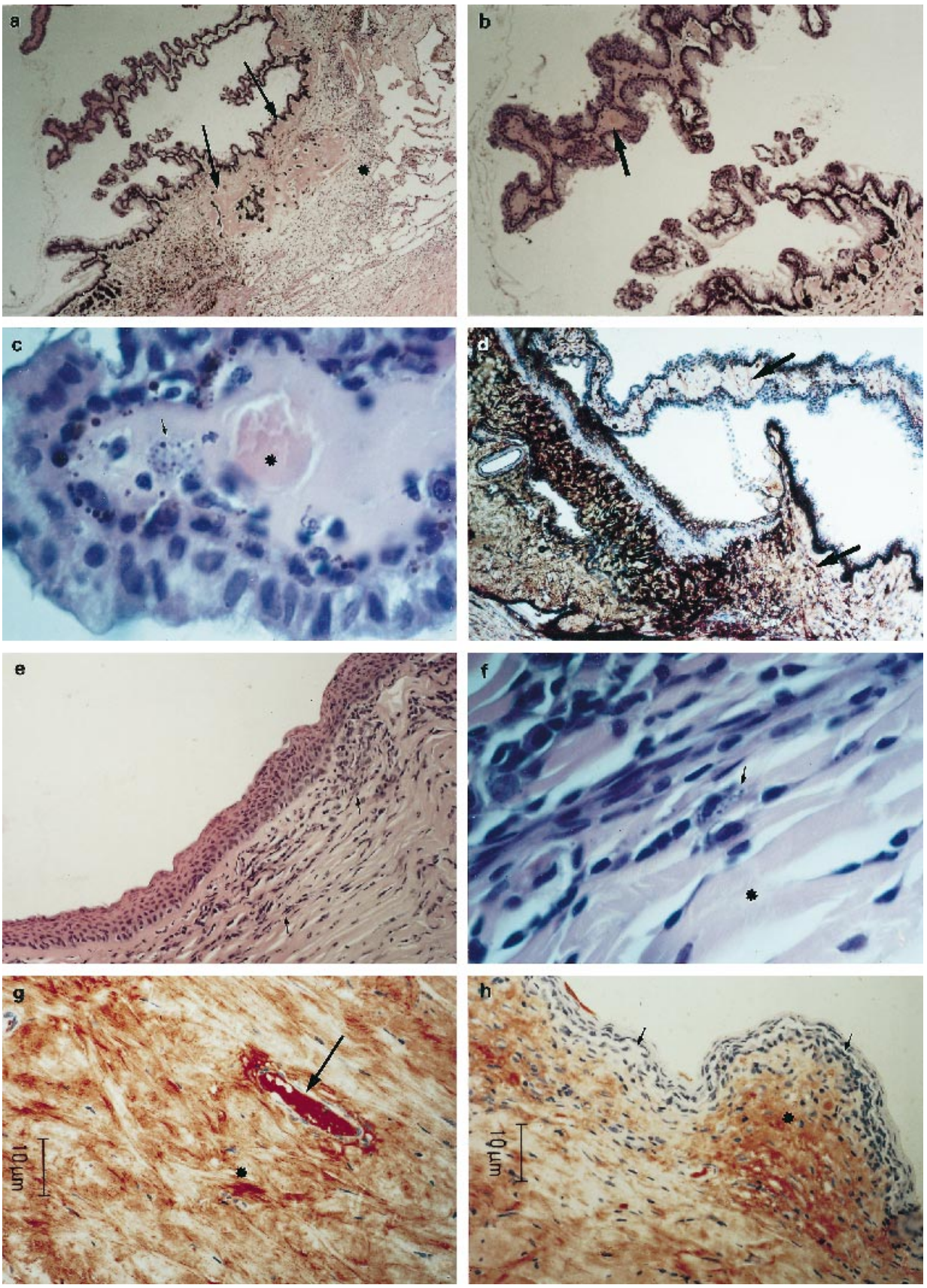
Each of these sera was allowed to react on separate sections of eyes from each group at room temperature for 15 min before washing in PBS.

RESULTS

The two dogs naturally infected by *Leishmania* showed the clinical and haematological characteristics typical of the disease such as weight loss, asthenia, lymphoadenomegaly, cutaneous lesions and ocular alterations like blepharitis and keratoconjunctivitis. Corneal opacity and intense iritis with pupilar deformation, were also present.

Specific anti-*Leishmania* antibody levels, expressed in optical densities (OD) were detected in the aqueous humour (Table 1). The table also shows the positive percentages in relation to controls. In both animals, the antibody measure

Figure 1 a. Ciliary processes and ciliary body. Abundant mononuclear cells infiltrate (asterisk), vessel dilation and presence of thrombi. Wide hyalinization zones with structure loss at basal areas of ciliary processes (arrows). (Haematoxylin & Eosin × 125). b. Ciliary processes. Homogeneous and acidofilous exudate together with vasculitis and thrombosis at apical zones. Some degree of desquamation grade of glandular cells and pigment cell loss (arrow). H&E × 250). c. Detail of apical zone of ciliary processes with epithelium cell alteration. Accumulation of *Leishmania infantum* amastigotes in oedema bosom (arrow). Detail of a thrombus (asterisk). (H&E × 450).
d. Thick and filamentous deposits of IgG at ciliary processes and body level (arrow). (Immunoperoxidase × 125). e. Sclera-limbocorneal transaction zone. General dilation of capillaries with shabby colagen fibres. Intense lymphocytes, plasmatic cells and macrophage infiltrate, distributed in focal and diffuse form all over the connective tissue (arrows). (H&E × 250). f. Detail of the anterior zone with dilation of lymphatic vessels, interstitial oedema and lymphocytes (asterisk), plasmatic cells and macrophage exudate with *L. infantum* amastigotes inside (arrow). (H&E × 450). g. Immunoperoxidase staining of sclera with IgG deposits (asterisk) and presence of thrombosed vessels with immunoglobulin accumulation (arrow). (Immunoperoxidase × 250). h. Oedema in epithelium with separation of strata and protuberance close to IgG deposits (arrows). These deposits were intense in the basal zones of the epithelium. Positive immunoreaction at thrombosed capillaries (asterisk). (Immunoperoxidase × 250).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was different in the sera while it was practically identical in the aqueous humour.

From the histological point of view, the infection by *Leishmania* showed the existence of a remarkable lesional set of symptoms at different ocular structures.

Microscopically, the ciliary body and ciliary processes exhibited intense inflammatory foci (Fig. 1a–d). Areas with abundant round cells, lymphocytes and plasmatic cell infiltrate were found in the ciliary body. Deep vascular alterations with lymphatic vessel dilation and some thrombosis phenomena were also evident in these tissues. The ciliary processes showed a certain degree of desquamation of glandular cells and pigment cell loss. The apical zones of the processes showed vessels with thrombi inside and a homogeneous and acidophilous exudate that absorbed the connective tissue. Groups of amastigote forms of *Leishmania* were also seen in these areas. The most intense inflammation was seen in the insertion zone of the ciliary processes with wide hyalinization zones. The histochemical analysis showed IgG deposits in the ciliary processes and in the insertion area between these and the ciliary body. Complement deposits were not observed in any of the ocular structures.

In the sclera and the sclerocorneal limbus (Fig. 1e–h) an intense vasculitis with dilation of the lymphatic vessels and thrombosis was evident. Interstitial edema and a mononuclear infiltrate formed by lymphocytes, plasmatic cells and macrophages with intracytoplasmatic *Leishmania* amastigotes were observed. Immunoglobulin deposits were present in the connective tissue particularly in basal zones of the ephitelium. Immunoglobulin deposits were formed in the thrombi of the affected vessels.

In the iris (Fig. 2a–d) lymphocytes and plasmatic cells together with *Leishmania* infected macrophages were present. Vasculitis, thrombosis and immunoglobulin deposits at the thrombus level and the supporting connective tissue were also observed.

The most intense inflammatory process was located in the lacrimal duct (Fig. 2e–h) with abundant tissue of granulation, focal and diffuse infiltrate of lymphocytes, plasmatic cells and numerous macrophages with amastigote forms inside. The epithelium showed a generalized necrosis and partial loss of this. Immunoglobulin deposits were diffusely distributed all over the lacrimal duct.

None of those immunopathological pathologies were detected in control eyes.

DISCUSSION

The immunological and histological results present in this paper indicate the existence of an ocular leishmaniasis in dogs naturally infected by *L. infantum*. In previous studies reported to date, keratoconjunctivitis and keratouveitis have been described as the most usual symptoms (McConnell *et al*. 1970, Roze 1986, Molleda *et al*. 1993) which frequently mask an irididocyclitis (Longstaffe *et al*. 1983).

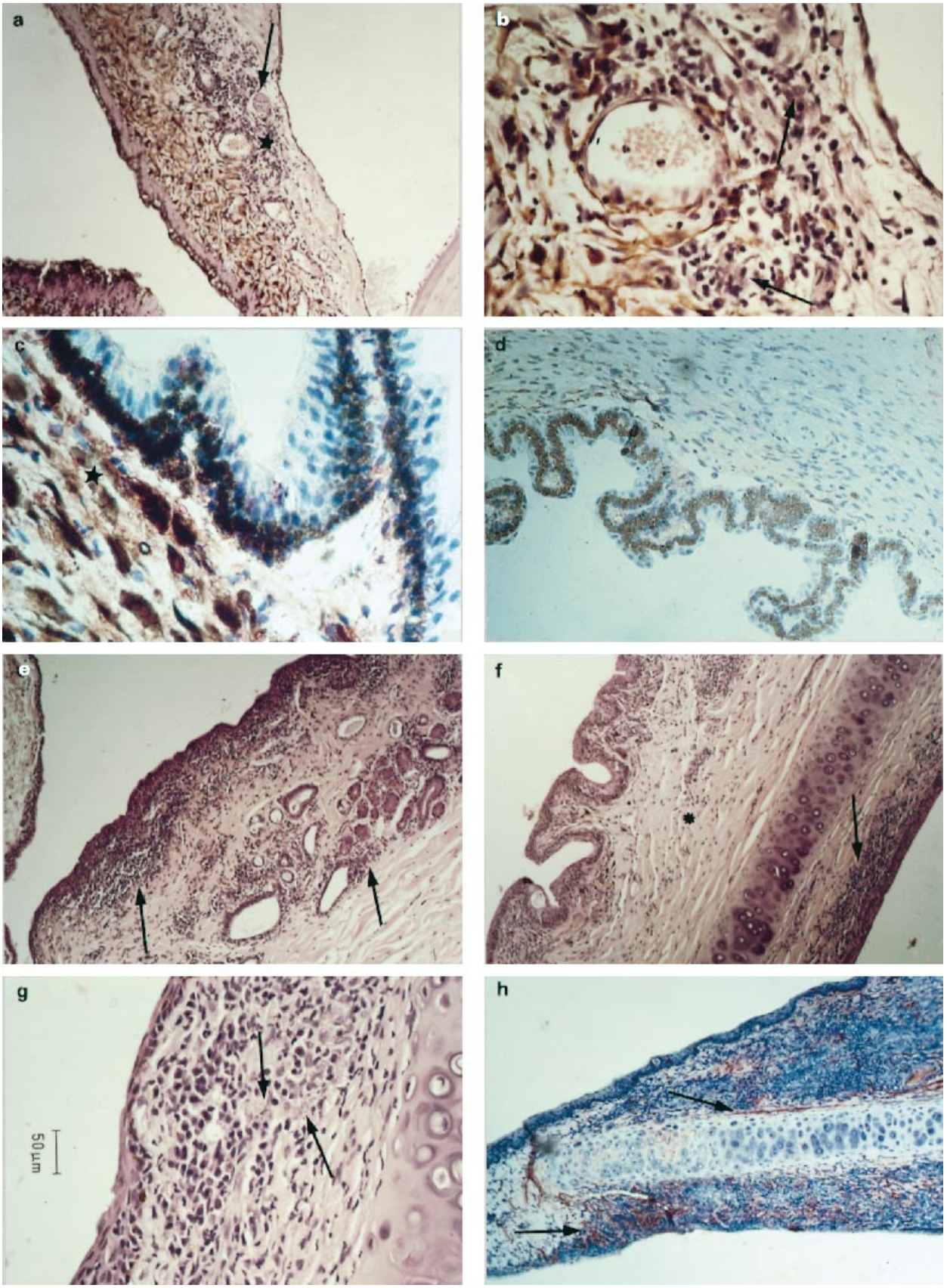
The ELISA data have shown the existence of specific anti-*Leishmania* IgGs in the serum and aqueous humour of the affected dogs. The detection of antibodies in the anterior chamber of the eye was independent of antibody levels in the respective sera and their presence can be related to the uveitis process in the sick animals.

In other fluids like urine, Kohanteb, Ardehali & Rezai (1987) detected the existence of antibodies in all the patients with visceral leishmaniasis. In the renal filtration barrier, immunoglobulins and immune complex deposits are frequently described in glomerular capillaries and mesangial matrix in patients with visceral leishmaniasis (Weisniger *et al*. 1978), in canine leishmaniasis (Nieto *et al*. 1992a) and experimental leishmaniasis in hamsters (Sartori *et al*. 1987).

Our results show the presence of *Leishmania* amastigote forms at different ocular structures (lacrimal duct, ciliary body and processes, iris and sclera) and the appearance of a serious inflammatory process mainly at the blood-aqueous humour barrier.

The multiorganic distribution of *L.* (*d.*) *infantum* with parasitic microgranulomas described in organs of the mononuclear phagocytic system (Keenan *et al*. 1984, Kirmse, Mahin & Lahrech 1987) and others such as lungs, heart, pancreas (Longstaffe *et al*. 1983), digestive system (Mugai *et al*. 1983), etc., bring about serious organic disfunctions. The polyclonal stimulation of B lymphocytes (Carvalho *et al*. 1983, Galvao-Castro *et al*. 1984) also

Figure 2 a. Iris. Accumulation of melanocytes in the interior part of the iris (star). External part with an intense infiltrate of mononuclear cells, dilation of vessels, thrombosis and cellular margination (arrow). (H&E × 250). b. Detail of iris with intense lymphocytes, plasmatic cells and macrophages with *Leishmania* amastigote infiltrates (arrows). (H&E × 450). c. Immunoglobulin deposits irregularly distributed all over iris connective tissue (star). (Immunoperoxidase × 450.) d. Control dog. (Immunoperoxidase × 250.) e. Lacrimal duct. Generalized necrosis of the epithelium. Granulation with abundant lymphocytes and plasmatic cells in the connective tissue (arrows). (H&E × 250.)
f. Bilateral epithelial necrosis (arrow) and focal and diffuse infiltrate with lymphocytes and plasmatic cells (asterisk). (H&E × 250.) g. Detail of lacrimal necrosis with partial epithelium loss. In the connective tissue, lymphocytes, abundant plasmatic cells and macrophages with *L. infantum* amastigote forms inside (arrows). (Immunoperoxidase × 250.) h. View of the lacrimal duct with diseminated immunoglobulin deposits all over the conective tissue (arrows). (Immunoperoxidase × 125.)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enhances production of specific and nonspecific antibodies against *Leishmania* with IC production (Ceci *et al*. 1985, Brandonisio *et al*. 1990) which are deposited in certain organs such as kidneys and skin (Sartori *et al*, 1987, Nieto *et al*. 1992a).

In canine visceral leishmaniasis the ocular inflammatory process may have two origins: on the one hand, inflammation can be a consequence of the anterior uveitis with intense leukocyte margination and *Leishmania* forms similar to the one described in skin (Brenner *et al*. 1984, Ginel *et al*. 1993). On the other hand, the inflammation can be a consequence of an immunomediated hypersensibility type III response derived from *Leishmania* antigen and from specific and nonspecific immunoglobulin deposits as it occurs at the glomerular filtration barrier (Sartori *et al*. 1987, Nieto *et al*. 1992), through which great amounts of immunoglobulins (Kohanteb *et al*. 1987, Sartori *et al*. 1987) and soluble antigen (Kohanteb *et al*. 1987) are eliminated.

Vasculitis with an increase in vascular permeability, is often attributed to the presence of ICs in many other systemic diseases such as lupus erythematosus (Atkins *et al*. 1972) and rheumatoid arthritis (Moore *et al*. 1982). Char *et al*. (1979) showed a correlation between the relative concentrations of ICs and the severity of ocular inflammation in patients with diffuse uveitis and chronic iridocyclitis. However, other studies (Hylkema *et al*. 1983) proved that the mere presence of intraocular ICs cannot be held solely responsible for the induction of uveitis and iridocyclitis since they were produced in he eyes of mice with no resultant inflammatory reaction.

Our results suggest that the presence of free and intracytoplasmatic amastigote forms in the inflammatory focus together with IC deposits formed by immunoglobulins and *Leishmania* antigens may be the two possible origins of the different oculopathies in visceral leishmaniasis caused by *L. infantum*. Char *et al*. (1979) observed that IgG was the predominant immunoglobulin in these immune complex deposits in uveitis.

In the same way, we can consider this organic site (uvea) as a blood-aqueous humour barrier liable to bear this kind of pathology because it is a place subjected to a high sanguine turbulence and pressure. Preliminary studies indicate that during visceral leishmaniasis the persistent fever (Hernández-Rodriguez *et al*. 1987), with a tissue CO_2 increment caused in part by the anaemia and the cholesterol metabolism alteration having a rise in low density lypoproteins (LDL-cholesterol) (Nieto *et al*. 1992b), may lead to a vascular permeability alteration and IC deposits.

In conclusion, the histological and immunological alterations may define the existence of an 'ocular leishmaniasis' worthy of further studies.

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