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Research paper

T lymphocyte immunophenotypes in the cerebrospinal fluid of dogs with visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a disease causing several clinical manifestations in dogs, including neurological disorders. Nevertheless, there are few studies related to the evaluation of the brain alterations during VL. Evidences of the involvement of cerebral barriers in infected dogs was reported, including the presence of brain inflammatory infiltrate, with a predominance of CD3+ T cells. Therefore, the aim of this study was to determine the immunophenotypes of T lymphocytes in the cerebrospinal fluid (CSF), as well as in peripheral blood, and to correlate with brain alterations in dogs with VL. We detected elevated percentages of double negative (DN) and double positive (DP) T cells in the CSF, with a predominance of TCRαb. In the histopathological analysis, we observed a predominance of lymphoplasmacytic infiltrate, mainly in leptomeninges, ranging from mild to intense, and we observed a positive correlation between the intensity of inflammation in the subependymal area and the DN T cells of the CSF. Thus, the DN T cells seem be acting as villains of the immune system through pro-inflammatory mechanisms. Further, the proportion of the different population of CSF T cells did not differ from those observed in the blood, which provides us with more evidence of blood-CSF barrier breakdown. Together, the results provide more explanation to the inflammation observed in the brain of dogs with VL, which the DN T cells contribute to the origin and progression of the neurological disease. This study provides insight into the immunophenotypes of T lymphocytes in the CSF during canine visceral leishmaniasis.

1. Introduction

Visceral leishmaniasis (VL) is an anthropozoonosis caused by *Leishmania infantum* (Syn *L. chagasi*; Mauricio et al., 2000). In Brazil, dogs are the main hosts of the parasite and can present several clinical manifestations, from subclinical to systemic disorders.

Chronically infected dogs may rarely develop neurological clinical disorders; nevertheless, the pathogenesis of the cerebral form of the VL has still not been elucidated. Infected dogs may present tetraplegy, generalised seizures, walking in circles, vestibular and cerebellar signs, myoclonia and motor incoordination (Font et al., 2004; Ikeda et al., 2007; José-López et al., 2012).

Brain lesions have been described in some reports; the main findings related to canine visceral leishmaniasis were meningitis and choroiditis (Nieto et al., 1996; Viñuelas et al., 2001; Melo et al., 2009, 2013; Melo and Machado, 2011) and the deposition of antigens and immunoglobulins in the central nervous system (CNS) (Garcia-Alonso et al., 1996; Melo et al., 2015a). Multiple brain infarcts were also described in two infected dogs (José-López et al., 2012).

A limited number of reports have pointed to parasite detection together with lesion descriptions. Parasite migration to the meninges (Viñuelas et al., 2001), choroid plexus (Nieto et al., 1996; Márquez et al., 2013) and brain parenchyma (Márquez et al., 2013) was described in a chronically infected dog. Recently, we have detected the presence of *L. infantum* DNA in brain areas (Grano et al., 2014) and up-regulation of the gene expression of some toll-like

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receptors (Melo et al., 2014) within the brain of naturally infected dogs.

The choroid plexi (CP) in the blood-CSF (cerebrospinal fluid) barrier and can prevent the entrance of toxic molecules and drugs into the CNS (Liddelow, 2015). Several bacterias, parasites and viruses such as *Neisseria meningitidis*, *Streptococcus suis, Trypanosoma brucei*, Sendai virus, Measles virus, Coxsackievirus B3 (CVB3), AIDS (HIV-1) Echovirus 30 (EV30) and leukaemia (HTLV-1) viruses present tropism for the CP (Levine, 1987; Strazielle and Ghersi-Egea, 2000; Schwerk et al., 2015). Thereby, these structures can act as an entry route for the *Leishmania* parasite within the brain. As the brain is a site that is inaccessible for many diagnostic procedures and since there is a proximity of the CSF to brain lesions, the CSF can act as a predictive tool and reflect alterations in the CNS (Duque et al., 2002).

In a previous study performed by our research group, it was observed that dogs with VL presented brain inflammatory infiltrate with a predominance of CD3+ T lymphocytes (Melo et al., 2009), but a low percentage of CD4+ and CD8+ T lymphocytes (Melo et al., 2015b). This suggests that another subset of T cells in the brain might be involved in the triggering of brain inflammation. Furthermore, the leukocyte populations of the CSF differ from those of the blood in healthy dogs, requiring a deeper evaluation of both compartments (Duque et al., 2002).

There are papers describing T lymphocytes immunophenotypes in dogs, especially in the blood (Tipold et al., 1998; Itoh et al., 2009; Alexandre-Pires et al., 2010; Watabe et al., 2011; Lima et al., 2012), although there are only a few conflicting papers describing the function of double-negative (CD4− CD8− = DN) and double-positive (CD4+ CD8+ = DP) T cells. In dogs, DP T cells present an activated phenotype and may have unrecognised functions in *in vivo* immunity and infection, as well as in inflammatory diseases such as allergy, chronic infection, autoimmunity or cancer (Buttlar et al., 2015). TCRαβ+DN T cells have been connected to autoimmune conditions and present a pro-inflammatory profile. These cells were reported to be increased in several autoimmune/inflammatory disorders (Bleesing et al., 2001; Crispín et al., 2008; Alunno et al., 2013), produce pro-inflammatory cytokines (Crispín et al., 2008), and on the other hand also have regulatory properties (D'Acquisto and Crompton, 2011). With regard to DN cells, there are only a few papers reporting their function, which appear to be conflicting. The DN T cells have potential for a pathogenic role during autoimmunity, acting in the development of disease, as well as for homeostatic role in suppressing excessive immune responses that are deleterious to the host (D'Acquisto and Crompton, 2011). Nevertheless, there are no studies reporting the presence of these cells in the CSF of dogs infected by *Leishmania*.

Therefore, in view of the paucity of data regarding brain inflammation during VL, and in view of the possible role of T lymphocytes during infection, the aim of this study was to determine the immunophenotypes of T lymphocytes in the CSF of infected dogs and to compare with the same cell populations in peripheral blood. Brain lesions were also evaluated in order to correlate with the T lymphocytes populations in the CSF.

2. Material and methods

2.1. Animals

Seventeen naturally infected dogs, eleven male and six female, ranging in age from 1 to 8 years-old, were selected from the Veterinary Teaching Hospital of UNESP, São Paulo State University and from the Zoonosis Control Centre in the municipality of Araçatuba, São Paulo State, Brazil, which is an area endemic for VL. VL diagnosis was achieved using a routine ELISA (enzyme-linked immunosorbent assay) according to Lima et al. (2005). The cut-off point was determined previously with serum from 38 healthy dogs from a non-endemic area, using as reference the mean added by 3 times the standard deviation obtained for the group. The samples were analyzed in duplicate and a blank well (PBS + 0.05% Tween 20® solution) was included in all plates. The ELISA results were confirmed by popliteal lymph node fine-needle aspiration. Of the infected dogs, 64.7% (n = 11) presented anti-*L. infantum* IgG antibody titres higher than the cut-off value in sera, ranging from 0.289 to 1.095 (Fig. 1). Moreover, we detected *Leishmania* amastigotes in all of the dogs through popliteal lymph node fine-needle aspiration.

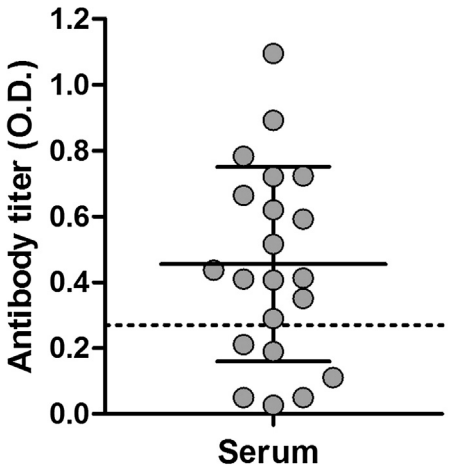

Fig. 1. Scatter plot presenting serum reactivity (IgG) against *Leishmania* antigens evaluated by ELISA. Anti-*L. infantum* anti-antibody titers were determined by optical density (O.D.; absorbance at 492 nm). Horizontal lines indicate the mean and SD. The dotted lines represent the lower limit of positivity (cut-off): 0.270.

Dogs were euthanised with the owners' permission, in compliance with state law (São Paulo et al., 2006). None of the animals were previously vaccinated against VL. All animals were symptomatic, with at least three clinical signs. 82.35% (n = 14) of the dogs presented dermatological alterations (seborrhea, generalised alopecia, nasal hyperkeratosis and hypotrichosis), 76.47% (n = 13) presented generalised lymphadenopathy, 52.94% (n = 9) presented onychogryphosis, 52.94% (n = 9) presented cachexia, 29.41% (n = 5) presented ophthalmic alterations (conjunctivitis and ocular discharge) and 11.76% (n = 2) presented temporalis muscle atrophy. Nevertheless, they did not present a history of neurological signs and were also serologically negative for toxoplasmosis and neosporosis, as assessed by indirect immunofluorescence assays.

2.2. Sampling

The dogs were anesthetised with pentobarbital (Hypnol®). Peripheral blood samples (4 ml) were obtained from the cephalic vein into tubes with sodium heparin and 5–6 ml of CSF was collected from the cerebello-medullary cistern. Following that, the animals were euthanised with potassium chloride. Necropsies were performed immediately after euthanasia. The brain was collected and separated into two hemispheres, one of which was placed in 10% buffered-formalin. After fixation, fragments of some areas (hippocampus, diencephalon, lateral ventricle choroid plexus, midbrain, brainstem, cerebellum and fourth ventricle choroid plexus) were embedded in paraffin and submitted to histological procedures and haematoxylin-eosin (H–E) staining.

The inflammatory lymphoplasmacytic infiltrate was evaluated according to intensity using a ponderal index divided into four grades: Grade 0 (absence of inflammation); Grade 1, mild inflammation (slight inflammatory cell infiltrate mainly at the lep-