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IMMUNOHISTOCHEMICAL CHARACTERIZATION OF MONONUCLEAR CELLS AND MHC II EXPRESSION IN THE BRAIN OF HORSES WITH EXPERIMENTAL CHRONIC *Trypanosoma evansi* INFECTION*

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ABSTRACT:- LEMOS, K.R.; MARQUES, L.C.; AQUINO, L.P.C.T.; ALESSI, A.C.; ZACARIAS, R.Z. **Immunohistochemical characterization of mononuclear cells and MHC II expression in the brain of horses with experimental chronic *Trypanosoma evansi* infection.** [Caracterização imunoistoquímica de células mononucleares e expressão de CMH II no sistema nervoso central de equinos com infecção crônica experimental por *Trypanosoma evansi*]. *Revista Brasileira de Parasitologia Veterinária*, v. 16, n. 4, p. 186-192, 2007. Departamento de Medicina Veterinária, Centro Politécnico, UNICENTRO. Rua Simeão Camargo Varela de Sá, 03, Guarapuava, PR 85040-080, Brasil. E-mail: krlemos@yahoo.com.br

An histochemical and immunohistochemical study was carried out to evaluate the mechanisms of immune response of horses experimentally infected by *Trypanosoma evansi*. For this purpose the HE histochemical stain and the avidin biotin peroxidase method were used. To determine the presence and immunoreactivity of immune cells we used anti-major histocompatibility complex II antibodies. Cellular infiltration phenotype was characterized with the aid of anti-CD3 antibody for T lymphocytes and by anti-BLA 36 antibodies for B lymphocytes. Macrophages were marked with an antibody against myeloid/histocytes antigen (clone Mac387). Lesions in the CNS of experimentally infected horses were those of a wide spread non suppurative encephalomyelitis and meningomyelitis. The severity of lesions varied in different parts of the nervous system, reflecting an irregular distribution of inflammatory vascular changes. Lymphoid perivascular cuffs and meningeal infiltrations were of predominantly composed of T and B cells. The parasite, *T. evansi*, was not identified in these horses tissues.

KEY WORDS: *Trypanosoma evansi*, trypanosomiasis, MHC II, T and B lymphocytes, macrophages.

RESUMO

Este estudo objetivou caracterizar a resposta imune celular no sistema nervoso central (SNC) de equinos com infecção crônica experimental por *Trypanosoma evansi*. Para este propósito, foram utilizados os métodos histoquímicos (HE) e imunoistoquímicos do complexo avidina-biotina peroxidase (ABC). O fenótipo do infiltrado celular foi caracterizado com

o auxílio de anticorpos anti - CD3, para linfócitos T e anti-BLA36 para linfócitos B. Os macrófagos foram marcados com anticorpo antiantígenos da linhagem mielóide/histiócitos (Clone Mac387). A lesão no sistema nervoso central (SNC) dos equinos infectados com *T. evansi* foi caracterizada como meningoencefalite e meningomielite não supurativa. A gravidade das lesões variou em diferentes segmentos do SNC, refletindo distribuição irregular das alterações vasculares. A distribuição de células T e B e antígenos do complexo maior de histocompatibilidade classe II foram avaliados dentro do SNC de equinos cronicamente infectados com *T. evansi*. O infiltrado perivascular e meníngeo eram constituídos predominantemente por células T e B. Macrófagos foram raramente visualizados. *T. evansi* não foi identificado no parênquima do SNC dos equinos.

PALAVRAS-CHAVE: *Trypanosoma evansi*, tripanossomíases, CMH II, linfócitos T e B, macrófagos.

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INTRODUCTION

African trypanosomes are protozoans that cause diseases in humans and animals (SILEGHEM et al., 1994). *Trypanosoma evansi* is the most widely distributed of all species of trypanosome, and has the greatest economical impact on mammals, particularly on the domestic ones (SILVA et al., 1995; MUÑOZ; CHAVES, 2001).

It causes a disease of subacute or chronic course known as "Mal das Cadeiras", characterized mainly by ataxia of the pelvic limbs (MARQUES, 1996). It brings about impaired locomotion associated to central nervous system lesions (CNS) ((MARQUES, 1996; CADIOLI, 2001). In the advanced phases of the evolution of experimental trypanosomiasis in equines and donkeys, weakness of the pelvic limbs and impaired locomotion were observed, besides walking in circles, dysmetria and stiff neck, which are symptoms attributed to myositis, meningomyelitis, encephalitis and plexochoroiditis (MARQUES, 1996; CADIOLI, 2001).

The basic lesions in the tissues of animals infected by *T. evansi* are inflammatory (IKEDE et al., 1983; UCHE; JONES, 1992; JUBB; HUXTABLE, 1993; MARQUES, 1996) and are characterized by perivascular cuffing of inflammatory cells, obstruction of capillaries and veins, besides neuronal destruction and demyelination foci at various degrees (HOLMES, 1987; ANTHOONS et al., 1989).

Cell infiltrates are of fundamental importance in the pathogenesis of inflammatory lesions of the CNS. Specific mechanisms of leukocyte recruitment are required in the infectious encephalitis because the entrance of immune cells is normally limited by the presence of the blood brain barrier (BBB), and also because of the complex structure of the endothelial cells, microglia and astrocytes (DECKERT-SCHLUTER et al., 1994; MERRIL; BENVENISTE, 1996).

The mechanisms regulating the susceptibility of African trypanosomomes have been enigmatic, Uzona et al. (1998) suggest that the enhanced induction and secretion of IFN- α during *T. congolense* infections contribute to the relative susceptibility to the disease. Shi et al. (2005) in experimental African trypanosomiasis, concluded that a subset of a pathogenic, MHC class II-restricted CD4⁺ T cells mediates early mortality in infected BALB/c mice via excessive synthesis IFN- γ .

The aim of this study was to characterize, through immunohistochemistry, the cells that participate in the inflammatory infiltrate and the expression of antigens of major histocompatibility complex type II in the CNS of equines experimentally infected with *T. evansi*.

MATERIAL AND METHODS

Biological samples

The study was conducted with six CNS came from equines experimentally infected with *T. evansi*. Three clinically healthy equines constituted the control group. All equines inoculated with *T. evansi* were observed until they presented symptoms

of CNS disturbance, characterized by motor incoordination of the pelvic limbs, which occurred 67 days after inoculation (DAI) in equine 3, 77 DAI in equines 4 and 6, 78 DAI in equines 1 and 2, and 124 DAI in equine number 5. The animals in the control group (equines 7, 8 and 9) did not present any clinical symptoms and were observed up to the 125th DAI (MARQUES, 1996).

Immunohistochemical evaluations of the CNS of equines

The immunohistochemical method used was the avidin-biotin peroxidase complex (ABC). The protocol of the immunohistochemical reactions was initiated by the deparaffinization in xylene and re-hydration in alcohol. Endogenous peroxidase was blocked at room temperature with specific blocker Dako S2001; the material was then incubated for 30 minutes (PBS-BSA 5%) to block unspecific proteins. Two washes with Tris HCL added with Tween 20 were carried out between all incubations. The material was revealed with DAB (diaminobenzidine) for 5 minutes (Reagent 54-10-00 KPL) and counterstained with Harris hematoxylin 1:2.

Anti-HLA antibody (Clone TaL.1B5) (Dako M0746) was used to demonstrate MHC II antigens, diluted at 1:200, for 12-18 hours. The sections were pre-treated with target retrieval solution (Dako -S1699), diluted at 1:10, for 30 minutes in a steam pot (95°C) and later incubated with goat anti-mouse biotinylated antibody (Dako E0433), diluted at 1:200, for 45 minutes.

Tissue macrophages were detected using anti-lineage myeloid/histiocyte antigen (Clone - Mac387 - Dako M 0747), diluted at 1:200, for 12-18 hours. Goat anti-mouse biotinylated antibody (Dako E0433) was used as binding antibody, diluted at 1:200, and incubated for 45 minutes. To demonstrate T lymphocytes, the enzymatic antigen reactivation process was carried out with the use of pronase (Dako S2013) for 15 minutes. Subsequently, anti-T CD3 lymphocyte antibody was employed (Dako A0452), diluted at 1:100, for 2 hours, associated with goat anti-rabbit binding antibody (Dako E0432), at 1:300, for 45 minutes. After antigen reactivation by heat with the use of microwaves (720W) in citrate solution of pH 6.0, the B lymphocyte specific marker (Novo Castra – NCL-BLA36), diluted at 1:25, was applied to the sections for 12-18 hours. Posteriorly, it was associated with goat anti-mouse antibody (Dako E0433) 1:200 for 45 minutes.

RESULTS

The perivascular and submeningeal inflammatory infiltrate in the animals inoculated experimentally with *T. evansi* was composed of mononuclear cells. Among them, CD3⁺ T lymphocytes were identified, which presented immune marking of all cytoplasm by the specific antibody. This cell phenotype represented 49% of the inflammatory infiltrate.

Perivascular inflammatory infiltrates, with cells marked by the anti-CD3 antibody, were seen in the parenchyma of the spinal cord, mesencephalon, cerebellum, cerebellar cortex, in the subependymal portions, and in the nervous parenchyma

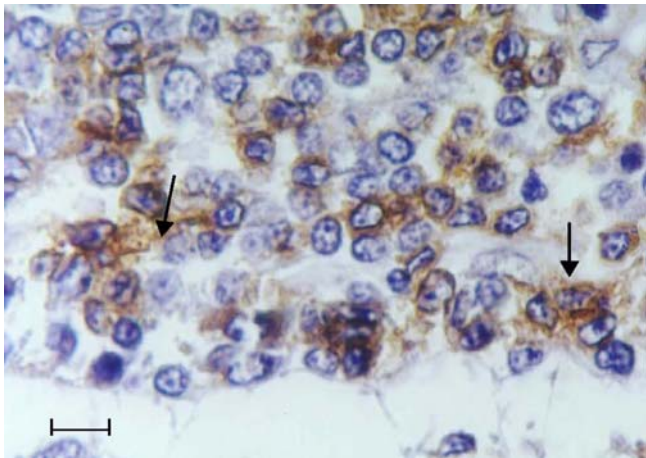


Figure 1. Photomicrograph of the inflammatory infiltrate. Note the characteristic marking of the CD3+ cells with pericytoplasmic (arrows). (— = 10µm). ABC Method. Experimental chronic *Trypanosoma evansi* infection.

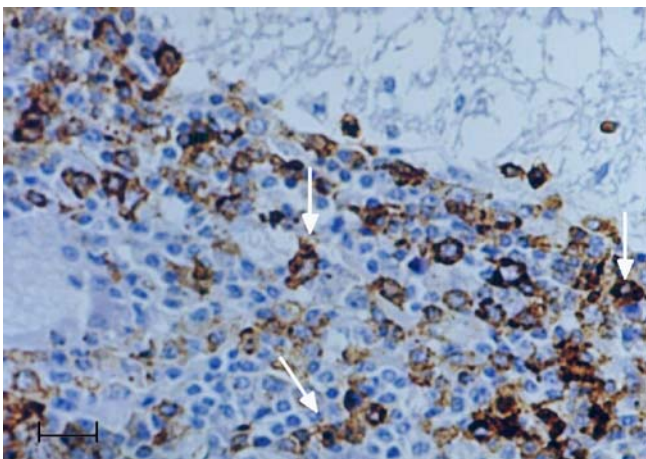


Figure 2. Photomicrograph of CNS perivascular infiltrate. Note the discreet brown color in the cytoplasm of the cells with B lymphocyte phenotype (arrows) (— = 25 µm). ABC Method. Experimental chronic *Trypanosoma evansi* infection.

of the caudate nucleus (Figure 1). Positive markings were observed in the meningeal and perivascular inflammatory infiltrate of equines inoculated with *T. evansi*.

Characteristic immune marking was observed with the use of anti-B lymphocyte antibody. The cells of B lymphocyte phenotype in the submeningeal inflammatory infiltrate presented weak marking with discreet brown staining in the cytoplasm. The perivascular inflammatory infiltrate observed in the CNS of equines inoculated with *T. evansi* was also composed of cells immune marked by anti-B lymphocyte antibodies (Figure 2). The inflammatory infiltrate was 40.52% composed of B lymphocytes.

Anti-macrophage antibodies enabled the visualization of only discreet amounts of macrophages in the inflammatory infiltrate. The positive markings were characterized by the intense brown color in all cell cytoplasm. These cells were identified in the choroid plexus and in some blood vessels in

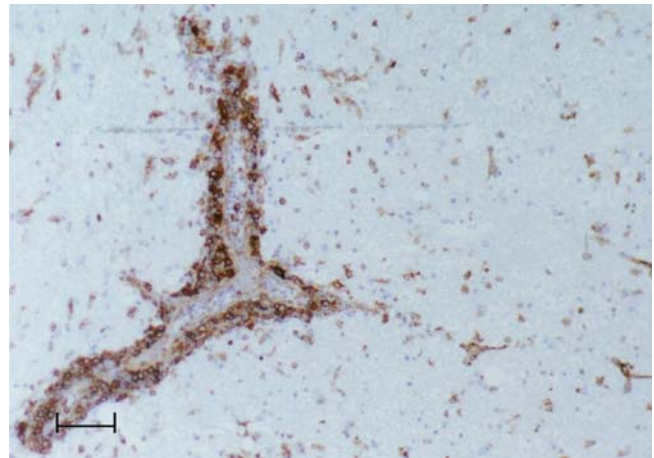


Figure 3. Photomicrograph of the thalamic region. Note the immune reactive ependymal cells. Anti-HLA antibodies. (— = 50µm). ABC Method. Experimental chronic *Trypanosoma evansi* infection.

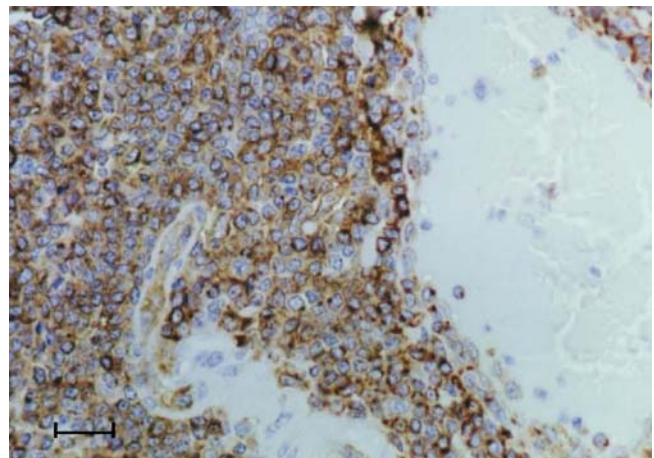


Figure 4. Inflammatory infiltrate with intense positive reaction to the antibody. Note the brown color in the cytoplasm and the blue counterstaining of the nucleus. Anti-HLA antibodies. (— = 25µm). ABC Method. Experimental chronic *Trypanosoma evansi* infection.

the parenchyma of the cortical region of the brain and spinal cord. However, they were not identified in the neuropil. Their participation in the inflammatory infiltrate of equines inoculated represented less than 1%.

The ependymal cells of the animals in the control group presented immune reactivity to the anti-MHC class II marker in a subliminal form. In the animals inoculated with *T. evansi*, these cells presented intense immune marking (Figure 3). Concurrently, the endothelial cells and T and B lymphocytes of the inflammatory infiltrate were stained by the anti-HLA antibody (clone Tal 1.B5) (Figure 4). In the control equines, the endothelial cells were less intensely stained.

DISCUSSION

In natural conditions of infection, in several endemic regions in the world, *T. evansi* induces neurological symptoms

and death in equines, bovines, camels and dogs (SEILER et al., 1981; SILEGHEM et al., 1994; TUNTASUVAN et al., 1997).

Equines (MARQUES, 1996), dogs (AQUINO, 1997), coatis (HERRERA, 1998), bovines (POCHINI, 2000) and mules (CADIOLI, 2001) experimentally infected with a strain isolated by Moreira and Machado (1985) in Brazil developed, in variable periods of time, symptoms and/or neurological lesions involving mainly the CNS. The distribution and the characterization of the lesions in equines based on conventional microscopy had been described previously by Marques (1996). In this study, the phenotypical characterization of the inflammatory cells participating in the CNS lesions of equines infected by *T. evansi* is represented mainly by T lymphocytes. Thus, these cells constitute the main cell elements present in the lesions. This fact had already been evidenced in the meninges, Virchow-Robin spaces and white matter of rats chronically infected by *T. brucei gambiense* (ANTHOONS et al., 1989). Galvão-Castro (1978) also reported the participation of T cells in the immune response and in the development of the lesions in rats infected by *T. b. brucei*. Greenwood et al. (1976) did not give importance to T cells in the immune pathogenesis of trypanosomiasis due to their absence in the cerebral spinal fluid (CSF).

The cell characterization of the inflammatory infiltrate in the CNS of the equines in the present study could validate the perspective of lesion caused by T lymphocyte because these cells may secrete cytokines, such as IFN- γ , which lead to alterations in the adjacent tissues and induce the expression of MHC II molecules. The aberrant MHC II expression may lead to an excessive activation of T lymphocytes because many more cells are able to present antigens (ABBAS et al., 2000). In cattle with trypanosomiasis, the activation of T cells and the production of cytokines are associated to the pathogenesis and are relevant in the immune response (LUTJE et al., 1996).

In the equines the observation of a high density of T lymphocytes positively stained by the anti-MHC II antibody associated with the inflammatory infiltrate characterize their active immune participation in the chronic phase of the disease. Activated equine T cells highly express MHC II molecules and when at rest they express them in a subliminal form (CREPALDI et al., 1986). In humans, only activated T cells express MHC II (JANEWAY; TRAVERS, 1997). Kipar et al. (1998) demonstrated the expression of MHC II in about 100% of brain lymphocytes and macrophages. The activation of T lymphocytes is fundamental for most of the immune responses, as they perform regulatory and effector functions. The expression of MHC class II in equines may occur in CD4+ and CD8+ T cells (BELL et al., 2001). It was not possible, however, to accurately confirm which subgroup phenotype was the most frequent in the equines infected by *T. evansi*.

Since immune marking was negative, possibly due to the unspecificity of the antibody used, T lymphocyte subgroups could not be described in this experiment. Consequently, phenotyping of those cell types could not be done. It is known,

however, that in chagasic encephalitis there is a predominance of CD8+ lymphocytes and that this distinguishes that disease from autoimmune encephalitis, from multiple sclerosis and from African trypanosomiasis, in which there is a predominance of CD4+ lymphocytes (SILVA et al., 1999). However, Bakhiet et al. (1990) reported that in the trypanosomiasis transmitted by tse-tse flies to rodents, the CD8+ T cells and IFN- γ produced are involved in the immunosuppression and in the pathogenesis of the disease. Uzonna et al. (1998) suggest that IFN- α has also been associated with enhanced host susceptibility to *T. brucei* infections, but for the other hand, also has been reported to mediated parasite control and host protection. Shi et al. (2005) suggest that the IFN- α -producing T cell are MHC II restricted and this mediates early mortality in infected mice and activated macrophages system, exerts than your pathological effect.

The overexpression of MHC II molecules in the cells of the vascular endothelium of CNS of the animals inoculated with *T. evansi* and in the cells of the inflammatory infiltrates probably directed the presentation of these antigens to the T CD4+ cells, thus directioning T_H1 or T_H2 response, as explained by Janeway and Travers (1997).

The activation of the CD4+ subgroup of T cells by the MHC II peptide complexes activate helper T cells (T_H2) necessary to stimulate B cells to produce antibodies (JANEWAY; TRAVERS, 1997). Thus, the presence of B cells in the inflammatory infiltrate of the CNS of equines infected by *T. evansi* indicates the activation of this cell subgroup.

The myeloid/histiocyte antigen (Clone Mac 387) enabled, in an unequivocal form, to identify macrophages in the CNS of the equines infected by *T. evansi* and thus verify that only discreet amounts of this cell type were present in the choroid plexus and in blood vessels distributed in the parenchyma of the cortical region of the brain and spinal cord. Macrophage participation in the inflammatory infiltrates was discreet, representing less than 1% of all the cells in the infiltrate. Gazzinelli et al. (1998), analyzing the immunity mediated by cells induced by *T. cruzi*, concluded that a great variety of pathogens and microbial products stimulate macrophages to produce proinflammatory chemotactic cytokines, essential events to initiate the inflammatory process and determine the nature of the immune response. This pattern of immune response is due to the interaction of the parasite within the macrophage, which induces the synthesis of cytokines, such as IL-12 and chemokines. Silva et al. (1999) described the profile of the inflammatory infiltrate in the acute phase of the experimental infection with *T. cruzi*, in which macrophages and CD8+ lymphocytes predominated. In the African trypanosomiasis, on the other hand, there is no development of parasites within the macrophages and these cells have a minor participation in those diseases. This suggests that these parasites are unable to produce a response through cytokines liberated by the macrophages, and are, thus, less potent to induce trypanocidal activity. So, they remain in the hosts in a great number, perpetuating their life cycle. Furthermore, there

is evidence of immune suppression induced by the macrophages in the African trypanosomiasis. However, it has not been determined whether this immune suppression is a result of the liberation of products from the parasites or of their state of activation (BANCROFT; ASKONAS, 1985).

In this study the presence of B lymphocytes was also observed in great proportion (40.52%) when compared to T lymphocytes, suggesting that this is the strain that induces the disease of subacute chronic course in equines. Bancroft and Askonas (1985) affirmed that there is no efficient response of spleen B cells to trypanosomes "in vitro", and therefore, there is no increase in antibody production, which suggests a suppressive action of macrophages and T cells. The presence of a great proportion of B lymphocytes in the inflammatory infiltrate of the CNS of equines does not necessarily characterize efficient cell and humoral immune response, especially because the course of this trypanosomiasis, except in rare cases of spontaneous cure, evolves to total emaciation and death (HÖRCHNER et al., 1983; WOO, 1977). The BLA36 antibody, used to demonstrate B lymphocytes, is associated to the phase of development of these cells, differing from their usual markers, i.e., antigens of hematopoietic lineage (IMAM et al., 1990).

The immune phenotyping of the inflammatory infiltrate, with the presence of a significant number of B cells, suggests that the vascular lesions associated with the infiltrate originate from the deposition of immune complexes in those areas. This, according to Abbas et al. (2000), characterizes type III hypersensitivity, which leads to the activation of leukocytes and complement system, resulting in tissue damage. Urquhart (1980) suggests that sensitized T cells may exert ancillary effect on the production of anti-trypanosome antibodies, leading to an increase in the deposition of immune complexes in the tissues. Holmes (1987) described the formation of immune complexes in the choroid plexus, which contribute to the alterations in the vascular permeability and integrity of the blood brain barrier (BBB). The presence of pronounced inflammatory infiltrate in the CNS of the inoculated equines, in association with the presence of phagocytes, represented by the microglia, suggests an important participation of the complement system in this disease, as the activation of this system triggers a response that results in the recruitment of inflammatory cells, opsonization and lysis of the agents (JANEWAY; TRAVERS, 1997).

In the inflammatory infiltrate of the CNS of the equines infected by *T. evansi*, T and B lymphocytes exhibited intense positive marking by the anti-MHC II antibody. This fact reinforces the hypothesis that these B lymphocytes do not secrete antibodies, since the cells of this lineage that differentiate in plasmocytes do not present MHC II on their surface anymore (JANEWAY; TRAVERS, 1997). Besides proinflammatory responses, B cells and, more specifically, immunoglobulin (Ig) G antibodies are crucial for parasite killing. Hence, parasitemia control is abolished in B cell-deficient mice, whereas IgM-deficient mice control the

infection as efficiently as do wild-type mice (MAJES et al., 2006).

The development of B lymphocytes is characterized, in relatively early phases, by the appearance of several cell surface proteins important in the activation of mature B lymphocytes by helper T cells, among them molecules of MHC class II (JANEWAY; TRAVERS, 1997). In the present study, the observation of positive immunohistochemical reaction to this antibody in the perivascular and meningeal inflammatory infiltrate clearly demonstrated that the B cells present expressed this protein on the cell surface, a fact that characterizes their function as memory B cells.

The role of the histocompatibility molecules in the susceptibility to trypanosome in the domestic animals has not been described yet. The control of chronic *T. congolense* trypanosomiasis was analyzed by Majez et al. (2006) demonstrated that interferon (IFN) gamma mediated immune activation is crucial for parasitemia control and that infections in major histocompatibility complex (MHC) class II-deficient mice indicate that this molecule is needed for initiation of IFN-gamma and subsequent tumor necrosis factor (TNF) production. The results demonstrated in this study evidenced that the number of cells presenting antigens and the density of expression of the class II histocompatibility molecules in the different populations in the CNS may influence the result of the immune response.

CNS endothelial cells of the non-infected equines expressed MHC II in a subliminal form. On the other hand, the analysis of this immune reaction in the inoculated equines demonstrated intense expression of these molecules in the endothelial cells of the microvasculature. Maesen et al. (1999) described this pattern of immune reaction in the CNS with acute and demyelinating inflammations and suggested that the increase in the expression of MHC II by the endothelial cells is associated to numerous alterations that characterize their activation, mediated by cytokines in the CNS, in the initial phase of the immune response. This fact, in particular, enables the presentation of antigens to CD4⁺ T lymphocytes by the endothelial cells. As previously discussed, the expression of MHC class II in equines may occur in CD4⁺ and CD8⁺ T cells (BELL et al., 2001), which makes the qualification of the subgroup phenotypes of the inflammatory infiltrate of the inoculated equines more subjective. Nonetheless, the expression of intense immune reactivity to MHC II antibody by the endothelial cells, as described, suggests that the T-lymphocytes participating in the inflammatory reaction of those animals are CD4⁺.

The determination of the "age" of the CNS lesions in the equines inoculated with *T. evansi* based on pathological criteria is not possible because this is a systemic disease with a chronic character, which only develops nervous symptoms at a later stage of development.

In the trypanosomiasis, the knowledge of the neuropathological mechanisms reveals complex relationships between the parasite and the host. The integrity of the BBB

seems to be a crucial factor in the pathogenesis of those diseases. This CNS dysfunction may be increased by the response of the host or by the direct action of the parasite. As demonstrated, it is observed that the basic host response to *T. evansi* is of inflammatory nature, with a predominance of CD3+ T lymphocytes and B lymphocytes in the infiltrates.

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