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Novel markers of inflammatory response and hepatic dysfunction in canine leishmaniasis

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ABSTRACT

Dogs are the main host of *Leishmania infantum*, and the clinical presentation may range from asymptomatic to systemic manifestations. The immune mechanisms in infected, but clinically healthy dogs, prevails Th1 response mediated by cytokines. In this sense, adenosine deaminase (ADA) and butyrylcholinesterase (BChE) are considered as key enzymes in several physiological processes, including the modulation of inflammatory process. Considering the variable immune response against *Leishmania* and the known participation of ADA and BChE, the aim of this study was to assess the relation between these two enzymes with the inflammatory response as well as hepatic function in dogs naturally infected with *L. infantum*. For this purpose, the activity of ADA and BChE was assessed in sera of 24 dogs naturally infected with *L. infantum*, plus 17 healthy dogs. The naturally infected dogs had clinical signs compatible with leishmaniasis and sera activities of ADA ($P < 0.01$) and BChE ($P < 0.05$) decreased, when compared to the healthy group. The reduction of ADA activity probably represented an effect on inflammatory response, especially due to the decreased hydrolysis of extracellular adenosine, might in order to protect against tissue damage and, also, setting a down-regulation on pro-inflammatory cytokines. BChE enzyme had no effect on modulating the immune response in leishmaniasis, but it decreased, a fact may related to deficiency of synthesis in the liver. Therefore, ADA and BChE activities reduced probably in order to protect against extra tissue damage and due failure in synthesis, respectively.

1. Introduction

Leishmaniasis is a complex disease caused by more than 20 different species of protozoans of *Leishmania* genus (Kinetoplastida, Trypanosomatidae). Leishmaniasis is transmitted by over 30 distinct phlebotomine sand flies, and it has four different types of clinical presentation: visceral leishmaniasis (VL), cutaneous leishmaniasis, muco-cutaneous leishmaniasis, and post-kala-azar dermal leishmaniasis (PKDL) [1,2]. VL, also known as kala-azar, is an anthropozoonosis caused by *Leishmania donovani* complex: *Leishmania infantum* (Syn. *chagasi*) in the Americas and in the Mediterranean basin, and *L. donovani* in Asia and Africa [1,3,4]. Domestic dogs are the main host of *L. infantum*, with clinical presentation ranging from asymptomatic to systemic manifestations, characterized by fever, anemia, progressive weight loss, hepatomegaly, splenomegaly, renal alterations, skin disease, ocular lesions, disorders of the cardiovascular, and respiratory system [5,6].

VL is an immunomediated disease, and studies already demonstrated a failure of the cellular response in symptomatic dogs. This failure is characterized by decrease of the lymphoproliferative response against *Leishmania* antigens, and decrease in number

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of $CD4^+$ T lymphocytes [7]. In infected, but clinically healthy dogs, Th1 immune response prevails, mediated by IL-12, IFN-γ and TNF-α. This response is in contrast to symptomatic animals, in which cytokines such as IL-4, IL-10 and TGF-β mediate the Th2 response, are the major effectors of response [6–10].

Adenosine deaminase (ADA) and butyrylcholinesterase (BChE) are key enzymes in several physiological processes [11–13]. ADA works as a key enzyme in the metabolism of purines, catalyzing the irreversible deamination of adenosine into inosine [14]. The participation of ADA/adenosine physiologically is wide, especially in neuromodulation, apoptosis, necrosis, cell proliferation, coagulation, and immune response [11,12,14,15]. While ADA is a purine-system enzyme, BChE is a nonspecific choline esterase (also called as "pseudo" choline esterase) hydrolyzing other esters, as well as choline esters. BChE is present in blood serum, pancreas, liver, and central nervous system [16]. Plasma and serum BChE levels are increased in protein-energy malnutrition, during stress, chronic and acute inflammation, and in other clinical conditions [17]. Therefore, considering the variable immune response against *Leishmania* and the participation of ADA and BChE on inflammatory response, the aim of this study was to assess the relation between ADA and BChE with inflammatory response and hepatic function in symptomatic dogs, naturally infected by *L. infantum*.

2. Materials and methods

2.1. Animals

Twenty-four (24) dogs, unspecific age and gender, living in Caruaru and Petrolina (cities of Pernambuco State) composed our main group. These dogs, previously diagnosed as serologic positive *Leishmania* sp (ELISA and RIFI), as well as PCR positive (samples of bone marrow, spleen, and lymph nodes) for the complex *L. donovani*. They were negative for *Leishmania braziliensis*. According to researchers [18], *L. infantum* is the main agent when PCR results are positive, especially in association with geographic localization of dogs, and presence of clinical signs. In addition, sera samples from 17 healthy dogs, negative for *L. infantum* (serologic and PCR), composed our negative control group.

Real-time PCR (qPCR) was carried out according to the technique describer by Ramos et al. [18], detecting and quantifying kinetoplast minicircle DNA with primers LEISH-1 (5′-AACTTTTCTGGTCCTCCGGGTAG-3′) and LEISH-2 (5′-ACCCCCAGTTTCCCGCC-3′), and the TaqMan-MGB probe (FAM-5′-GCAGAAAT-3′-non-fluorescent quencher-MGB). All assays were performed in triplicate, including controls in each run, a negative (DNA of a non-endemic area dog) and a positive.

Of these animals, 10 mL of blood was collected by venipuncture (cephalic vein) with 25 mm × 7 mm disposable syringe and needle (Becton Dickson, São Paulo, Brazil). Blood samples collected were immediately transferred to sterile test tubes without anticoagulant; the sera obtained were stored at −80 °C. All sera samples composed the material to assess cytokines levels and ADA and BChE activities.

2.2. Cytokine levels

Cytokine quantification, as tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and interleukins (IL-1, and IL-6) was assessed by Enzyme Linked Immuno Sorbent Assay (ELISA) using commercial Quantikine canine immunoassay kits according to manufacturer's instructions (R&D Systems, Minneapolis, Minnesota, USA), according to the manufacturer's instructions. The determination of cytokine concentration was by the intensity of the color, measured spectrophotometrically by a microplate reader. Quantifications of cytokines were performed in duplicate.

2.3. Hepatic function

Liver produces BChE; thus, the hepatic function was evaluated. Serum activity of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were assessed in analyzer Cobas Mira® (Roche Diagnostics, Basel, Switzerland) using commercial kits (Bioclin® – Belo Horizonte, MG, Brazil). Tests were performed in duplicate.

2.4. ADA and BChE activity in serum

The activity of ADA, in serum, was determined spectrophotometrically, according to the method described by Giusti and Gakis [19]. The reaction started by addition of adenosine (substrate) to a final concentration of 21 mMol/L; incubations were carried out for 1 h at 37 °C. The reaction stopped by adding 10^6 mMol/L 0.16 mMol/L of phenolnitroprusside/mL solution. The reaction mixtures were mixed immediately to 125 mMol/L; 11 mMol/L of alkaline hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulphate, at a concentration of 75 $\mu mol\,L^{-1}$ served as ammonium standard. The ammonium concentration is directly proportional to the absorption of indophenol at 650 nm. The specific activity was reported as $U\,L^{-1}$.

The BChE enzymatic assay in serum was determined by the method of Ellman et al. [20], using the substrate butyrylthiocholine. Pre-incubation of samples was done at 37 °C for 2 min, and reading performed for 2 min, at intervals of 20–20 s on a spectrophotometer (412 nm). The sample analysis was carried out in duplicate, and the enzyme activity was expressed in μmoles BcSCh/h/mg in protein.

2.5. Statistical analysis

Firstly, the normality test was used on the data obtained, showing normal distribution. Then, Student test was applied on the normal data. The relationship among cytokines levels, and activities of BChE and ADA was verified by linear correlation. Values with probability (P) less than 5% were statistically different. Data were presented as mean values ± standard error.

3. Results

3.1. Clinical signs

The dogs infected and symptomatic had a range of clinical signs, including cutaneous lesions, generalized lymphadenopathy, weight loss, muscle atrophy, intolerance to exercise, splenomegaly, epistaxis, diarrhea, and onychogryphosis.

3.2. Cytokines levels, ALT and AST activities

The results obtained for cytokine levels are displayed in Fig. 1. There was a significant ($P < 0.01$) difference between groups, standing out the increased levels of TNF-α, IFN-γ, IL-1, and IL-6 in dogs infected by *Leishmania*.

The results of liver function are in Table 1. The activity of ALT and AST increased significantly ($P < 0.01$) in dogs infected by *L. infantum*, when compared to healthy dogs.

3.3. ADA and BChE activities

ADA and BChE activities are displayed in Fig. 2. Sera activities of ADA ($P < 0.01$) and BChE ($P < 0.05$) were lower in dogs infected by *Leishmania*, when compared to the healthy animals.

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