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Humoral and cellular immune responses in dogs with inapparent natural *Leishmania infantum* infection ☆

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ABSTRACT

Molecular analysis, serology and immunophenotyping for T lymphocytes and their subsets, B lymphocytes and monocytes were performed on dogs naturally infected with *Leishmania infantum*. Animals were categorised as asymptomatic dogs I (AD-I), with negative serology and positive molecular results, and asymptomatic dogs II (AD-II), with positive serology and positive molecular results, and these were compared to symptomatic dogs (SD) and control dogs (CD).

AD-I exhibited immunophenotypic features similar to those of CD, including isotype profiles and concentrations of monocytes. Similar biomarkers were found in AD-II and SD, such as, higher levels of immunoglobulins IgG, IgG2, IgM and IgA and higher concentrations of eosinophils. High frequencies of T lymphocytes and $CD4^+$ T cells were observed in both AD-I and AD-II compared to SD, whereas $CD8^+$ T cells were higher only in AD-II compared with SD. Analysis of B lymphocytes revealed an increased frequency of this cell type only in AD-II animals compared with SD. Asymptomatic dogs appear to have a dichotomous infection spectrum that can influence the humoral and cellular immunological status during canine visceral leishmaniasis.

Introduction

Leishmaniasis is endemic in 88 countries in tropical and subtropical regions of the Old and New World, with more than 350 million people exposed to the infection (Desjeux, 2001). The estimated incidence is 2 million new cases per year, with 0.5 million being visceral leishmaniasis (VL) (Desjeux, 2004). Infected dogs have a high cutaneous parasite density and represent the main domestic reservoir of *Leishmania infantum*, contributing to the propagation of the disease (Deane and Deane, 1962). In Brazil over the past 10 years, more than 2 million dogs were screened and more than 160,000 seropositive dogs were eliminated; however, the incidence of human VL has not been reduced to an acceptable level (Lemos et al., 2008; Romero and Boelaert, 2010).

Canine visceral leishmaniasis (CVL) can be categorised into three distinct clinical forms on the basis of major features observed in seropositive infected dogs, which can be classified as asymptomatic dogs (AD), oligosymptomatic dogs and symptomatic dogs (SD) dogs (Mancianti et al., 1988; Reis et al., 2009). *L. infantum*-infected dogs also include animals in which the presence of parasites is confirmed through direct methods (such as PCR) and which have low-titre anti-*Leishmania* antibodies (Paltrinieri et al., 2010).

Oliva et al. (2006) identified subpatent infection with *L. infantum* in asymptomatic dogs that were intermittently positive by nested PCR but became negative for long periods of time. Seropositive asymptomatic dogs are important sources of amastigotes for infection of phlebotomines that contribute to the transmission of *L. infantum* (Marzochi et al., 1985; Molina et al., 1994; da Costa-Val et al., 2007). In endemic areas, 10–62% of apparently healthy and/or seronegative dogs were positive for *Leishmania* by PCR (Martin-Sanchez et al., 2001; Solano-Gallego et al., 2001; Lachaud et al., 2002; Andrade et al., 2006). Thus, asymptomatic dogs may play a role in the transmission of *Leishmania* parasites but cannot be detected by conventional serological tests, such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).

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The aim of this study was to use the humoral and cellular immune response to explore the dichotomy in dogs with asymptomatic and symptomatic infection with *L. infantum* and to identify features that could be used to identify resistant and susceptible profiles.

Materials and methods

Dogs and experimental design

Forty-one mongrel dogs of either gender from the endemic area of Belo Horizonte, Minas Gerais, Brazil, were selected on the basis of serological tests (IFAT and ELISA, Biomanguinhos/Fiocruz) for *Leishmania* spp. Dogs with an IFAT titre <1/40 were considered to be seronegative and dogs with an IFAT titre ⩾1/40 were considered to be seropositive and infected with *Leishmania* spp. Positive infection was confirmed by ELISA and PCR in at least one skin sample (Degrave et al., 1994) and the species of *Leishmania* responsible was determined by restriction fragment length polymorphism–PCR (Volpini et al., 2004).

The study was conducted from June 2008 to August 2009 after approval by the ethical committees for the use of experimental animals of the Federal University of Ouro Preto (CETEA/UFOP 032/2007), Federal University of Minas Gerais (CETEA/UFMG 020/2007) and the Municipal Health Secretariat of Belo Horizonte City Council, Minas Gerais State, Brazil (CEP-SMSA/PBH 001/2008).

Selection and clinical classification of dogs

Dogs were selected and clinically classified according to the presence/absence of clinical signs: (1) asymptomatic, with no signs suggestive of disease; (2) symptomatic, with characteristic clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogryphosis, cutaneous lesions, apathy and keratoconjunctivitis; and (3) control dogs, classified according to negative serological and molecular results and absence of clinical signs.

Haematology

Peripheral blood (5 mL) from the brachiocephalic vein was collected into tubes containing ethylene diamine tetraacetic acid (EDTA) at a final concentration of 1 mg/mL. Erythrocytes and leucocytes were quantified using an automatic cell counter (Model 2800 Vet, Mindray). Differential leucocyte counts were performed by examination of at least 200 leucocytes in Giemsa-stained blood smears by light microscopy.

ELISA for immunoglobulin isotype profile

ELISAs were performed to determine the anti-*Leishmania* immunoglobulin pattern using soluble *L. infantum* (MHOM/BR/1972/BH46) promastigotes antigen (SLA) (Rosário et al., 2005; Reis et al., 2006c). The protein concentration was quantified by the Lowry method, adjusted to 1 mg/mL and samples were stored at −70 °C. Ninety-six well microplates (MaxiSorp, Nalge Nunc) were coated with SLA at a concentration of 2 μg/well, left overnight at 4–8 °C and then washed. Serum samples were added to the wells at a dilution of 1:80, followed by washes after the addition of goat anti-dog IgG1 (anti-heavy chain specific) conjugated with peroxidase, IgM (anti-μ chain specific), IgA (anti-α chain specific) and IgE (anti-ε chain specific) or sheep anti-dog IgG and IgG2 (both anti-heavy chain specific) (Bethyl Laboratories). Wells were washed and then the substrate and chromogen (O-phenylenediamine, Sigma–Aldrich) were added. The absorbance was read on an automatic ELISA microplate reader (EL 800G PC, Bio-Tek) at 492 nm. The concentrations of conjugate were determined by a block titration method with positive and negative standard sera. The conjugates anti-IgG1, IgM, IgA, IgE were used at a dilution of 1:1,000, anti-IgG was used at a dilution of 1:8,000 and anti-IgG2 was used at a dilution of 1:16,000.

Immunophenotyping by flow cytometry

Immunophenotyping of peripheral blood by flow cytometry was performed as described by Reis et al. (2005). After a pre-fixation step, erythrocytes were lysed in 1 mL EDTA-treated whole blood by the slow addition of 13 mL fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson), followed by incubation for 10 min at room temperature (RT). After centrifugation (450 *g* for 10 min at RT), the pellet was resuspended in 500 mL phosphate-buffered saline (PBS) supplemented with 10% fetal bovine serum (PBS–10% FBS). Using 96-well U-bottom plates (Limbro Biomedicals), 30 μL prefixed leucocyte suspension were incubated at RT for 30 min in the dark with 30 μL monoclonal antibodies (mAbs) diluted previously in PBS–10% FBS.

The mAbs used in the study were diluted purified anti-canine CD5 (1:800, rat IgG2a, clone YKIX322·3), anti-canine CD4 (1:1,000, rat IgG2a, clone YKIX302·9) and anti-canine CD8 (1:800, rat IgG1, clone YCATE55·9) (Serotec). Undiluted fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD21 (5 μL, mouse IgG1, clone IOBla; Immunotech) and diluted phycoerythrin (PE)/Cy-5-conjugated mouse anti-human CD14 (50 μL, 1:200, mouse IgG2a, clone TÜK4; Serotec) were also used in direct immunofluorescence procedures. Cells were also incubated in the same conditions in the presence of 60 μL of diluted FITC-conjugated sheep anti-rat IgG polyclonal antibody (1:100 or 1:200; Serotec).

Before flow cytometric data collection and analysis were performed, labelled cells were fixed for 30 min with 200 μL FACS fix solution (10.0 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, 6.6 g/L sodium chloride; pH 7.2). Flow cytometric measurements were performed on a FACScan (Becton Dickinson) and analysed using CellQuest software (10,000 events acquired per sample). The results were expressed in absolute counts (cell number/mm^3) through the product of the percentage of positive cells ($CD5^+$, $CD4^+$, $CD8^+$ and $CD21^+$) within gated lymphocytes by absolute lymphocyte counts. Absolute counts for T lymphocyte subsets were also calculated as the sum of absolute values of $CD4^+$ plus $CD8^+$ cells. The absolute counts for monocytes were the products of $CD14^+$ cells within ungated leucocytes by the total leucocyte counts.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. The normality of the data was assessed using the Kolmogorov–Smirnoff test. Considering the non-parametric nature of all data sets, Kruskal–Wallis tests were used to investigate differences between the four groups, followed by Dunn's test for pairwise comparisons. Differences were considered to be significant at $P < 0.05$.

Results

Reclassification according to serological, molecular and clinical features

Dogs with no clinical signs and negative serological and molecular results were included in the control group (CD, $n = 7$). Seronegative dogs without clinical signs but positive molecular results for *L. infantum* were classified as asymptomatic dogs I (AD-I, $n = 8$). Dogs with positive serology and molecular results for *L. infantum* but no clinical signs were classified as asymptomatic dogs II (AD-II, $n = 10$). Dogs with clinical signs and positive serological and molecular results were classified as symptomatic dogs (SD) ($n = 16$) (Table 1).

Haematology

There were lower concentrations of eosinophils in AD-II and SD compared with AD-I and CD and lower concentrations of lymphocytes in SD compared with AD-II (Table 2). Symptomatic *L. infantum* infection was associated with decreased erythrocyte counts and haematocrits in AD-II compared with AD-I and CD ($P < 0.05$) and decreased haemoglobin concentrations in SD compared to other groups ($P < 0.05$).

Anti-Leishmania immunoglobulin isotypes

Concentrations of IgG, IgG1, IgG2, IgM, IgA and IgE in AD-I were similar to CD (Fig. 1). There were increased IgG, IgG2, IgM and IgA concentrations in AD-II and SD compared with AD-I and CD

Table 1
Serological and molecular status of dogs categorised according to clinical status with and without natural infection by *Leishmania infantum*.

Groups	Serodiagnosis IFAT and ELISA		Molecular diagnosis PCR–RFLP		Total
	Positive	Negative	Positive	Negative	
Control (CD)	0	7	0	7	7
Asymptomatic I (AD-I)	0	8	8	0	8
Asymptomatic II (AD-II)	10	0	10	0	10
Symptomatic (SD)	16	0	16	0	16
Total	26	15	34	7	41

IFAT, Indirect fluorescent antibody test; ELISA, Enzyme-linked immunosorbent assay; PCR–RFLP, Polymerase chain reaction–restriction fragment length polymorphism.