J. Comp. Path. 2006, Vol. 135, 100–107

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER
www.elsevier.com/locate/jcpa

Relationship between Canine Visceral Leishmaniosis and the *Leishmania (Leishmania) chagasi* Burden in Dermal Inflammatory Foci

R. C. Giunchetti[*,†], W. Mayrink[‡], O. Genaro[‡,§], C. M. Carneiro[*,‖], R. Corrêa-Oliveira[†], O. A. Martins-Filho[¶], M. J. Marques[‡], W. L. Tafuri[*] and A. B. Reis[*,†,‖]

[*] *Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas/NUPEB, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais,* [†] *Laboratório de Imunologia Celular e Molecular, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais,* [‡] *Laboratório de Leishmanioses, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais,* [‖] *Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais and* [¶] *Laboratório de Doença de Chagas, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brasil*

Summary

The skin is the first point of contact with organisms of the genus *Leishmania* from sand fly vectors, and apparently normal skin of sick dogs harbours amastigote forms of *Leishmania chagasi*. In relation to canine visceral leishmaniosis (CVL), the ear skin was examined in 10 uninfected dogs (UDs) and in 31 dogs dogs naturally infected with *L. chagasi*. The infected animals consisted of 10 symptomless dogs (SLDs), 12 mildly affected dogs (MADs) and nine affected dogs (ADs). A higher parasite burden was demonstrated in ADs than in SLDs by anti-*Leishmania* immunohistochemistry ($P<0.01$), and by Leishman Donivan Unit (LDU) indices ($P = 0.0024$) obtained from Giemsa-stained impression smears. Sections stained with haematoxylin and eosin demonstrated a higher intensity of inflammatory changes in ADs than in SLDs ($P<0.05$), and in the latter group flow cytometry demonstrated a correlation ($P = 0.05/r = 0.7454$) between the percentage of $CD14^+$ monocytes in peripheral blood and chronic dermal inflammation. Extracellular matrix assessment for reticular fibres by staining of sections with Masson trichrome and Gomori ammoniacal silver demonstrated a decrease in collagen type I and an increase in collagen type III as the clinical signs increased. The data on correlation between cellular phenotypes and histological changes seemed to reflect cellular activation and migration from peripheral blood to the skin, mediated by antigenic stimulation. The results suggested that chronic dermal inflammation and cutaneous parasitism were directly related to the severity of clinical disease.

Keywords: dog; kala azar; *Leishmania chagasi*; parasitic infection

Introduction

Visceral leishmaniosis (VL; kala azar) is endemic in 87 countries, and approximately 90% of VL cases recorded worldwide occur in Bangladesh, Brazil, India and Sudan. Brazil is responsible for 90% of the VL records from the American continent (Monteiro *et al.*, 1994).

One of the first epidemiological surveys in Brazil was conducted by Chagas *et al.* (1938) in the region of Abaeté, state of Pará, where infection rates of 1.48% in man and 4.49% in dogs were found. Later, Deane and Deane (1962) reported the role of the dog and the fox

[§] In memorium.

Correspondence to: A.B. Reis, Laboratório de Imunopatologia, Núcleo de Pesquisa em Ciências Biológicas, Universidade Federal de Ouro Preto, Campus Universitário, Morro do Cruzeiro, Ouro Preto, MG – Brasil, CEP 35.400-000 (e-mail: alexreis@nupeb.ufop.br).

0021-9975/$ - see front matter
doi:10.1016/j.jcpa.2006.06.005

(*Disicyon vetulus*) as domestic and sylvatic reservoirs, respectively.

Earlier reports of canine visceral leishmaniosis (CVL) described various macroscopical skin lesions (e.g., desquamation, alopecia, pustular dermatitis, ulcerative dermatoses and nodular disease, the type of which depended on the immune response (Adler and Theodor, 1932; Cunha, 1938; Torres, 1941; Ferrer *et al.*, 1988). The skin was considered by Abranches *et al.* (1991) to be an important reservoir compartment for parasites in healthy and sick *Leishmania*-infected dogs and the important role of dogs in VL transmission is supported by the high parasite loads found in the skin of infected animals (Deane and Deane, 1962).

Histopathological changes in the skin of *Leishmania*-infected dogs consist of variable degrees of focal or diffuse inflammatory infiltrate in the dermis, and variable numbers of plasma cells, macrophages (parasitized or not by amastigotes of *Leishmania chagasi*), lymphocytes and isolated neutrophils (Torres, 1941; Santos *et al.*, 2004; Solano-Gallego *et al.*, 2004). Changes in the extracellular matrix (ECM) in *Leishmania amazonensis*-infected mice are characterized by decreased collagen type I, increased collagen type III, and reduced fibronectin and laminin (Abreu-Silva *et al.*, 2004).

Histological tissue changes in CVL are probably triggered by the type of host immune response. Symptomless VL dogs are believed to produce a T-helper (Th) 1-mediated response. Clinically affected VL dogs, on the other hand, show a Th 2-mediated response, their peripheral blood mononuclear cells being unable to produce interferon (IFN)-γ in the presence of parasite antigens (Pinelli *et al.*, 1994). Bourdoiseau *et al.* (1997) reported the occurrence of immunosuppression associated with diminished numbers of $CD4^+$ T lymphocytes and $CD21^+$ B lymphocytes.

Investigations on cutaneous immunopathology in CVL might contribute to a better understanding of events related to kala azar morbidity and to the human disease (Nieto *et al.*, 1999; Moreno and Alvar, 2002). The present study was therefore designed to investigate the relationship between CVL and parasite burden as seen in the ear skin of dogs with different clinical forms of *L. chagasi* infection.

Materials and Methods

Animals

Thirty-one dogs naturally infected with *L. chagasi* and 10 uninfected dogs (UDs; controls) were obtained from the Zoonosis Control Centre, Belo Horizonte City Council. The dogs, of either sex, were aged 2–6 years. The UDs were confirmed as negative by parasitological examination and by an indirect fluorescent antibody test (IFAT) for *anti-Leishmania* IgG (Biomanguinhos Kit; FIOCRUZ-RJ, Brazil), titres of <40 indicating freedom from VL. The VL dogs, selected on the basis of IFAT titres of >40, were classified clinically according to signs of infection (Mancianti *et al.*,1988) as: symptomless dogs (SLDs; $n = 10$); mildly affected dogs (MADs; $n = 12$), with a maximum of three clinical signs; or affected dogs (ADs; $n = 9$), with more than three clinical signs. The study was approved by the Ethical Committee for the use of Experimental Animals, Universidade Federal de Minas Gerais.

Collection and Examination of Ear Skin Samples

The dogs were euthanatized by an intravenous overdose of barbiturate. Samples of ear skin were fixed in 10% neutral buffered formalin for (1) routine histopathological examination of sections stained with haematoxylin and eosin (HE), Masson trichrome and Gomori ammoniacal silver, and (2) anti-*Leishmania* immunohistochemistry.

Evaluation of parasite density in terms of the Leishman Donovan Unit (LDU) index was carried out by light microscopy on Giemsa-stained impression smears prepared from fragments of ear skin, the LDU index being the number of *Leishmania* amastigotes per 1000 nucleated cells (Stauber, 1956).

Parasite density was also evaluated immunohistochemically, as described by Tafuri *et al.* (2004). Briefly, serum from a dog naturally infected with *L. chagasi* (IFAT titre $>1{:}40$), diluted 1 in 100 in 0.01 M phosphate-buffered saline (PBS), was applied as the primary antibody. The slides were then incubated with biotinylated anti-mouse and anti-rabbit antibody (LSAB2 Kit; Dako, Carpinteria, CA, USA), which cross-reacts with canine serum immunoglobulins (Tafuri *et al.*, 2004), and subsequently with the streptavidin–peroxidase complex (LSAB2 Kit; Dako). The reaction was "visualized" with diaminobenzidine (DAB; Sigma, St Louis, MO, USA) and hydrogen peroxide. Finally, the slides were dehydrated, cleared, counterstained with Harris's haematoxylin, and mounted under coverslips.

The dermal inflammatory pattern and the cell population were evaluated histologically on HE-stained sections. The inflammatory infiltrate was graded according to Solano-Gallego *et al.* (2004), as follows: −, no inflammatory infiltrate; +, isolated foci of inflammatory cells; ++, isolated to coalescing areas of inflammatory infiltrate; +++, diffuse areas of inflammatory infiltrate.

Parasite density, assessed semi-quantitatively in sections labelled immunohistochemically for amastigotes, was based on the average number in five fields (×400) in areas with inflammatory infiltrate, in accordance