J. Comp. Path. 2018, Vol. 158, 56–65

Available online at www.sciencedirect.com

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INFECTIOUS DISEASE

Histological and Immunological Description of the Leishmanin Skin Test in Ibizan Hounds

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Summary

The leishmanin skin test (LST), a delayed-type hypersensitivity (DTH) reaction to *Leishmania infantum*, can specifically identify dogs that have made a cell-mediated immune response to *L. infantum* infection. The Ibizan hound appears to be more resistant to *L. infantum* infection than other breeds of dog. The aim of this study was to describe the histological and immunohistochemical changes induced by the LST in Ibizan hounds living in an area highly endemic for leishmaniosis. The majority of dogs were apparently healthy, lacked serum antibody to *L. infantum* and blood parasitaemia, but had marked specific interferon gamma production after in-vitro blood stimulation with *L. infantum*. Leishmanin (3×10^8 killed promastigotes of *L. infantum*/ml) was injected intradermally and biopsy samples were obtained from a positive reaction at 72 h from nine Ibizan hounds. A moderate to intense, perivascular to interstitial dermatitis and panniculitis characterized the inflammatory response at the injection site. In addition, three samples had diffuse inflammation in the deep dermis and panniculus. Oedema and necrosis were present in the deep dermis and panniculus. Congestion and haemorrhage were observed in five biopsies. T lymphocytes ($CD3^+$) and large mononuclear cells ($lysozyme^-$) were the most prevalent cells. $CD3^+$ cells were significantly more numerous than $CD20^+$ B cells and $lysozyme^+$ cells. B cells were sparsely distributed, especially in the deep dermis and panniculus. Rare neutrophils and macrophages ($lysozyme^+$) were observed with few eosinophils. Toll-like receptor (TLR)-2 protein was expressed in large mononuclear cells mainly located in the superficial dermis. *Leishmania* immunohistochemistry was negative and quantitative polymerase chain reaction was positive in all cases. The intradermal injection of killed *L. infantum* promastigotes in Ibizan hounds causes similar histological and immunohistochemical findings to those described for human subjects and are indicative of a DTH response. Moreover, TLR2 protein is expressed in inflammatory cells similar to findings in clinically affected skin biopsy samples.

Keywords: dog; *Leishmania infantum*; leishmanin skin test; Toll-like receptor 2

Introduction

Canine leishmaniosis (CanL) is a zoonotic vector-borne disease caused by *Leishmania infantum* in the Mediterranean basin. It has a range of disease manifestation in dogs ranging from mild to overt severe fatal disease (Baneth *et al.*, 2008; Solano-Gallego *et al.*, 2009). In addition, subclinical infections are common (Baneth *et al.*, 2008). The presence or absence of disease and its clinical variability is determined by the host's immune response (Solano-Gallego *et al.*, 2009). In fact, both innate and adaptive immune responses play a role in the outcome of *Leishmania* infection. However, only the adaptive immune response has been extensively investigated in dogs (Hosein *et al.*, 2017). The balance between the protective T-helper 1 (Th1) cellular response and the humoral immune response mediated by T-helper 2 (Th2) cells determines the clinical manifestation of the infection. A predominantly Th1 immune response

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0021-9975/$ - see front matter
https://doi.org/10.1016/j.jcpa.2017.11.004

is characterized by production of cytokines such as interleukin (IL)-2, tumour necrosis factor (TNF)-α and interferon gamma (IFN-γ), which induce anti-*Leishmania* activity by apoptosis of parasites in macrophages via nitric oxide and reactive oxygen species metabolism and is therefore capable of controlling infection and progression of disease. On the other hand, Th2 cells induce IL-4, IL-5, IL-10 and transforming growth factor (TGF)-β and correlate with antibody production and disease progression (Hosein *et al*., 2017).

The humoral immune response is detected by means of serological methods including quantitative techniques such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), and qualitative techniques such as rapid tests (Solano-Gallego *et al*., 2014). Unfortunately, there are few and poorly standardized assays to evaluate *Leishmania*-specific cellular immune responses. These include the leishmanin skin test (LST) or Montenegro's skin test and cytokine production measurement, such as detection of IFN-γ, in lymphocyte proliferation assays or in stimulated whole blood from dogs (Cardoso *et al*., 1998; Solano-Gallego *et al*., 2001; Fernández-Bellon *et al*., 2005; Strauss-Ayali *et al*., 2005; Rodríguez-Cortés *et al*., 2010; Solano-Gallego *et al*., 2016b). The LST consists of the intradermal inoculation of *Leishmania* antigen and the elicitation of a delayed-type hypersensitivity (DTH) reaction in a previously infected dog (Solano-Gallego *et al*., 2000, 2001). Positive response to the intradermal injection of *Leishmania* antigen is widely used as a clinical indicator for evidence for the presence of a parasite-specific cellular immune response in infected dogs. Clinically, a positive LST is associated with absence of disease or mild clinical disease and therefore a good clinical outcome (Solano-Gallego *et al*., 2000; Ordeix *et al*., 2005; Lombardo *et al*., 2014). However, dogs with moderate disease, before and after treatment, and vaccinated dogs may have a positive reaction as well (Ferrer *et al*., 2003; Bourdoiseau *et al*., 2009). By contrast, the response is low or absent in non-infected dogs or dogs with severe disease (Ferrer *et al*., 2003). Although this test has been proposed by some authors to be a predictor of a good clinical outcome of the infection (Solano-Gallego *et al*., 2000; Ordeix *et al*., 2005), little is known regarding the histopathological or immunological reaction in resistant dogs.

Although the innate immune response has been poorly studied in leishmaniosis, it is well established that it instructs the development of long lasting pathogen-specific adaptive immune responses (Hosein *et al*., 2017). In this sense, Toll-like receptors (TLRs), one of the most important pattern recognition receptor families, are important in the early host defence against the pathogen and activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response (Kumar *et al*., 2009). Although the role of TLRs in the pathogenesis of CanL has not been fully addressed, it would seem that there is an association between TLR2 and the pathogenesis of cutaneous lesions in CanL. In fact, it has been revealed recently that there is lower expression of TLR2 in skin biopsy samples from dogs with mild disease (i.e. papular dermatitis) compared with dogs with moderate or severe disease (Esteve *et al*., 2015). Moreover, TLR2 upregulation in blood and skin seems to be associated with disease progression in dogs (Hosein *et al*., 2015; Montserrat-Sangrà *et al*., 2016) and reduction in TLR2 transcription has been described with treatment and clinical improvement (Montserrat-Sangrà *et al*., 2016).

The Ibizan hound has been reported to be resistant to *Leishmania* infection (Solano-Gallego *et al*., 2000). In fact, this dog breed rarely manifests clinical disease and mounts significant cellular response to the infection demonstrated by a high prevalence of positive LST reactions as well as a potent *Leishmania*-specific IFN-γ production and low or no humoral response when compared with other breeds from the same geographical area (Solano-Gallego *et al*., 2000; Martínez-Orellana *et al*., 2017).

The literature regarding the histopathological study of positive reactions to intradermal injection of *Leishmania* antigen in dogs is scarce (Genaro *et al*., 1992; Tafuri *et al*., 1993). Therefore, the aim of this study was to investigate the histological and immunological changes induced by the LST in Ibizan hounds living in an area highly endemic for leishmaniosis.

Materials and Methods

Dogs

Nine Ibizan hounds, living in an area highly endemic for leishmaniosis (Island of Mallorca, Spain) with a positive LST were enrolled in this study (Table 1). Briefly, two males and seven females, with a median age of 16 months (range 6–84 months) were included. Three dogs had mild clinical signs suggestive of leishmaniosis characterized by a persistent papulocrusting dermatitis on the inner aspect of pinnae (Fig. 1) in the absence of haematological and biochemical abnormalities including a normal serum electrophoresis. All but one dog had negative results by quantitative serology for the detection of *L. infantum*-specific