LONGITUDINAL STUDY OF DOGS LIVING IN AN AREA OF SPAIN HIGHLY ENDEMIC FOR LEISHMANIASIS BY SEROLOGIC ANALYSIS AND THE LEISHMANIN SKIN TEST

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Abstract. The literature contains few longitudinal studies that have assessed areas endemic for canine leishmaniasis and over the same time interval Leishmania-specific cellular and humoral immunity in healthy dogs. Fourteen dogs, three mixed breed and 11 Ibizian hounds, living in an area of Spain that was highly endemic for leishmaniasis were followed-up over a three-year period by serologic analysis and the leishmanin skin test (LST). All but one of these dogs remained clinically healthy during the study period. Serocconversion was observed in four dogs. The three mixed breed dogs had a negative reaction in the LST in the first and third years. The general trend in the Ibizian hounds was an increase in the diameter of the LST reaction at both the 48- and 72-hour readings in the third year. This study demonstrates that in addition to an increase in Leishmania-specific humoral immune response in Ibizian hounds, a parallel increase in cellular immune response was observed.

INTRODUCTION

Leishmaniasis is an important cause of morbidity and mortality in humans and dogs, the latter of which is the main peridomestic reservoir in the Mediterranean basin and in South America. Following studies on Leishmania major infection in mice, the existence of protective Leishmania-specific cellular immunity has also been reported in dogs. Different methods for determining a cellular immune response in humans and mice have been commonly used. An in vivo test frequently used is the leishmanin skin test (LST), while two in vitro tests frequently used are the lymphocyte proliferation assay and the interferon-γ production test done on peripheral blood mononuclear cells. The same methods have been recently applied to the dog.

A positive LST result in dogs indicates that they have a specific cellular immune response to the Leishmania antigen, while sick dogs fail to show a detectable cellular immune response because of the anergic state of these animals, as has been shown in previous studies. However, there is still little information on the LST when conducted with L. infantum promastigotes in dogs. Presumably, the immune response to L. infantum in the dog is a combined humoral and cellular immune response composed of the whole spectrum of immunity from resistant dogs with a predominantly cellular response to susceptible dogs with an exaggerated humoral response. Based on previous cross-sectional studies, approximately half of the dogs living in an endemic area had Leishmania-specific cellular immunity, either alone (one-third) or associated with production of IgG antibodies to Leishmania (two-thirds). The other half of the dogs, which showed negative results in a cellular immunity test, including one-third who had not been previously infected and one-fourth of those infected showing only a humoral immune response, presumably will eventually develop clinical disease.

Several investigators have demonstrated serocconversion in healthy dogs. However, the literature contains few longitudinal studies that assessed both cellular and humoral immunity on healthy dogs over a long period of time. The aim of this study was to follow-up dogs living in a area of Spain highly endemic for leishmaniasis (the Island of Mallorca) by means of serologic analysis and an LST over a three-year period to assess simultaneously the dynamics of both Leishmania-specific cellular and humoral immunity.

MATERIALS AND METHODS

Dogs. Fourteen dogs living outdoors in the village of Felanitx (Mallorca, Spain), an area endemic for leishmaniasis, were included in the study. Three of them were mixed breed dogs and the remaining 11 were Ibizian hounds. Their ages in 1998 ranged from one to seven years, with a mean ± SD age of 2.8 ± 2.2 years.

In 1998 and 2001, all dogs were clinically examined and blood samples were collected for routine leishmaniasis serologic analysis and general biochemical testing (total protein concentration and its electrophoresis pattern, urea nitrogen, and creatinine). In 1999, the 11 Ibizian hounds were clinically examined and blood samples were obtained for analysis. All dogs were subjected to the LST in 1998 and 2001 after blood samples were collected. In 1998, serology and the LST were conducted in December in all mixed breed dogs and in May in all Ibizian hounds. All dogs were evaluated again in January 1999 and October 2001.

Diagnostic tests and examinations of the dogs were consistent with governmental policy, and followed ethic guidelines approved by Comissió d’Ètica en Experimentació Animal i Humana of Universitat Autònoma de Barcelona. Due to the importance of leishmaniasis in endemic areas, it is recommended to conduct serologic analysis for leishmaniasis at least once a year in dogs living in these areas. All handling, care, and diagnostics of the dogs were done with the explicit permission of the owners and in conjunction with local veterinarians.

Sera. Blood was obtained by cephalic or jugular venipuncture. Serum samples were kept at −40°C until tested.

Enzyme-linked immunosorbent assay (ELISA). An ELISA was performed as previously described. Briefly, microtiter plates were coated with 20 µg/mL of L. infantum antigen in 0.1 mL of coating buffer (0.1 M carbonate-bicarbonate, pH 9.6), per well and incubated overnight at 4°C. One hundred microliters of dog sera per well diluted 1:400 in phosphate-buffered saline (PBS), 0.05% Tween 20 (PBST) and 1% dried

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skimmed milk was added and incubated for one hour at 37°C. The plates were then washed three times with PBST and once with PBS, and 100 μL/well (0.06 μg/mL) of protein A conjugated to horseradish peroxidase (Sigma, St. Louis, MO) was added. This conjugate was incubated for one hour at 37°C, and the plates were then washed as above. The substrate solution (0.4% 3,3′-diaminobenzidine, 0.4 μg/mL, Sigma) and H2O2 (0.4 μg/mL) in 0.1 M phosphate/citrate buffer, pH 5.0, was then added (200 μL/well) and samples were developed for 20 minutes at 24°C. The reaction was stopped by the addition of 50 μL of 3 M H2SO4. Absorbance values were read at 492 nm in an automatic microELISA reader (Anthos 2001; Anthos Labtec Instruments, Salzburg, Austria).

Results was quantified as units related to a positive serum used as a calibrator and arbitrarily set at 100 units. The cutoff value was 35 units (mean + 2 SD of 32 dogs from non-endemic areas). Results less than this cutoff were considered uncertain if \( \geq 23 \) units (mean + 2 SD) and negative if \( < 23 \) units.

**Leishmanin skin test.** The leishmanin reagent was an inactivated suspension of \( 3 \times 10^7 \) *L. infantum* (World Health Organization code MHOM/FR/78/LEM 75) promastigotes/mL in leishmanin diluent (0.4% phenol-saline). One hundred microliters of the solution was injected intradermally in the skin of the groin. Skin reactions were recorded after 48 and 72 hours. An induration and erythematous area > 5 mm in diameter was considered positive at any of the two time readings. Leishmanin diluent (100 μL) was used as control.

**Statistical analysis.** Differences between groups were analyzed by means of an unpaired Student’s *t*-test. *P* values < 0.05 were considered significant.

**RESULTS**

**Clinicopathologic findings.** All Ibizian hounds and two mixed breed dogs were clinically healthy over the three-year period. In the first year of the study (1998), one mixed breed dog (3a) showed clinical signs compatible with canine leishmaniasis (ulceration in one eyelid, bilateral blefaritis, desquamation, and lymphadenopathy). This dog had high levels of antibodies to *Leishmania* and was treated with allopurinol. In 2001, the dog was clinically healthy and showed only a scar in the affected eyelid.

The average biochemical test values were in the normal ranges for all dogs in 1998, 1999 (only Ibizian hounds were tested), and 2001, with the exception of elevated levels of total protein in 1998 and β-globulin throughout the three-year study period. Levels of β-globulin were elevated in 11 of the 14 dogs in 1998, 7 of 11 dogs in 1999, and 8 of 14 dogs in 2001.

**Serologic analysis.** Serologic and LST results are shown in Table 1. In 1998, only two (3a and 16i) of the 14 dogs studied had specific IgG antibodies to *Leishmania*. In 1999, only one (16i) of 11 dogs had a positive result. In 2001, 6 (3a, 2i, 3i, 15i, 16i, and 17i) of 14 dogs were positive, one (1a) was uncertain, and seven were negative. Seroconversion was observed in 2001 in four dogs (2i, 3i, 15i, and 17i).

**Leishmanin skin test.** The three mixed breed dogs (1a, 2a, and 3a) had a negative LST result at both 48 and 72 hours in 1998 and 2001. Conversely, the Ibizian hounds had a higher number of positive LST results at both 48 and 72 hours in 2000 than in 1998. The diameter of the LST result at 48 hours increased in 2001 when compared with the diameter of LST result at 48 hours in 1998 (mean ± SD = 19 ± 7 mm versus 7 ± 6 mm; *P* = 0.021), and this increase was observed in all but three dogs (3i, 15i, and 31i). Similar findings were observed when comparing the 72-hour readings for 2001 and 1998 (mean ± SD = 24 ± 10 mm versus 12 ± 6.7 mm; *P* = 0.011), and the increase in the diameter of the LST result was observed in all dogs, except for a slight decrease recorded in dog 31i.

The Ibizian hounds were classified as seropositive (2i, 3i, 15i, 16i, 17i), those who were seropositive at least once during the study, and seronegative (6i, 14i, 29i, 30i, 31i, 32i), those who were never seropositive during the study. There was no statistically significant difference between seropositive and seronegative dogs in the diameters of LST readings at 48 or 72 hours in 1998 and 2001 (*P* > 0.05).

**DISCUSSION**

This study demonstrated that in areas where leishmaniasis is highly endemic and dogs living outdoors are exposed to *Leishmania*-infected phlebotomine sand flies each season, such as on the Island of Mallorca, there was an increase over time in the *Leishmania*-specific immune response of...
these dogs.\textsuperscript{11} Here we demonstrate that this increase over time of a \textit{Leishmania}-specific immune response in dogs is not only due to humoral immunity, as has been described extensively,\textsuperscript{10,12} but also due to cellular immunity. In addition, the development of humoral immunity does not appear to interfere with or lead to a decrease or lack of specific cellular immunity in healthy dogs. In contrast, Brazilian dogs showed a slight decrease in LST positivity after 13\textsuperscript{12} or 19 months\textsuperscript{14} of follow-up.

Several explanations can be postulated for the increase over time of \textit{Leishmania}-specific cellular immunity in dogs. The persistence of a parasite load could be one mechanism that permits the constant stimulation of memory T cells leading to protection from reinfection\textsuperscript{16} that may occur each season to dogs living in endemic areas. Another explanation can be related to the evasion mechanisms and the chronic infection that \textit{Leishmania} induces in the mammalian host.\textsuperscript{17,18} resulting in a slower development of the immune response over time, as demonstrated with longer seroconversion time periods needed\textsuperscript{19,20} when compared with other microorganisms, such as some bacteria\textsuperscript{21} or viruses.\textsuperscript{22} This same scenario can occur with cellular immunity, in which a longer period of time may be needed for it to develop properly. In a healthy human population, the measurement of cutaneous delayed-type hypersensitivity (DTH) to a battery of ubiquitous antigens (multitest cell-mediated immunity [CMI] system; Merieux Institute, Inc., Miami, FL) is an accepted means of assessing cell-mediated reactivity that is unrelated to the chance of exposure to an infectious agent. It has long been established by this method that the size of reaction increases with age from childhood to young adult, then decreases in the elderly. In human medicine, such an increase in cutaneous DTH (multitest CMI system) is associated with general skin reactivity rather than to immune mechanisms.\textsuperscript{23–26} Such baseline information is not available for dogs in veterinary medicine, but it is noteworthy to consider that age \textit{per se} can be a factor in the variation of cellular immunity.

Our results showed seroconversion at the end of the study in 4 of 11 dogs that had a positive LST result three years earlier when the study began. If the LST result is an indicator of \textit{Leishmania} infection or exposure, we conclude that seroconversion occurred at least one year after infection was detected. In the past decade, experimental infections of dogs with \textit{L. infantum} have shown that seroconversion occurs between one and three months after infection.\textsuperscript{19} Conversely, seroconversion in naturally infected dogs has been recently estimated to occur an average of 94 days after infection.\textsuperscript{20} However, our results are consistent with those of others,\textsuperscript{27} and support the fact that the time of seroconversion after natural infection probably occurs much later, or never, in some dogs.

The LST has been used in dogs mainly for cross-sectional studies. The only longitudinal study to date using the LST reported that dogs with leishmaniasis showed negative results on the LST at the time of diagnosis, but six of seven showed a positive result on the LST after a one-year treatment period.\textsuperscript{28} The clinical improvement in this treated group paralleled the increase in its LST diameter. In the only treated dog (3a) that we followed in the present study, the LST result remained negative and the levels of \textit{Leishmania}-specific IgG decreased slightly over the three-year period, even though clinical improvement was observed. Obviously, many factors can account for this difference, such as type and duration of the treatment, concomitant infections or diseases, reinfections, and length of the follow-up period.

For longitudinal studies, such as vaccine or new treatment trials, humans and dogs must be tested by the LST at least twice. In humans, some studies have reported that the LST could lead to an immune response.\textsuperscript{29,30} while others found that the LST did not induce a specific immune response.\textsuperscript{31,32} These discrepancies may be due to differences in the parasite species used for leishmanin antigen manufacturing or in the protein concentration of the leishmanin. In dogs, it is not known whether the LST can induce an immune response. Our study shows that in a natural setting, some dogs (1a, 2a, and 3a) continued to show negative results on the LST three years after the first LST, suggesting that repeated injections of leishmanin may not be immunogenic to dogs. However, further studies are needed to address this very important question.

It is well known that there is a marked increase in γ-globulins, together with smaller increases in α and β-globulins, in dogs with patent leishmaniasis.\textsuperscript{33} However, in our study, the only consistent clinicopathologic parameter altered throughout all three years of the study was the elevation of levels of β-globulins. There are different proteins included in the β-globulin fraction, with most of them related to acute-phase proteins. The increase in β-globulin has never been studied extensively in dogs with leishmaniasis. Recently, a study described an increase in serum concentrations of some acute-phase proteins in \textit{L. infantum}-infected symptomatic and asymptomatic but seropositive dogs.\textsuperscript{34} Our results confirm that the parasite induces an acute-phase response in the host seen as an increase in levels of β-globulins.

In conclusion, this study demonstrates for the first time that over time, and parallel to the increase of a \textit{Leishmania}-specific humoral immune response in dogs, there is also an increase in the cellular immune response.

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