Chapter 3

Evaluation of the efficacy of two leishmanins in the detection of cellular immunity in asymptomatic dogs

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Abstract

There are few studies in dogs concerning leishmanin skin test. We evaluated and compared the efficacy of two leishmanin preparations for detection of dog *Leishmania* cellular-mediated immunity. Clinically healthy dogs living in an endemic area were studied. Leishmanin preparation 1 ($3x10^8$ promastigotes/mL) was superior to leishmanin preparation 2 ($5x10^6$ promastigotes/mL), measured as the percentage of positive reactions and the diameter of the induced induration. Leishmanin skin test is a valuable tool, although the results show that the degree of response, as it has been shown in human beings, is depending on the preparation used.

Keywords: Leishmania infantum, leishmanin skin test, dog.

Introduction

The widely held opinion that dogs invariably occupied the anergic pole of the leishmanial disease spectrum (Slappendel, 1988) changed when specific cellular immunity was demonstrated in asymptomatic dogs naturally infected with *Leishmania* (Cabral *et al*., 1992; Cabral *et al*., 1998; Cardoso *et al*., 1998; Pinelli *et al*., 1994). These findings argue that canine leishmaniosis may display a wide disease spectrum similar to that seen in human infection, where clinical disease represents one pole and asymptomatic infection the other (Badaró *et al*., 1986).

The delayed-type hypersensitivity reaction in leishmaniosis, generally known as the Montenegro or leishmanin skin test (LST) (Montenegro, 1926), is a useful tool for the evaluation of cell-mediated immunity in *Leishmania* infection in human beings (Pearson & Sousa, 1996) and in dogs (Cardoso *et al*., 1998; Pinelli *et al*., 1994). During active infection in human visceral leishmaniosis, the leishmanin skin test is negative while it is positive when subclinical infection (self-healing), early stage of visceral leishmaniosis or after succesful treatment (Cahill, 1971; Manson-Bahr, 1961). It is probable that something similar occurs in the canine population (Cardoso *et al*., 1998). Contrary to what occurs in humans, where the concentration of promastigotes and characteristics of leishmanin preparations are important in the results of LST (Akuffo *et al*., 1995; Alimohammadian *et al*., 1993), there are few studies concerning LST in dogs.

The aim of the present study was to evaluate and compare the efficacy of two leishmanin preparations for detection of dog *Leishmania* cellular-mediated immunity.

Material and Methods

Both leishmanins used were inactivated suspensions of *Leishmania infantum*, kindly provided by Dr. Jorge Alvar (Instituto de Salud Carlos III, Madrid, Spain). *Madrid leishmanin* (ML) was prepared in the Instituto de Salud Carlos III, Madrid, Spain, and contained $3x10^8$ promastigotes/mL of *L. infantum* LEM 75 in 0.4% phenol-saline with a protein content of 7.3 mg/dL. *Roma leishmanin* (RL) was prepared in the Istituto Superior de Sanita, Roma, Italy, and contained $5x10^6$ promastigotes/mL of *L. infantum* LEM 75 in 0.5% phenol-saline with a protein content of 2.4 mg/dL. Controls were diluent, saline, and purified protein derivative (PPD).

LST was carried out in a village located in the island of Mallorca (Spain), where canine leishmaniosis is endemic. Thirty-one Ibizian hounds clinically healthy and living outdoors were studied. This breed was selected because the Ibizian Hound presents a predominant cellular immune response (Solano-Gallego *et al*., 2000). Efficacy was evaluated by subcutaneous injection of 0.1 mL of leishmanin, ML in the right groin and RL in the left groin. The largest diameter of the induced induration and its perpendicular diameter were measured and averaged at 48 and 72 h. Indurations ≥ 5 mm were considered positive. Five non-infected healthy dogs and five dogs suffering from patent leishmaniosis served as controls.

Blood was collected from all dogs and anti-*Leishmania* IgG antibodies were assessed by means of ELISA as described elsewhere (Solano-Gallego *et al*., 2000).

Statistical analysis was assessed by Student's t test (paired and unpaired) using the SPSS program. A $P<0.05$ was considered significant.

Results

The results are shown in Table 1. At 48 h, 16 out of 31 dogs tested positive to ML with a mean induration of 8.12 mm. In contrast, only 1 out of 31 dogs tested positive to RL with an induration of 6 mm. At 72 h, 25 out of 31 dogs tested positive to ML with a mean induration of 14.6 mm, while only 4 out of 31 dogs tested positive to RL with a mean induration of 4 mm. The majority of the dogs testing positive to ML at 48 h increased their reaction at 72 h. Two dogs tested positive at 48h but negative at 72 h. ML induced significantly more positive responses both at 48 h ($P < 0.0001$) and at 72 h ($P < 0.0001$) than RL. ML induced significantly more positive responses and more induration at 72 h than at 48 h ($P < 0.001$). There was a non-significant tendency for the ML to induce a stronger response at 72h (14.6±1.4 mm) than at 48 h (8.12±1.7 mm) ($P = 0.210$). All control animals tested negative to ML and RL at any time reading. Diluent, saline and PPD controls were negative in all the animals tested. In terms of sensitivity, measured as the percentage of positive reactions, and potency, measured as the diameter of the induced induration, ML was superior to RL.

Table 1. Percentage of positive DTH and diameter of reaction to Madrid leishmanin and to Roma leishmanin measured at 48 h and 72 h in asymptomatic dogs.

Leishmanin	Time readings	Percentage of positive DTH	Diameter of reaction
Madrid	48 hr	51.6%	8.12 ± 1.7
Madrid	72 hr	80.6%	14.6 ± 1.4
Roma	48 hr	3.0%	0.8 ± 0.4
Roma	72 hr	12.9%	1.4 ± 0.7

[a] mm ± standard error

Serology was positive in 15 out of 31 Ibizian hounds (mean O.D at 490nm = 0.35; SD =0.57; low levels) and in all dogs with clinical patent disease (mean = 2.67, SD = 0.58; high levels) and negative (mean = 0.100, SD = 0.04) in non-infected dogs.

Discusssion

The main explanation for the low percentage of a positive LST with RL could be the lower concentration of promastigotes per mL and the lower protein content compared with ML. This explanation is not in agreement with Cardoso *et al.* (1998), which described good sensitivity in the dog in concentrations ranging from $3x10^6$ promastigotes/mL to $3x10^8$ promastigotes/mL but using the same leishmanin preparation. However, other authors have found sensitivity and potency to be different for leishmanin preparations manufactured in different laboratories when studied in human beings (Akuffo *et al*., 1995; Alimohammadian *et al*., 1993). Findings comparable to what we report in this study. Another explanation could be the degradation of protein antigens during storage. Nevertheless, both leishmanin preparations were stored at 4 ^oC and leishmanin has been shown to be stable at this temperature and not to lose sensitivity or specificity for a number of years (Badaró *et al*., 1990; Weigle *et al*., 1991).

The few studies in the literature concerning LST in dog (Cardoso *et al*., 1998; Pinelli *et al*., 1994) and some of the humans studies (Alimohammadian *et al*., 1993; Weigle *et al*., 1991) measure the leishmanin concentration in promastigotes per mL. Possibly, the protein content together with the promastigotes per mL concentration would be a better measurement to compare studies carried out in other laboratories with different leishmanin preparations.

In field work, sometimes it is not feasible to measure skin reactions on more than one occasions. Since more and larger reactions were recorded at 72 hr, it appears that this is the optimum time to make readings in the dog. Moreover, the best concentration to detect cellular immunity in the dog is $3x10^8$ promastigotes per mL.

In conclusion, leishmanin skin test, if well standardized, can be a valuable tool for diagnosis and prognosis, epidemiological studies, and as a measure of vaccine efficacy in the dog.

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Chapter 4

The Ibizian hound presents a predominantly cellular immune response against natural *Leishmania* infection

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Abstract

Veterinarians working in the Balearic Islands (Mallorca), an endemic region of canine leishmaniosis, have reported very few cases of leishmaniosis in Ibizian hounds while concurrently observing that dogs of other breeds had a high incidence of clinical canine leishmaniosis. To further investigate this observation, two populations of dogs from the Balearic Islands were examined for the presence of *Leishmania*-specific cellular immunity using a delayed type hypersensitivity test (DTH) to leishmanin and for the presence of *Leishmania*-specific humoral immunity using an ELISA. Fifty-six asymptomatic dogs, 31 Ibizian hounds and 25 dogs belonging to other breeds were examined. Seventy-seven per cent of the dogs demonstrated a specific immune response against *Leishmania*, either humoral or cellular. This finding suggests that the infection rate (77%) was higher than previously considered. For Ibizian hounds 81% were DTH positive while only 48% of the other dogs were DTH positive. A statistical association between Ibizian hounds and positive DTH response was found. A specific humoral response was found in 48% of Ibizian hounds and in 56% of the other dogs. No statistical association relative to the *Leishmania*-specific IgG1 and IgG2 levels were found between the two groups. The Ibizian hound has been reported to be more resistant to *Leishmania* infection and we found that the Ibizian hound mounts a significant cellular response to infection. Thus, the Ibizian hound may be an interesting canine model for the investigation of protective anti-*Leishmania* immune response.

Key words: Dog; cellular immunity; *Leishmania infantum*.

Introduction

Canine leishmaniosis is a systemic severe disease caused by the protozoan parasite *Leishmania infantum* (Gramiccia *et al*., 1989). The disease is endemic in the Mediterranean basin, but occasionally, can be diagnosed outside of this region in dogs that have been living previously in endemic areas (Longstaffe & Guy, 1985). It was considered that dogs infected with *L. infantum* develop a humoral immune response that is not immuno-protective (Slappendel & Ferrer, 1998). In fact, most patients demonstrate hypergammaglobulinemia (Abranches *et al*., 1991a; Vitu *et al*., 1973) and high anti-*Leishmania* titers (Abranches *et al*., 1991a; Liew & O'Donnell, 1993).

Recent studies have demonstrated that some dogs develop a specific anti-*Leishmania* cellular immune response which is effective in protective, leading to a natural immunological resistance (Cabral *et al*., 1992; Cabral *et al*., 1998; Pinelli *et al*., 1994). Several studies have demonstrated a high seroprevalence in the Mediterranean canine populations without detectable clinical signs (Abranches *et al*., 1991b; Acedo-Sánchez *et al*., 1996; Acedo-Sánchez *et al*., 1998; Fisa *et al*., 1999). The percentage of dogs naturally resistant is not well established. Pinelli et al. (1994) found that 6 out of 10 beagle dogs developed a protective immune response when experimentally infected by *Leishmania*. According to Cabral *et al*. (1998) and to Barbosa Santos *et al*. (1998), 40% of dogs living in endemic areas display a cellular immune response, either alone or associated with a humoral response. The DTH to leishmanin has proven to be an useful tool to detect animals with an strong cellular immune response that protects the animals from infection so that most of them would be resistant (Cardoso *et al*., 1998; Pinelli *et al*., 1994). According to Deplazes *et al*. (1995) the titre of specific IgG1 and IgG2 could help to identify susceptible dogs. Dogs with IgG1 and IgG2 titres that remain elevated for prolonged periods of time would be considered most susceptible.

Studies in mice have demonstrated that the type of immune response against *Leishmania* infection is genetically controlled (Behin *et al*., 1979; Launois *et al*., 1998; Mitchel, 1983). However, no studies have been published demonstrating different infection rates between genetically different canine breeds. Veterinarians practicing in the Balearic Islands, an endemic focus of canine leishmaniosis, have observed that Ibizian hound very rarely develop clinical leishmaniosis. The Ibizian hound is originally from North Africa and has lived in the Balearic Islands for over two thousand years. Being exceptionally well suited for hunting, the Ibizian hound has become a very common breed in the Balearic Islands (Gounot, 1990). Consequently, it is likely that individuals resistant to leishmaniosis have been naturally selected for resistance to *Leishmania*.

Using these clinical observations, our working hypothesis was that the Ibizian hound is a breed genetically resistant to canine leishmaniosis due to a cellular immune response against the parasite. To investigate this hypothesis, we compared the cellular and humoral specific immune response against *L. infantum* infection in Ibizian hounds and in dogs of other breeds from the island of Mallorca.

Materials and methods

Animals

We used for this study two groups of dogs living outdoors in the village of Felanitx (Mallorca, Spain), an endemic area of leishmaniosis. The first group comprised 31 Ibizian hound dogs. Their ages ranged from 6 months to 10 years, with an average of 3 years. The second group consisted of 25 dogs of different breeds. Their ages ranged from 7 months to 9 years, with an average of 3 years. All dogs were clinically healthy.

Sera

Blood samples were taken by cephalic or jugular venepuncture before leishmanin skin testing was performed. After centrifugation, the sera were kept at –80 ^{o}C.

Delayed-type hypersensitivity (DTH) reactions

Dogs were tested for DTH reaction to leishmanin. Leishmanin reagent was an inactivated suspension of $3x10^{8}$ *L. infantum* promastigotes per mL in 0.4% phenol-saline, kindly provided by Dr. Alvar (Instituto de Salud Carlos III, Madrid, Spain). One hundred µL of the solution was intra-dermally injected in the skin of the groin. Skin reactions were recorded after 72 h and an induration or eritematous area >5 mm in diameter was considered positive in agreement with Pinelli *et al*. (1994) and (Cardoso *et al*., 1998). Purified protein derivative (2500TU/ml) (Central Veterinary Institute Lelystad, The Netherlands) and leishmanin diluent as well as saline solution (0.1 mL of each) served as controls.

ELISA

Leishmania infantum (WHO code MHOM/ FR/ 78/LEM 75) antigen was kindly provided by Dr. Montserrat Portús (Universitat de Barcelona, Spain). Promastigotes were harvested by centrifugation (1300 x g, 10 min, 4^{o}C) and washed with phosphate buffer saline (PBS pH 7.4) (Sigma). The parasites were resuspended in PBS and disrupted by sonication. Microtitre plates were coated with 20 µg mL^{-1} of *L. infantum* antigen in 0.1 mL of coating buffer (0.1M carbonate-bicarbonate, pH 9.6) and incubated overnight at 4^{o}C. One hundred µL per well of dog sera, diluted 1:400 for IgG1, IgG2 and IgG in PBS-0.05% Tween 20 (PBST)-1% dried skimmed milk, were incubated for 1 h at 37^{o}C. After washing three times with PBST and one time with PBS, 100 µL per well at different dilution of anti-dog IgG1 (1:2000) or anti-dog IgG2 (1:5000) or anti-dog IgG (1:20000) conjugated to horseradish peroxidase (Bethyl laboratories, Montgomery, TX, USA) were added. These conjugates were incubated for 1 h at 37^{o}C, then the plates were rewashed. The substrate solution (ortho-phenylene-diamine, 0.4 mg/mL) (Sigma) and H_2O_2 (0.4 µL/mL) in 0.1M of phosphate/citrate buffer pH 5.0, was added at 200 µL/well and developed for 20 min at 24^{o}C. The reaction was stopped with 50 µL of H_2SO_4 3M. Absorbances values were read at 490 nm in an automatic microELISA reader (Bio-tek instruments EL 312e microplate).

Sera from 28 non-infected *L. infantum* dogs living in an endemic region were tested to set up a cut-off for IgG, IgG1 and IgG2 specific ELISA determinations. Cut-off absorbance values were established as the mean plus 3 standard deviations, resulting in 0.236 for IgG (mean 0.099, standard deviation 0.0456), 0.229 for IgG1 (mean 0.098, standard deviation 0.0438), 0.128 for IgG2 (mean 0.0624, standard deviation 0.022). All determinations included the serum from a sick dog with a confirmed infection as positive control and the serum from a healthy dog as a negative control.

Statistical analysis

Association between variables and differences among groups were analyzed by means of Chi-square and Student's t test respectively. The rate of infection was analysed between two groups of dogs with two samples Z-Test. A $P<0.05$ was considered significant.

Results

Rate of infection

The rate of infection was calculated using all animals that were seropositive in IgG antibodies and/or positive in DTH test. The percentage was 77% for all dogs investigated in this study (43 out of 56 asymptomatic dogs). The percentage of infection in the Ibizian hound dogs was 81% (25 out of 31) and 72% (18 out of 25) in the dogs of other breeds. We concluded that the percentatges of infection are not different between the two groups (two samples Z test, $P=0.2152$).

DTH skin reactions to leishmanin

The results of DTH skin testing can be seen in Table 1. Twenty-five out of 31 (81%) Ibizian hounds had a positive reaction to leishmanin. Only 12 out of 25 (48%) of the other dogs had a positive response to the intradermal inoculation of leishmanin. No reactions were observed when purified protein derivative (PPD), saline solution and diluent alone were inoculated. According to these results, we concluded that the groups of dogs and DTH are associated (Chi-square test of association, $P=0.010$). The percentages show in Table 1 suggested that Ibizian hounds group are more likely than other breeds group to develop a positive DTH. However, either Ibizian hounds or other testing positive did not differ in the intensity of DTH reaction as measured by reaction diameter (Student's t test, $P=0.936$).

Table 1. Relationship between DTH to leishmanin and the two groups of dogs studied.

	DTH to leishmanin		
	+	−	Total
Ibizian hound	25	6	31
Other breed	12	13	25
Total	37	19	56

Relationship between specific IgG antibodies and groups of dogs

Among the 31 samples from Ibizian hound dogs, 15 presented positive titres of specific anti-*Leishmania* IgG and 16 negative. In the different breed dogs, 25 samples were analyzed and 14 were found positive and 11 negative. We concluded that the specific IgG antibodies and the groups of dogs are not associated (Chi-square test of association, $P=0.571$).

Relationship between specific IgG1 and IgG2 antibodies and groups of dogs

Out of the 31 Ibizian hounds, 15 were positive for IgG1 and 21 were positive for IgG2. Of the 25 other dogs, 7 were positive for IgG1 and 18 were positive for IgG2. We concluded that the specific IgG1 and IgG2 antibodies and the groups of dogs are not associated (Chi-square test of association, P=0.120 and P=0.730 respectively).

Relationship between DTH skin reactions to leishmanin and specific IgG, IgG1 and IgG2 antibodies.

The relationship between DTH and IgG, IgG1 and IgG2 antibodies in the studied dogs can be seen in Table 2. Statistical analysis did not find any association between DTH *versus* IgG (Chi-square test of association, P=0.059, P=0.577 and P=0.288 respectively).

The relationship between DTH and the specific immunoglobulins for the Ibizian hound group can be seen in Table 3 and for the non-Ibizian hound group in Table 4.
We found, when we analysed the relationship between DTH and IgG, six Ibizian hounds (19%) and 7 dogs of different breed (28%) were negative on both serology and DTH. Ten Ibizian hounds (32%) and 4 dogs of different breeds (16%) presented a positive DTH test and negative serology. Fifteen Ibizian hounds (48%) and 8 dogs of other breeds (32%) were both seropositive and positive in DTH test. Six dogs of other breeds (24%) and none of the Ibizian hound dogs were seropositive and negative in DTH test.

Statistical analysis did not find any association between either DTH and IgG, IgG1 and IgG2 in other dogs group (Chi-square test of association, P=0.529, P=0.901 and P=0.443 respectively). However, in the Ibizian hound group, the statistical analysis demonstrated association between DTH and IgG (Chi-square test of association, P=0.029) but did not find any association between DTH and IgG1 (Chi-square test of association, P=0.930) or IgG2 (Chi-square test of association, P=0.583).

Statistical analysis did not find any association between DTH and antibody response when they were grouped in four classes: IgG1 and IgG2 positive, IgG1 and IgG2 negative, IgG1 positive and IgG2 negative, and IgG1 negative and IgG2 positive in any of the two groups of dogs studied (Chi-square test of association, total of dogs P=0.243, Ibizian hounds P=0.240 and other dogs P=0.477).

Table 2. Relationship between DTH to leishmanin and the specific IgG, IgG1 and IgG2 antibodies in all dogs studied (56).

		IgG1		**IgG2**		**IgG**	
		+	−	+	−	+	−
DTH	+	16	21	28	9	23	14
	−	6	13	11	8	6	13
Total		22	34	39	17	29	27

Table 3. Relationship between DTH to leishmanin and the specific IgG, IgG1 and IgG2 antibodies in the Ibizian hounds.

		IgG1		IgG2		IgG	
		+	−	+	−	+	−
DTH	+	12	13	18	7	15	10
	−	3	3	3	3	0	6
Total		15	16	21	10	15	16

Table 4. Relationship between DTH to leishmanin and the specific IgG, IgG1 and IgG2 antibodies in the other breed of dogs.

		IgG1		IgG2		IgG	
		+	−	+	−	+	−
DTH	+	4	8	10	2	8	4
	−	3	10	8	5	6	7
Total		7	18	18	7	14	11

Discussion

The results of this study show that the rate of infection between dogs living in an endemic area is considerably higher than previously described (Ben said *et al*., 1992). If we consider that dogs presenting a specific cellular *and/or* humoral immune response have been infected, the infection rate is very high (77%). The proportion of positive dogs is approximately the same for both groups: 81% in the Ibizian hound (25 out of 31) and 72% (18 out of 25) in the dogs of other breeds. These data agree with that published recently by Cabral *et al*. (1998), who found a rate of *Leishmania* infection of 65% in dogs living in Portugal. Berrahal *et al*. (1996) also found a rate of infection of 80% in dogs living in France using PCR (polymerase chain reaction) to detect the parasite in skin and conjunctival samples. Until now, most epidemiologic studies have been based only on serologic surveys. Consequently, these studies did not detect positive DTH and negative serologic dogs, thus underestimating the rate of infection (Dye *et al*., 1993; Tesh, 1995).

Dogs with a positive DTH skin test against leishmanin subsequent are considered to have been exposed to the parasite and potentially resistant to *Leishmania* infection (Pinelli *et al*., 1994). According to our results, 48% of dogs of other breeds living in an endemic area are positive by DTH. The remaining 52% (negative in the intradermal test) include dogs, which have not been infected (28%) and infected dogs with an exclusive humoral immune response (24%) which probably will develop clinical disease. In summary, in an endemic area we find two groups of dogs: sick and asymptomatic. The asymptomatic dogs are divided into three groups: resistant dogs, dogs that will develop clinical disease and non-infected dogs. Presumably, the immune response to *L. infantum* is a mixed humoral and a cellular response. Thus, a whole spectrum of immune responses exists: from Resistant dogs appear to present a predominantly cellular response and susceptible animals present an exaggerated humoral response.

Results obtained from the Ibizian hound group suggests that this breed constitutes a special group of dogs regarding the immune response against *Leishmania* infection. Most (25 out of 31, over 81%) showed a positive DTH to leishmanin. Six dogs that were negative to the DTH, were also negative in the serology for IgG, which might indicate that they have not been exposed to the parasite. A statistically significant association was found between Ibizian hounds and DTH positive. However, dogs of other breed that were positive for DTH were as intense as the DTH response of Ibizian hounds, suggesting that dogs of other breeds are as capable of responding as are Ibizian hounds, but as a group the Ibizian hound responds more uniformly with a positive DTH response. Consequently, we consider the Ibizian hound more *Leishmania* resistant than other canine breeds.

Several authors have suggested that the presence of anti-*Leishmania* antibodies alone is not a conclusive sign of disease progression (Gicheru *et al*., 1995; Nieto *et al*., 1999). Titres of specific IgG1 and IgG2 should be a better indicator of disease status than total IgG (Deplazes *et al*., 1995). The analysis of IgG subclasses in the infected dogs provides evidence of a direct correlation between induction of high levels of IgG1 anti-*Leishmania* antibodies and the appearance of clinical symptoms of the disease (Nieto *et al*., 1999). Results in asymptomatic dogs are in agreement with these findings. Although some animals (six non-Ibizian hounds) were seropositive for IgG and negative in the DTH, none presented high levels of either IgG1 or clinical symptoms compatible with leishmaniosis.

Deplazes et al. (1995) found that sick dogs initially presented with high levels of both *L. infantum* specific IgG1 and IgG2. They clinical improved after a long treatment with N-metilglucamine antimoniate (Glucantime®, Rhône-Mérieux), and upon improvement the level of IgG1 decreased and IgG2 levels remained constant. These data suggest a shift in response, from Th2 to Th1, as in the infection of *Leishmania major* in mice where the IgG1 is associated with Th2 type response and IgG2 is associated with Th1 type response (Reed & Scott, 1993). In the current study, dogs with positive DTH skin reactions had a polymorphic humoral immune response ranging from seronegative to positive titres levels of either IgG1 or IgG2. An association between DTH test and either of the IgG subclasses was not found.

In human studies, the *Leishmania* protective IgG subclasses are not well defined. Rodriguez *et al*. (1996) reported levels of parasite specific IgG1, IgG2, IgG3 and low levels of IgG4 in cutaneous leishmaniosis. Skeiky *et al*. (1997) reported elevated levels of IgG4 in diffuse cutaneous leishmaniosis. Shiddo *et al*. (1996) found high levels of all four IgG subclasses in visceral leishmaniosis. In humans, IgG1 and IgG3 can fix complement, while IgG2 is less effective and IgG4 does not fix complement (Brekke *et al*., 1995). Cell-mediated immunity undoubtedly represents the primary mechanism of resistance to *Leishmania* infection, there seems to be sufficient evidence to suggest that a humoral mechanism, including a functional complement system and an appropiate antibody response, may constitute indispensable elements of an effective protective response at least in human beings (Ulrich *et al*., 1996). The contribution of antibodies and complement in a protective role in leishmaniosis, possibly via mechansims of lysis of some *Leishmania* species and the ability to enhance macrophage mediated parasite killing, requires further study.

Data collected in this study confirm our hypothesis that the Ibizian hounds as a group present a more uniform cellular response to infection than do dogs of other breeds. Thus, the Ibizian hound appears to be a very interesting canine model for the investigation of immunological mechanisms involved in a self-limited *Leishmania* infection and in the search for a vaccine against canine leishmaniosis.

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Chapter 5

Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology

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Abstract

We studied and compared the prevalence of *Leishmania* infection and the seroprevalence and the prevalence of canine leishmaniasis in an area where canine leishmaniasis is endemic. One hundred dogs living on the island of Mallorca (Spain) were studied. In this study, we clinically examined each dog for the presence of symptoms compatible with leishmaniasis, determined the titre of anti-*Leishmania* antibodies, and investigated the presence of *Leishmania* DNA by PCR in skin, conjunctiva, and bone marrow samples of each dog. The prevalence of the disease and the seroprevalence were 13 and 26%, respectively. In 63% of the dogs, *Leishmania* DNA could be detected by PCR in at least one of the tissues studied. The results of positive PCR in the bone marrow, the conjunctiva, and the skin were 17.8, 32, and 51%, respectively. The prevalence of the infection, 67%, was calculated using all animals that were seropositive and/or positive by PCR with any tissue. The results showed that the majority of dogs living in an area where canine leishmaniasis is endemic are infected by *Leishmania* and that the prevalence of infection is much greater than the prevalence of overt *Leishmania*-related disease.

Introduction

Canine leishmaniasis is a severe systemic disease of dogs caused by the protozoan parasite *Leishmania infantum.* Clinical manifestations of the disease include nonpruritic skin lesions, such as exfoliative dermatitis and ulcerations, local or generalized lymphadenopathy, loss of weight, poor appetite, ocular lesions, epistaxis, lameness, renal failure, and diarrhea (Ciaramella *et al*., 1997; Ferrer *et al*., 1988; Koutinas *et al*., 1999; Slappendel, 1988). Leishmaniasis is a zoonotic disease for which dogs are considered the chief reservoir of the parasite. The disease is endemic in the Mediterranean basin, where seroprevalence ranges between 10 and 37% (Fisa *et al*., 1999; Sideris *et al*., 1999). There are, however, several studies suggesting that the rate of infection is higher than the figures found by serological investigations. A survey performed using the PCR and immunoblotting techniques found that most dogs living in southern France had been exposed to *Leishmania* (Berrahal *et al*., 1996). These results agree with another study that found a rate of *Leishmania* infection of 65% for dogs living in Portugal by using serology and cell-mediated tests (Cabral *et al*., 1998).

The percentage of infected dogs living in an area where canine leishmaniasis is endemic has major public health implications. It was demonstrated that infected, but asymptomatic, dogs were sources of the parasite for phlebotomine vector sandflies and as a consequence play an active role in the transmission of *Leishmania* (Molina *et al*., 1994).

The present study was designed to investigate and compare the prevalence of *Leishmania* infection, the seroprevalence and the prevalence of the disease in a canine population living in an area where canine leishmaniasis is endemic. One hundred dogs living on the island of Mallorca (Spain) were included in this study. Veterinarians clinically examined all dogs, and the titre of anti-*Leishmania* antibodies was determined. The presence of *Leishmania* DNA in each dog was investigated by PCR with three tissues: skin, eye conjunctiva, and bone marrow.

Materials and methods

Animals

The study was carried out on the Island of Mallorca, an endemic area of canine leishmaniasis. The subjects of the study were 100 dogs from different breeds and ages, which had to be euthanatized in the Animal Pound of Palma de Mallorca for reasons related to city sanitation policy.

Sampling

Prior to sampling and euthanasia, all dogs were examined to detect clinical symptoms compatible with canine leishmaniasis. The dogs were then premedicated with acepromacine maleate and anesthetized intravenously with sodium thiopental.
Blood was collected by cephalic or jugular venepuncture, and the serum samples for detecting and quantifying specific antibodies to *Leishmania* were stored at -80^{o} C. Three types of tissues for PCR were sampled: bone marrow, skin, and eye conjunctiva. Bone marrow aspirates were obtained from the costochondral junctions by using a 22 gauge needle. Cutaneous samples were collected from the upper part of the muzzle by

punch biopsy with a diameter of 5 mm and with each biopsy weighing approximately 30 mg. Conjunctiva samples were obtained using scissors, with each biopsy weighing approximately 30 mg. Samples were stored at –20° C before DNA extraction. After sampling was completed, dogs were euthanatized using an overdose of parenteral barbiturates.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Riera *et al*., 1999). Briefly, microtitre plates were coated with a 20-μg mL^{-1} concentration of *L. infantum* antigen in 0.1 mL of coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) and incubated overnight at 4°C. One hundred microliters of dog sera per well was diluted 1:400 in phosphate-buffered saline (PBS)-0.05% Tween 20 (PBST)-1% dried skim milk and was incubated for 1 h at 37°C. After washing three times with PBST and once with PBS, 100 μL of anti-dog immunoglobulin G (IgG) (1:20,000) conjugated to horseradish peroxidase (Bethyl laboratories, Montgomery, Tex.) was added. This conjugate was incubated for 1 h at 37°C, and then the plates were rewashed. The substrate solution (ortho-phenylene-diamine, 0.4 mg/mL) (Sigma) and H_2O_2 (0.4 μL/mL) in 0.1 M of phosphate-citrate buffer (pH 5.0) was added at 200 μL/well and developed for 20 min at 24°C. The reaction was stopped with 50 μL of H_2SO_4 3M. Absorbance was read at 490 nm in an automatic microELISA reader (EL 312e microplate; Bio-tek instruments).

Sera from 28 dogs not infected with *Leishmania infantum* that were living in a region where it is endemic were tested to set up a cut-off for IgG-specific ELISA determinations. The cut-off absorbance was established as the mean plus three standard deviations, resulting in 0.236 for IgG (mean, 0.099, standard deviation, 0.0456). All determinations included the serum from a sick dog with a confirmed infection as positive control and the serum from a healthy dog as a negative control.

DNA isolation

Bone marrow

Samples of DNA were prepared as previously described (Roura *et al*., 1999). Briefly, 115-μL of bone marrow samples were washed and centrifuged three times in tris-EDTA buffer (pH 8·0), and the leucocyte pellet was incubated in 0.1 mL of lysis buffer (50mM potassium chloride, 10 mM tris-HCl [pH 8·4], 0.5% Tween-20 and 100 μg of proteinase K mL) at 56°C overnight. Proteinase K was inactivated by incubating the samples at 95°C for 10 minutes before using them in the PCR.

Conjunctiva and skin tissue

Conjunctiva and skin biopsies were digested overnight in the presence of sodium dodecyl sulfate and proteinase K at final concentrations of 2% and 0.2 mg/mL, respectively, in 1 mL of TE buffer (Tris 50mM [pH 8.0], 20mM EDTA). Afterwards, DNA was isolated by double phenol-chloroform extraction (Sambrook *et al*., 1989).

PCR

Leishmania specific oligonucleotide primers SP176 (5'- TCTTGCGGGGAGGGGGTG-3´) and SP177 (5´-TTGACCCCCAACCACATTTTA-3´) were used to amplify a 120-base-pair fragment of *Leishmania* kinetoplast DNA minicircles (Rodgers *et al*., 1990). PCR was conducted in a 50-µL final reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate, 0.3 µM concentrations of each primer, 3 µl of supernatants of digested tissue, and 1.25 U of *Taq* polymerase (Ecogen). Reactions were carried out in an automatic thermocycler (Perkin Elmer) with a thermal cycling profile of 94°C for 3 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and finally 72°C for 5 min, and at that point the thermocycler maintained a constant temperature of 4°C. Positive controls containing 10 ng of genomic of *Leishmania* DNA and a negative control without template DNA were included. Amplified fragments were analyzed by electrophoresis in a 2.5% agarose gel containing ethidium bromide (0.5 µg/mL) at 100 V for 1 h. A ϕX174 HaeIII (DNA MWM V; Boehringer Mannheim) was used as a molecular weight marker.

To ensure that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation, or PCR inhibition, sample DNA was also amplified for β-actin by using a forward primer (5´-ACCTGGAGTTCGAGGYTCGGA-3´) and a reverse primer (5´-AAGTAACCCTGGTTGGTGAAGCAG-3´) (Helfand *et al*., 1999). When samples did not yield amplification products, they were extracted again until amplification products were obtained.

Results

Thirteen animals presented one or more clinical signs of canine leishmaniasis while 87 dogs were asymptomatic. Twenty-six of the dogs were seropositive. Eleven of them also showed clinical signs while 15 were clinically healthy dogs. Two dogs with clinical signs of leishmaniasis were seronegative.

The presence of parasite DNA was detected in 63 dogs for at least one of the three tissues investigated. The results of the PCRs with the different tissues were as follows: 17 out of 95 dogs (17.8%) had positive bone marrow, 32 out of 100 dogs (32%) had positive conjunctiva and 51 out of 100 dogs (51%) had positive skin. *Leishmania* DNA was detected in all animals presenting clinical signs.

Results for clinical status, serology, and PCR, including the three tissues sampled for each dog, are shown in Table 1.

The prevalence of the infection, 67%, was calculated by adding all animals that were seropositive and/or positive for PCR with any tissue.

Table 1. Clinical status, serology, and PCR results, including the three tissues sampled for each dog, in a canine population living in an area of Mallorca where leishmaniasis is endemic

	Serology	Presence of *Leishmania* DNA			Clinical status		
Group no.	ELISA	Bone marrow	Conjunctiva	Skin	No. of Symptomatic dogs	No. of Asymptomatic dogs	Total no. of dogs
1	+	+	+	+	9	4	13
2	+	-	-	+	2	6	8
3	+	-	-	-	0	4	4
4	+	+	-	-	0	1	1
5	-	-	+	+	0	10	10
6	-	-	-	+	2	17	19
7	-	-	+	-	0	9	9
8	-	+	-	+	0	1	1
9	-	+	-	-	0	2	2
10	-	-	-	-	0	33	33

Discussion

According to our results, the prevalence of canine leishmaniasis in Mallorca and the seroprevalence are 13 and 26%, respectively. These results are in agreement with those obtained by various authors throughout the Mediterranean basin (Deplazes *et al*., 1998; Sideris *et al*., 1999; Zaffaroni *et al*., 1999). For canine leishmaniasis, serology is considered to be a sensitive and useful technique and is well correlated with clinical signs. However, the meaning of asymptomatic but seropositive dogs (15 out of 26) is difficult to explain without a follow-up study. Undoubtedly, this condition indicates previous contact with the parasite, but we do not know whether these dogs are immune resistant animals or whether they will subsequently develop the disease (Cabral *et al*., 1998).

The prevalence of infection that we found in Mallorca (67%) is very high, although lower than the 80% found in France using a smaller number of dogs (Berrahal *et al*., 1996). Thus, our study and others confirm that the prevalence of *Leishmania* infection has been underestimated (Berrahal *et al*., 1996; Cabral *et al*., 1998). These data are useful for epidemiological studies and would provide a better estimation of the level of transmission of the parasite.

The frequency of DNA parasite detection was different in different tissues. The low percentage of positive bone marrow PCR (17%) suggests that a hematogenous dissemination to the bone marrow takes place only in part of the animals. Consequently, the detection of *Leishmania* DNA in bone marrow by using PCR is not an adequate method to detect *Leishmania* infection in dogs. Our results, therefore, disagree with those of other authors who found bone marrow PCR a better diagnostic method than serology (Ashford *et al*., 1995). On the other hand, half of the dogs studied were positive to parasite DNA detection in the skin. This result indicates that skin is the

major tissue reserve of parasites in dogs and that PCR in skin biopsy is a sensitive method to detect infection. This finding concerns the biology of the parasite because the skin is the most accessible tissue for the vector. Furthermore, cutaneous samples were collected from the upper part of the muzzle where most sandflies take their blood meal (Killick-Kendrick & Killick-Kendrick, 1999).

In the compartmental mathematical model of canine leishmaniasis, it was assumed that asymptomatic dogs were not infectious for sandflies (Dye *et al.*, 1992; Hasibeder *et al.*, 1992). However, other authors showed that infectivity of dogs presenting *Leishmania* infection is not exclusively linked to the symptomatic stage of the disease**:** they found that three out of five asymptomatic but seropositive dogs transmitted the parasite to the sandfly vectors (Molina *et al.*, 1994). Our results support the idea that asymptomatic dogs must be considered infectious for sandflies. We showed that 54% of healthy dogs living in an area where canine leishmaniasis is endemic must be considered asymptomatic carriers of *Leishmania*. Further studies are needed to ascertain the potential of asymptomatic dogs to transmit *Leishmania* to vector sandflies.

Studies of the immune response against the *Leishmania* parasite in the dog have revealed that T lymphocytes and the cytokines they produce play a crucial role in determining whether an infection with this intracellular pathogen results in either protective immunity or progressive disease (Pinelli *et al.*, 1995; Pinelli *et al.*, 1994; Pinelli *et al.*, 1999). In the population of our study, it would have been interesting to study the cellular immune response using a field tool such as leishmanin skin test. This was not, however, possible for ethical reasons, as we would have needed to previously manipulate animals not destined for research. However, it has been reported that 40% of the asymptomatic dogs living in Portugal (Cabral *et al.*, 1998) and that 48% of the asymptomatic dogs living in Mallorca (Solano-Gallego *et al.*, 2000) had demonstrable parasite-specific cellular immunity. We found that 37% of asymptomatic dogs were PCR positive in the skin and/or conjunctiva and were seronegative. Probably, dogs with suitable cellular immunity could control the spread of *Leishmania* parasite and live in equilibrium with the parasite in the skin and mucosal regions. This fact may explain the high rate of infection due to the whole spectrum of immune response to *Leishmania* in the canine population (Cabral *et al.*, 1998). In human beings, a strong association between the decrease in the number of $CD4^+$ T lymphocytes and the increase in the infectivity of people coinfected with *L. infantum* and human immunodeficiency virus to the sandflies has been reported recently (Molina *et al.*, 1999).

The high prevalence of *Leishmania* infection in regions where leishmaniasis is endemic has to be taken into account in any campaign aimed at controlling canine leishmaniasis. In fact, some authors have demonstrated that removing seropositive dogs is an insufficient method to eradicate canine leishmaniasis (Ashford *et al.*, 1993; Dietze *et al.*, 1997).

Vaccines against *Leishmania* are a goal for the scientific community working on human and canine leishmaniasis. Potential vaccines could act by either destroying the parasite and/or preventing disease pathology. While the latter type of vaccine would prevent severe disease and morbidity among immunized dogs, the parasite transmission cycle would remain intact. This is especially important because of the high prevalence of *Leishmania* infection that we have shown, suggesting that asymptomatic dogs act as reservoirs for parasite transmission to sandflies. Thus, potential canine vaccines must

induce sterile immunity, eliminating amastigotes that reside in apparently healthy skin. This type of vaccine would both prevent canine leishmaniasis and result in decreased human disease.

In conclusion, this study demonstrates that the prevalence of *Leishmania* infection in an area of endemicity is higher than assumed, and that the main tissue reserve of the parasite in dogs is the skin. This information is essential for designing and implementing appropriate control measures and must be addressed when evaluating the efficacy of any vaccine.

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Chapter 6

Evaluation of the specific immune response in dogs infected by *Leishmania infantum*

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Abstract

The immune system plays a key role in *Leishmania* infection. Dogs that show clinically patent leishmaniosis have a predominantly humoral, non-protective immune response (T helper-2 like). In contrast, infected dogs, which do not develop the disease display a predominantly cellular, protective immune response (T helper-1 like). Our research was directed to assess the usefulness of immunological techniques in the evaluation of dogs infected by *Leishmania* and also to define the immune profiles of this population of dogs. The following parameters were studied: anti-*Leishmania* IgG1, IgG2, total IgG antibodies, delayed test of hypersensitivity (DTH) to leishmanin, lymphocyte proliferation assay and production of cytokines (gamma-interferon (IFN-γ) and tumor necrosis factor-alpha (TNF-α). We investigated three groups of dogs: (1) Healthy non-infected, (2) Infected without patent disease, and (3) Infected with clinically patent leishmaniosis (before and after treatment). Healthy, non-infected dogs were consistently negative for all assays performed. Infected animals without clinically patent disease showed two different immune-profiles. The majority showed low titres of anti-*Leishmania* antibodies with a positive DTH and a high production of IFN-γ. The remainder showed positive titres of anti-*Leishmania* antibodies with a negative or a weakly positive DTH. Before treatment, ill dogs presented high level of anti-*Leishmania* antibodies (mainly IgG2), negative DTH, no production of IFN-γ but a production of TNF-α. Clinical recovery was associated with a decrease in the titre of antibodies (initially, IgG1 titres were reduced followed by IgG2/total IgG) and also an increase of the diameter of the DTH. The combination of serology, DTH and measurement of cytokines constitutes a useful, clinically relevant method to evaluate the immune response to *Leishmania*.

Introduction

The last few years led to the revision and reformulation of some of the main paradigms of infectious diseases. First of all, the line between what has historically existed between infectious and non-infectious diseases has become somewhat blurred. Infectious agents are now considered as the cause of many diseases that previously had been considered as non-infectious. The role of *Helicobacter pylori* in the pathogenesis of gastric ulcer constitutes a good example (Maconi *et al*., 1999). Secondly, the relationship between infection and disease has become more complex. The simple scheme consisting of contagion, infection and disease has been demonstrated false in most diseases, at least in part. As an example, it has been well demonstrated that not all dogs infected by *Borrelia spp.* develop Lyme disease and that many remain asymptomatic (Greene, 1990). Finally, it seems that in most cases clinical healing is not associated with complete elimination of the infection. When techniques of high sensitivity (for instance PCR) are used to investigate the presence of infectious agents, most patients after treatment and clinical improvement remain infected for a long period after the end of the treatment. The huge advances in molecular diagnostic techniques and in immunology are responsible for these radical changes in the conception of infectious diseases of man and animals.

Canine leishmaniosis serves as an extraordinary example to describe the conceptual changes discussed previously. As in many other infectious diseases, we must alter or replace the old paradigm and begin to discuss and to spread the new paradigm. The major change is the difference between infection and disease. Different authors demonstrated that the rate of infection is much higher than the prevalence of the disease. One communication presented in the World Congress (Solano-Gallego *et al*., 2000b) demonstrates that, similar to the situation in man, only a small portion of dogs infected by *Leishmania* develop the disease that we call leishmaniosis.

Extensive researches done in experimental models suggest that the immune system plays a key role in *Leishmania* infection (Solbach & Laskay, 2000). Dogs showing clinically patent leishmaniosis have a predominantly humoral, non-protective immune response (T helper-2 like). In contrast, dogs which are infected but do not develop the disease have a predominantly cellular, protective immune response (T helper-1 like). Therefore, the evaluation of the type of immune response in a single patient appears to be a key prognostic parameter. Our research was directed to evaluate the usefulness of immunological techniques in the evaluation of dogs infected by *Leishmania* and also to define the immune profiles of these animals.

Material and methods

Animals

Three groups of dogs were studied:

Group 1: 28 healthy non-infected dogs (negative in PCR for *Leishmania* and in serology).
Group 2: 30 infected dogs (positive PCR for *Leishmania*).

Group 3: 25 infected dogs with clinically patent disease leishmaniosis (direct observation of parasite or positive PCR for *Leishmania*) and treated with Glucantime® (Rhone-Merieux) and allopurinol during at least one year.

All dogs included in the study lived in endemic areas and belonged to different breeds and ages.

Serology

ELISA test was performed as described by Solano-Gallego *et al*. (2000a). Briefly, microtitre plates were coated with 20 μg mL^{-1} of *L. infantum* antigen in 0.1 mL of 0.1M carbonate-bicarbonate buffer and incubated overnight at 4°C. One hundred μL per well of dog sera, diluted 1:400 in PBS-0.05% Tween 20 (PBST)-1% dried skimmed milk, were incubated for 1 h at 37°C. After washing, 100 μL per well of anti-dog IgG (1:20000), anti-dog IgG2 (1:5000) and anti-dog IgG1 conjugated to horseradish peroxidase (Bethyl laboratories, Montgomery, TX, USA) were added and incubated for 1 h at 37°C. The substrate solution was ortho-phenylene-diamine. The reaction was stopped with H_2SO_4 3M. Absorbance values were read at 490 nm in an automatic microELISA reader. The cut-off was established at 20U for IgG, 22U for IgG1 and 11U for IgG2 (mean + 4 standard deviations of 32 dogs from non-endemic areas).

Delayed type of hypersensitivity (DTH) to leishmanin

Leishmanin reagent was an inactivated suspension of $3x10^8$ *L. infantum* promastigotes per mL in 0.4% phenol-saline, kindly provided by Dr. Alvar (Instituto de Salud Carlos III, Madrid, Spain). One hundred μL of the solution was intra-dermally injected in the skin of the groin. Skin reactions were recorded after 72 h and an induration or eritematous area >5 mm in diameter was considered positive in agreement with Pinelli *et al*. (1994) and Cardoso *et al*. (1998). Purified protein derivative (2500TU/ml) (Central Veterinary Institute Lelystad, The Netherlands) and leishmanin diluent as well as saline solution (0.1 mL of each) served as controls.

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from heparinized venous blood samples of the different groups of dogs by standard ficoll-hypaque (Histopaque 1.077, Sigma, St.Louis, MO) density gradient centrifugation, as described by Böyum, (1968). The isolated cells were washed three times in phospate-buffered saline (PBS) and were resuspended at a final concentration of $1x10^7$ cells ml^{-1} in supplemented RPMI-1640 (Gibco, Paisley, UK), prior to use in experiments.

Lymphoycte proliferation assay (LPA)

PBMC were cultured in a flat-bottomed 96-well-microtiter plate, at a density of 10^5 cells per well in supplemented RPMI. The cells were incubated (37°C, 5% CO_2) for 3 days with 5μg/mL of phytohemagglutinin (PHA), and for 5 days with 10 μg/mL of leishmanial soluble antigen (LSA) or without antigen and pulsed during the last 8 h with 10 μM of BrdU. Optimal concentrations of antigens and mitogens were determined in kinetic experiments performed prior to the present study. The cell proliferation was

determined using a non-radioactive ELISA technique, according to the manufacturers instructions (Cell proliferation ELISA, BrdU (colorimetric), Boehringer Manheim, Germany). The data are reported, as the mean of optical density (O.D.) (450nm) of triplicate cultures.

Measurement of cytokine production by PBMC after stimulation

PBMC at $1x10^6$cells per mL in complete medium were cultured and incubated with 5 µg/mL PHA, 10 µg/mL LSA and with medium alone. Supernatants were collected after 48 or 72 h centrifuged and stored at –80°C until tested.

Tumor necrosis factor alpha (TNF-α) activity was assessed in cell supernatants using a cytotoxicity bioassay previously described by Brazis *et al*. (2000) employing the TNF-α-sensitive L929 cell line. Briefly, 50 µL/well of 1 x 10^6 L929 cells/mL were seeded into flat-bottom plates and cultured overnight in supplemented RPMI medium. The medium was then replaced with 50 µL/well of fresh medium containing 5 µg/mL of cycloheximide and 100 µg/mL of STI. Mouse recombinant TNF-α was used to construct a standard curve ranging from 2 pg/mL to 2,000 pg/mL. Fifty µL/well amounts of samples and standards were added, and plates were incubated for 18 h at 37°C. Then 10 µL/well of MTT (5 mg/mL) was added, and after a 4 h incubation, 50 µl of a solution of 50% N, N-dimethylformamide and 20% SDS was incubated overnight. The plates were read in an ELISA reader at 570 nm, and results were extrapolated from the standard curve constructed using recombinant murine TNF-α.

IFN-γ activity was assessed in cells supernatants using a bioassay based on the inhibition of the cytopathic effect caused by vesicular stomatitis virus (VSV) on Madin-Darby canine kidney cells, ATCC CCL 34 (MDCK2) previously described by Pinelli *et al*. (1995). In a 96-well flat bottom plate 50 µL of the test supernatant was added in twofold dilutions. The MDCK2 cells at a density of $1x10^5$ cells/well in supplemented RPMI-1640 medium were added incubated overnight at 37°C and 5% CO_2. The following day the supernatants were removed and to the attached cells, 50 µL of VSV at 10^5 pfu/mL was added to each well. After 24h, when untreated infected cells showed complete cytopathic effect, all cells were stained with 50 µL 0.75% crystal violet in 0.9 %formaldehyde for 15 minutes. The IFN-γ activity was expressed as standard units/mL which represent the reciprocal of the maximum dilution protecting 50% of the cell monolayer as estimated visually.

PCR Leishmania

PCR was used to detect the infection by *Leishmania* of all dogs studied. Samples of bone marrow and skin were used. *Leishmania* specific oligonucleotides primers SP176 (5´- TCTTGCGGGGAGGGGGTG-3´) and SP177 (5´-TTGACCCCCAACCACATTTTA-3´) described by (Roura *et al*., 1999), were used to amplify a 120-base pair fragment of *Leishmania* kinetoplast DNA minicircles (Rodgers *et al*., 1990). PCR was conducted as described elsewhere (Roura *et al*., 1999).

Statistical analysis

Differences between groups were evaluated by one way analysis of variance (ANOVA) using a Bonferroni test to establish post-hoc. A P-value of <0.05 was considered significant. All calculations were done using the SPSS statistical analysis package.

Results

Determination of specific anti-Leishmania antibodies (Table 1 and Figure 1)

The majority of group 2 dogs (healthy but infected) were positive to specific anti-*Leishmania* antibodies, but with low levels**.** The average absorbance for IgG, IgG1 and IgG2 was 56.2, 29.8 and 51.3 respectively. The number of positive animals for IgG, IgG1 and IgG2 was 23, 13 and 25; respectively. Dogs of group 3 (sick dogs before the treatment) were clearly positive with all techniques. Titres of total IgG and IgG2 were consistently high in all patients, but IgG1 titres were highly variable. IgG1 titres decreased rapidly with the majority negative one year after the diagnosis. IgG2 and IgG decreased in parallel during the period of the treatment. Differences between groups were statistically significant for all immunoglobulins (P=0.001).

Table 1. ELISA results (mean± SD) for IgG, IgG1 and IgG2 in the three groups studied

Group of dogs	IgG	IgG2	IgG1
1. Non-infected (n=28)	9.9±4.5	6.2±2.2	9±4.4
2. Healthy but infected (n=30)	56.2±62.8	51.3±65.8	29.8±23.7
3. Sick before treatment (n=25)	164±35	156±14	76±77
3. Sick after treatment (n=25)	111±41	121±40	22±18

ELISA values quantified as units (U) related to a positive serum used as calibrator that was considered to have 100 U.

Delayed type of hypersensitivity (DTH) to leishmanin (Table 2)

The majority of group 2 dogs (were positive for DTH to leishmanin (21 out of 30). On the contrary, dogs affected by leishmaniosis were negative but 6 out of 7 the patients become positive after the treatment. Differences between group 2 and group 3 before treatment were statistically significant (P<0.001). The clinical improvement in the treated group runs parallel to the increase in the intradermal reaction to leishmanin.

Table 2. Results of DTH to leishmanin (mean±SD) of the three groups studied.

Group of dogs	DTH to leishmanin (mm)
1. Non-infected (n=15)	0
2. Healthy but infected (n=30)	13.3±9.4
3. Sick before treatment (n=14)	1.1±1.9
3. Sick after treatment (n=7)	10.1±7.4

Lymphocyte proliferation assay (Table 3)

Healthy but infected dogs displayed good proliferative responses that were comparable in both PHA and LSA. Dogs with clinically patent leishmaniosis did not present proliferative response to LSA and showed also a lower proliferative response to PHA, which was not signficant when compared to group 1 (P=0.349) and group 2 (P=0.061). The proliferative response to PHA from group 1 and 2 were similar (P=1). The differences in proliferative responses to LSA between groups were statistically significant (P=0.003).

Table 3. Results of LPA (mean±SD) in the three groups studied

Group of dogs	Unstimulated	PHA	LSA
1. Non-infected (n=7)	0.098±0.034	0.852±0.383	0.070±0.043
2. Healthy but infected (n=7)	0.102±0.086	1.015±0.270	0.756±0.603
3. Sick before treatment (n=8)	0.110±0.056	0.554±0.352	0.108±0.075

Measurement of IFN-γ produced by PBMC after stimulation (Table 4)

PBMC from dogs of group 2 showed a high production of IFN-γ after stimulation with either PHA or LSA. In contrast, PBMC from dogs of group 3, showed a lower production of IFN-γ after stimulation either with PHA or LSA as compared to group 2 (P=0.042; P=0.029, respectively). Differences between groups for PHA and LSA were statistically significant (P=0.043; P=0.012, respectively).

Table 4. Results of IFN (IU/mL) (mean±SD) in the three groups studied

Groups of dogs	Unstimulated	PHA	LSA
1. Non-infected (n=9)	28.9±24.7	137.8±107.9	26.7±26.5
2. Healthy but infected (n=12)	23.3±29.3	176.7±163.1	196.7±221.4
3. Sick before treatment (n=7)	2.9±8	20±20	0
3. Sick after treatment (n=4)	10±11.5	60±23	10±20

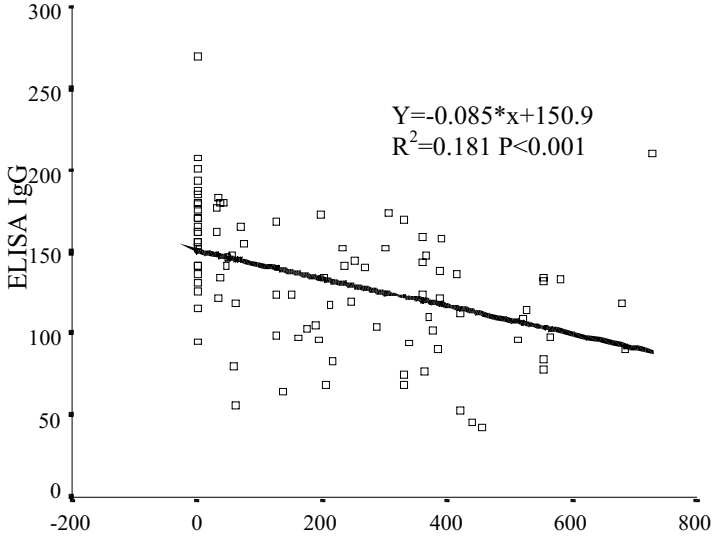
Measurement of TNF-α produced by PBMC after stimulation (Table 5)

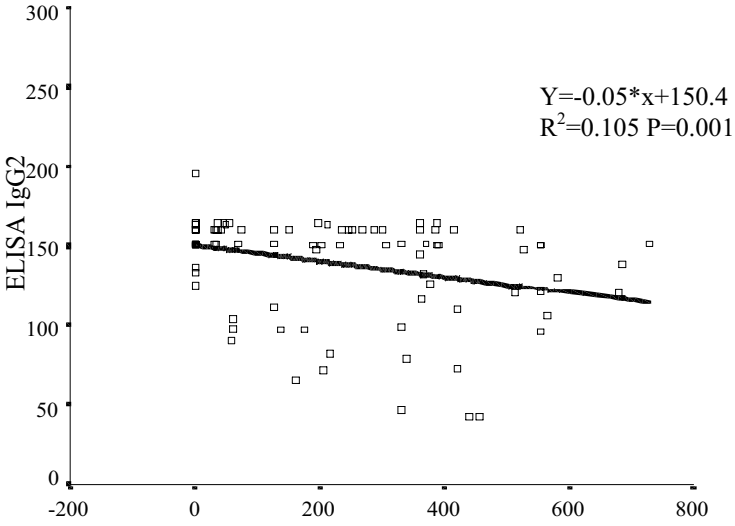
PBMC from all groups showed a similar production of TNF-α after stimulation with PHA (P=0.258). However, the production of TNF-α after stimulation with LSA was significantly higher in group 3 (ill dogs) than group 1 and group 2 (P=0.001; P=0.006, respectively).

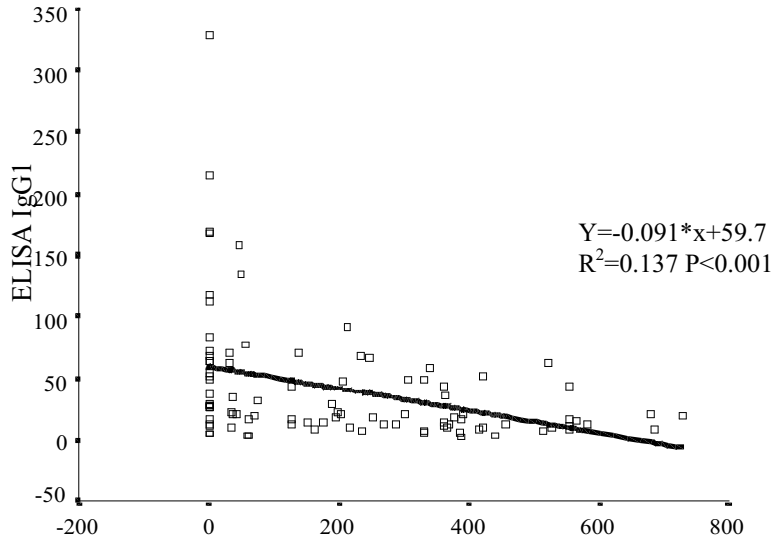
Table 5. Results of TNFalfa (pg/mL) (mean±SD) in the three groups studied.

Groups of dogs	Unstimulated	PHA	LSA
1. Non-infected (n=9)	0	1.4±1.3	0
2. Healthy but infected (n=12)	0.2±0.5	4.7±4	1.6±1.5
3. Sick before treatment (n=7)	0.9±1.2	4±3.8	5.6±3.1

Fig. 1. Anti-*Leishmania* specific IgG, IgG1 and IgG2 antibodies in 25 treated dogs.







Discussion

Based on the results we obtained using these techniques, it is possible to define the pattern of immune response of a single patient. We accomplished these results by combining techniques, which assess the humoral response (serology) with techniques that evaluate the specific cellular immunity (Cabral *et al*., 1998; Rhalem *et al*., 1999a) i.e., DTH or production of IFN-γ by PBMC. In practice, DTH is possibly more accommodating because of the complexity of current techniques available to measure the production of IFN-γ. In the near future, the development of better techniques to measure the production of canine cytokines (i.e., IL-4 and IFNγ) based on ELISAs might allow for easier evaluation of the type of immunity against *Leishmania* occuring in a patient undergoing examination.

Dogs living in an endemic area of leishmaniosis can be grouped in different categories. First, dogs can be divided in asymptomatic (healthy) and ill dogs. Dogs with clinically patent leishmaniosis are infected animals that have an inefficient immune response against *Leishmania* (Pinelli *et al*., 1994). Asymptomatic dogs, however, constitutes a rather heterogeneous group of animals (Cabral *et al*., 1998). This group includes healthy non-infected animals (PCR negative, seronegative, DTH negative, no production of IFN-γ), infected dogs which control the parasite by means of a cellular immune response (PCR positive, low titre of specific IgG antibodies and DTH/IFN-γ positive) and infected dogs which are incubating the disease and that will be in the future symptomatic (PCR positive, seropositive, low DTH and low production of IFN-γ). It is necessary, as stated before, to use several techniques to properly classify a single patient in one of the four groups.

Dogs affected by canine leishmaniosis show severe immunologic abnormalities. They present a predominantly humoral immune response characterised by the production of high amounts of specific anti-*Leishmania* IgG2 antibodies and a variable production of IgG1antibodies. The cellular immune response against Leishmania, however, is very weak or absent in these patients, as demonstrated by the low DTH, the low production of IFN-γ and the lack of proliferative responses of PBMCs. These results are similar to those described by (Pinelli *et al*., 1994) in dogs experimentally infected and indicate that these animals present a Th2 helper like immune response (non-protective and permitting a wide dissemination of the parasite). The production of TNF-α by PBMCs of these patients was elevated and highly variable. It is difficult to give a unique interpretation to this finding. TNF-α is a pro-inflammatory cytokine that provides a rapid form of host defence against infection (Nacy *et al*., 1991) but which also has many undesirable effects on the host (Blackwell, 1999). High production of TNF-α could play a role in the protection against *Leishmania* but could also be responsible of some of the clinical signs and lesions of leishmaniosis such as cachexia, fever, or weight loss as have also been reported in humans (Da-Cruz *et al*., 1996; Morsy *et al*., 1995; Pearson *et al*., 1992; Ribeiro-de-Jesus *et al*., 1998). But such results must be interpreted with caution because these dogs appear to be immudepressed as demonstrated by the low proliferative responses of PBMCs when stimulated by PHA. Some authors have previously described the anergic state to mitogens of dogs suffering from leishmaniosis (De Luna *et al*., 1999; Moreno *et al*., 1999) and have also found low figures number of blood $CD4^+$ cells (Moreno *et al*., 1999).

The treatment has a noticeable effect on the immune response of ill dogs. However, the effect is highly variable and difficult to predict. Some patients show a decrease in the titre of IgGs which runs parallel to the recovery of the cellular immunity and to the clinical recovery (Rhalem *et al*., 1999b). Other dogs, however, retain the humoral immune response and fail to recover the cellular immunity; these are the patients with the worst prognosis. Probably, as it has been demonstrated in *Leishmania major* infection in mice (Nabors *et al*., 1995), the treatment due to a reduction of the parasite burden could be the inductor for a switch from Th2 to Th1-like immune response. Obviously, many factors influence this evolution of the immune response during treatment, as type and duration of the treatment, concomitant infections or diseases, re-infections, and the status of the cellular immune response at the beginning of the treatment.

As expected, non-infected dogs were completely negative to all techniques performed. However, they were immunocompetent as their PBMCs respond with an intense proliferation when stimulated with mitogens (PHA).

Healthy, infected dogs can be divided also into two groups: (1) Dogs that clearly presented a specific-*Leishmania* cellular immunity demonstrated by a positive DTH, proliferation and production of IFN-γ and low specific-*Leishmania* humoral immunity and (2) Those animals that failed to have a specific-*Leishmania* cellular immuntiy and instead they showed only humoral immunity. The appearance of resistant dogs was reported in the last decade demonstrating cellular immunity in naturally and experimentally infection and associating this cellular immunity as Th1 like immune response (Cabral *et al*., 1992; Pinelli *et al*., 1994). However, the term resistant must be interpreted with caution as the parasites probably remain in these individuals in equilibrium without producing pathology. However, this is not an unchangeable situation and several factors can break the equilibrium and lead to clinical disease. This situation is similar to that of human (Schubach *et al*., 1998a; Schubach *et al*., 1998b) and animals (Aebischer *et al*., 1993; Belkaid *et al*., 2000; de Rossell *et al*., 1992) after healing, which persist infected but clinically healthy during long periods of time. The mechanisms of persistence of the parasite are currently being investigated (Bogdan *et al*., 1996; Bogdan & Rollinghoff, 1998). Some authors have hypothesised that *Leishmania* could persist in low nitric oxide producing cells as fibroblasts (Bogdan *et al*., 2000).

The integration of data obtained in broad epidemiological and immunological studies (Berrahal *et al*., 1996; Cabral *et al*., 1998; Solano-Gallego *et al*., 2000a; Solano-Gallego *et al*., 2000b), are changing the understanding of canine leishmaniosis. Infection and disease are no longer synonymous and the diagnosis has to be established by combining several techniques. Diagnosis and prognosis can be accurately established if, together with the detection of the parasite, the type of immune response of the patient, humoral *versus* cellular, is evaluated.

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