Chapter 7

Discussion

Introduction

As discussed previously, there has been a change of attitude concerning the perception of *Leishmania* infection in dogs. Infection and disease are no longer considered synonymous and there exists a wide range of immune responses of infected dogs living in endemic areas. Our findings, and others, have corroborated that *Leishmania* infection in the mammalian host is complex and we are long away from a complete understanding of it. We hope that this thesis will help to clarify some unresolved issues and also bring some new ideas about this infection.

We have grouped the discussion in four parts: 1) humoral immunity, 2) *Leishmania*-specific cellular immunity, 3) the spectrum of immunoresponses and immunoprofiles, and 4) prevalence of *Leishmania* infection, seroprevalence and prevalence of leishmaniosis.

Immunology

Humoral immunity

Humoral immunity has been studied extensively in infectious diseases. The development of highly sensitive and specific serologic methods (ELISA, IFA) coupled with the specific features of serum (ease in obtaining and storaging) has led to a greater understanding of antibody responses to infectious agents. During the last 20 years, canine leishmaniosis has been studied mainly through serology to determine the level of antibody production manifested during the disease. However, in the last decade the interpretation of specific anti-*Leishmania* antibodies has become more complex because the presence of anti-*Leishmania* antibodies *per se* does not correlated directly with disease progression (Gicheru *et al*., 1995; Nieto *et al*., 1999). Epidemiological studies have reported that the level of endemicity is directly related to the high number of dogs with a low titre of specific antibodies which reflect disparate clinical-immunological situations such as the early phases of infection or the natural resistance of the host (Abranches *et al*., 1983; Dye *et al*., 1992; Fisa *et al*., 1999). Sera with titres above the cutoff are rarely found when analyzing animals from non-endemic areas (Seguí, 1991).

Our work focused on the main immunoglobulin, IgG, produced in canine leishmaniosis (Abranches *et al*., 1991; Martínez-Moreno *et al*., 1995). We studied the production of specific total IgG and its subclasses (IgG1 and IgG2) in a large canine population. The results are described in chapters 2, 4, 5 and 6.

Various studies have described the levels of specific *Leishmania* IgG subclasses (IgG1 and IgG2) in ill, asymptomatic and treated dogs, sometimes with conflicting results (Bourdoiseau *et al*., 1997; Deplazes *et al*., 1995). The varying immunoresponses of infected animals and the small number of animals studied may account for this controversy. Our studies of several large cohorts of dogs have helped to clarify this aspect of the disease. The levels of total IgG and IgG2 were highly correlated in all groups studied (asymptomatic, symptomatic and naturally and experimentally infected treated dogs) and, as expected, they were significantly higher in symptomatic than in asymptomatic dogs. However, the correlation between total IgG and IgG1 was low (chapter 2).

In naturally and experimentally infected symptomatic dogs, we found that total IgG and IgG2 were high, as reported by previous authors (Bourdoiseau *et al*., 1997; Cavaliero *et al*., 1999; Deplazes *et al*., 1995), whereas IgG1 levels were extremely variable, ranging from background to high levels. Cavaliero and coworkers (1999) reported that IgG1 concentrations at the beginning of treatment were not as high as described by other authors (Deplazes *et al*., 1995). One report postulated that the levels of specific-*Leishmania* IgG1 and IgG2 antibodies could be prognostic indicators of cure and disease. IgG1 was associated with the development of the disease while IgG2 was associated with asymptomatic infection (Deplazes *et al*., 1995). Our results showed the presence of high levels of anti-*Leishmania* IgG2 antibodies in all severely affected animals. Thus, in contrast with the results previously described (Deplazes *et al*., 1995), the presence of high levels of IgG2 is not a marker of asymptomatic infections (chapter 2).

The high levels of specific anti-*Leishmania* IgG may be related to the dissemination of the parasite both in symptomatic and asymptomatic dogs because we detected parasite DNA in bone marrow, conjunctiva and skin samples in those animals and not in others having low or background levels (chapter 5). In addition, Zerpa and coworkers (2000) found positive *Leishmania* PCR results from bone marrow, lymph node and spleen in most dogs (79%) with strong serological reactivity.

Treatment of ill dogs induces clinical improvement, often accompanied by a decrease in the specific antibody levels (Fernández-Pérez *et al*., 1999; Lanotte *et al*., 1979; Mancianti *et al*., 1988; Riera *et al*., 1999) and a significant increase in the percentage of $CD4^+$ T cells and a decrease of γδ T-cells and $sIgG^+$ B-cells (Moreno *et al*., 1999). However, in other cases clinical improvement has not been associated with a decrease in the titre of specific antibodies (Ferrer *et al*., 1995). In our study (chapter 2), anti-parasite IgG and IgG2 levels decreased but very slowly while IgG1 levels dropped quickly in responsive dogs after extended treatment (Deplazes *et al*., 1995), whereas the decrease in unresponsive dogs was less marked or only temporary. In addition, IgG1 levels at diagnosis were significantly higher in responsive than in unresponsive dogs. Furthermore responsive dogs also showed a larger significant decrease in all immunoglobulins, similar to that observed in experimentally infected dogs. Thus, a low levels of IgG1 after treatment is only a good prognostic indicator if dogs presented high levels of this immunoglobulin before chemotherapy.

In our studies on the asymptomatic population, dogs with positive DTH reactions had polymorphic humoral immune responses ranging from background to medium levels of IgG1, IgG2 or total IgG. An association between DTH reaction and either of the IgG subclasses or total IgG was not found (chapter 4). We found asymptomatic seronegative dogs for IgG that had variable IgG1 responses. We were unable to explain the cause of this finding (possibly a crossreaction with other proteins or an early phase of the infection). On the contrary, asymptomatic seropositive animals for IgG had a low IgG1 response. This low IgG1 response remained throughout the infection even when IgG and IgG2 responses significantly increased in asymptomatic dogs that seroconverted (chapter 2). As our studies have shown (chapters 2, 4 and 5), antibody IgG production is not synonymous with illness but is indicative of exposure to the parasite. In contrast, the absence of antibodies is not equated with non-infection as demonstrated by the fact that seronegative dogs presented parasite DNA in some tissues, mostly skin and conjunctiva.

In human studies, the *Leishmania* protective IgG subclasses are not well defined. Rodríguez and coworkers (1996) reported levels of parasite specific IgG1, IgG2, IgG3 and low levels of IgG4 in cutaneous leishmaniosis. Skeiky and coworkers (1997) reported elevated levels of IgG4 in diffuse cutaneous leishmaniosis. In visceral leishmaniosis, high levels of all four IgG subclasses are described (Anam *et al*., 1999a; Chatterjee *et al*., 1998; Shiddo *et al*., 1996). In humans, IgG1 and IgG3 can fix complement, while IgG2 is less effective and IgG4 does not fix complement at all (Brekke *et al*., 1995).

Cell-mediated immunity undoubtedly represents the primary mechanism of resistance to *Leishmania* infection, and there seems to be sufficient evidence to suggest that a humoral mechanism may constitute indispensable elements of an effective protective response in human beings (Ulrich *et al*., 1996) and dogs. However, the contribution of antibodies and complement in a protective role in leishmaniosis, possibly via mechanisms of lysis of some *Leishmania* species and the ability to enhance macrophage mediated parasite killing, requires further study.

The predominance of specific IgG isotypes should be carefully studied to determine any association with Th1- or Th2-like activity. The immunological mechanisms that regulate the susceptibility or resistance to visceral parasitism by *Leishmania* are still unclear. The polarized Th1-Th2 response reported in the infection by *L. major* in mice (Locksley & Reiner, 1995; Reed & Scott, 1993) is not so evident as it is in human visceral leishmaniosis (Anam *et al*., 1999b; Mary *et al*., 1999), in *L. infantum*-infected mice (Honoré *et al*., 1998; Kaye *et al*., 1991; Miralles *et al*., 1994), or in *L. infantum*-infected dogs (Cabral *et al*., 1998). A mixed Th1-Th2 response or other T-cell clones may possibly be involved. Other accessory cells and signals, such as $CD8^{+}$T cells, diverse antigen-presenting cells, variable interleukin interactions and chronicity may be key factors in the modulation of the IgG isotype response in dogs.

The main findings regarding the humoral response are:

1. Antibody IgG response is not synonymous with illness but is indicative of exposure to the parasite. In contrast, the absence of antibodies is not equated with non-infection as demonstrated by seronegative dogs that present parasite DNA in some tissues, mostly skin and conjunctiva.
2. Asymptomatic dogs with positive DTH reactions have polymorphic humoral immune responses ranging from background to medium levels of IgG1, IgG2 or total IgG. An association between DTH reaction and either of the IgG subclasses or total IgG was not found.
3. Asymptomatic, seronegative dogs for IgG have variable IgG1 responses, but asymptomatic, seropositive animals for IgG have low IgG1 responses. This low IgG1 response remains throughout the infection even when IgG and IgG2 responses significantly increase in asymptomatic dogs that seroconvert.
4. The high levels of specific anti-*Leishmania* IgG may be related to the dissemination of the parasite in symptomatic and asymptomatic dogs demonstrated by the detection of parasite DNA in bone marrow, conjunctiva and skin samples in those animals and not in others having low or background levels.
5. In ill dogs, the levels of specific anti-*Leishmania* IgG and IgG2 antibodies are very high, and the levels of specific anti-*Leishmania* IgG1 antibodies are highly variable.
6. The main IgG subclass that plays a role in patent canine leishmaniosis is IgG2. High levels of IgG2 are not markers of asymptomatic infections.
7. The IgG2 levels are correlated with total IgG. IgG1 levels are not correlated nor with IgG2 levels neither total IgG levels.
8. The mean levels of total IgG, IgG2 and IgG1 significantly decrease in ill dogs after extended treatment. Anti-*Leishmania* IgG and IgG2 levels decrease slower than IgG1 levels which drop more quickly. In responsive-treated animals the decrease of all immunoglobulins is significant more notable than in unresponsive-treated dogs. A low level of IgG1 after treatment is only a good prognostic indicator if dogs presented high levels of this immunoglobulin before chemotherapy.

Leishmania-specific cellular immunity

In the last few years, the knowledge gained from studying *L. major* infection in mice has led to the discovery of the existence of protective *Leishmania* specific cellular immunity in the dog. Different methods for determing the cellular immune response in humans and mice such as LST — an *in vivo* test— and LPA or IFN-γ production by PBMC —*in vitro* tests— are commonly used. The same methods have been applied recently to the dog (Abranches *et al*., 1991; Cabral *et al*., 1992; Cabral *et al*., 1998; Cardoso *et al*., 1998; Martínez-Moreno *et al*., 1995; Pinelli *et al*., 1995; Pinelli *et al*., 1994).

In this thesis, we used several techniques to detect cellular immunity in the dog: LST, LPA and IFN-γ production. We found these three methods useful in detecting a cellular immune response in healthy, infected dogs. But we also found, in agreement with other authors, that dogs with patent canine leishmaniosis failed to demonstrate cellular immunity (Cardoso *et al*., 1998; Moreno *et al*., 1999; Pinelli *et al*., 1995; Pinelli *et al*., 1994; Rhalem *et al*., 1999a).

We studied the LST technique before we were ready to use it in our experiments. To better ensure accuracy and standarization of the LST, in detecting cellular immunity, we ran parallel experiments using two different preparations. The details are described in chapter 3. The results can be summarized as follows: 72 hr was the optimum time to take readings in dogs and the best concentration of promastigotes to detect cellular immunity in the dog was $3x10^8$ promastigotes per mL. After that, we used the above conditions in our studies (chapters 4 and 6). LST has proven to be a useful tool in detecting those dogs with a detectable cellular immune response. While at the same time, sick dogs failed to have a detectable cellular immune response because of the anergic state that these animals presented as described in previous studies (Cardoso *et al*., 1998; Pinelli *et al*., 1994). In treated dogs, the clinical improvement runs parallel to the increase in the LST as we describe in chapter 6. Based on our results, we believe that LST should be used, in veterinary medicine, for both diagnostic and prognostic requirements.

Proliferation of canine lymphocytes is usually measured by the ^{3}H-thymidine incorporation assay. However, this technique has a number of disadvantages, including the need for expensive, special equipment and the production of radioactive waste. The method we used —the BrdU assay— (Huong *et al*., 1991; Magaud *et al*., 1988) was better suited to our needs. It is based on the incorporation of BrdU as pyrimidine analogue in place of thymidine into newly synthesized DNA of proliferating cells. BrdU is subsequently detected by a monoclonal anti-BrdU-antibody. Recently, Wagner and coworkers (1999) demonstrated that the BrdU method is the most suitable nonradioactive alternative for the measurement of proliferation of canine lymphocytes. Therefore, the absolute values of both methods are comparable only with respect to their tendency due to ^{3}H-thymidine incorporation is evaluated as counts per minute in a scintillation counter and the incorporation of BrdU is measured by OD spectrophotometrically. This fact must be taken into account when we compare studies of LPA measured by these different incorporation assays. At present, there has not been any study that compares BrdU and ^{3}H-thymidine assays with respect to the efficacy of each assay in detecting *Leishmania*-specific cellular immunity in the dog.

IFN-γ production, measured by bioassay, also seems to be a sensitive method to detect cellular immunity. In our study (chapter 6), we obtained satisfactory results comparable to the findings of Pinelli and coworkers (1995). However, this technique is tedious and time-consuming. We must develop easier, field deployable, techniques that allow researchers to detect canine IFN-γ when working with a large number of dogs.

There are not studies regarding which cellular immunity detection method is best suited to analyze different groups of animals living in endemic areas. We need to clarify which technique is the most sensitive method to detect cellular immunity in the dog. It must be said that LST — an *in vivo* test— is involved with both localized and generalized immune responses. Conversely, LPA or IFN-γ production —*in vitro* tests— are involved with only generalized immune responses.

Finally, the presence or absence of *Leishmania*-specific cellular immunity must be considered in conjunction with serology to give the researcher a complete picture of the dynamics that are occuring between both responses. Both humoral and cellular immunity detection methods are useful tools in broad epidemiological and immunological studies as well as diagnostic and prognostic tools for patient care.

The main findings regarding *Leishmania*-specific cellular immunity are:

1. LST, LPA (BrdU assay) and IFN-γ production are useful methods to detect *Leishmania* cellular immunity in the dog.
2. In treated dogs, the clinical improvement runs parallel to the increase in the DTH reaction
3. LST can be used as a valuable tool for diagnosis and prognosis in veterinary medicine.
4. Cellular immunity methods are useful tools in broad epidemiological and immunological studies and in the diagnosis and prognosis for the individual patient.

The spectrum of immune responses and immunoprofiles

The widely held opinion that dogs invariably occupy the anergic pole of the leishmanial disease spectrum (Ferrer *et al*., 1988b; 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 suggest that canine leishmaniosis may display the wide disease spectrum similar to that seen in human infection where clinical disease represents one pole and asymptomatic infection the other pole (Badaró *et al*., 1986). Our results about the wide range of immune responses are detailed in chapters 4 and 6.

Our first proposal (chapter 4) was to investigate the spectrum of immune responses in a canine population living in an endemic area. According to our results, 48% of dogs from different breeds (n=25) living in an endemic area are positive by DTH, either alone (33%) or associated with anti-*Leishmania* IgG antibody production (67%). The remaining 52% (negative for LST) included dogs, which have not been previously infected (28%) and infected dogs with an exclusive humoral immune response (24%), which probably will develop clinical disease in the future. These results are in agreement with those obtained by Cabral and coworkers (1998). Our study design was similar to that of Cabral and coworkers (1998) study except they used LPA instead of LST to detect specific cellular immunity. Cabral and coworkers (1998) also found that 40% of dogs (n=49) living in endemic areas display a cellular immune response, either alone (40%) or associated with a humoral response (60%). The remaining 60% (negative in LPA) included dogs, which had not been infected (35%) or infected dogs with an exclusive humoral immune response (25%).

According to our results, dogs living in an endemic area can be divided into two groups: sick or 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, the spectrum of immune responses goes from resistant dogs that present a predominantly cellular response to sick dogs that present a predominantly humoral response.

Results obtained from the Ibizian hound group suggests that this breed constitutes a special group of dogs with respect to their immune response to *Leishmania* infection. Most dogs (25 out of 31, over 81%) showed a positive DTH, either alone (40%) or

associated with a humoral response (60%). Six dogs that were negative to the DTH were also negative serologically for IgG, which might indicate they have not been exposed to the parasite. A statistically significant association was found between Ibizian hounds and a positive DTH reaction. However, dogs of other breed that were positive for DTH were as intense as the DTH reaction of Ibizian hounds, suggesting that dogs of other breeds are as capable of responding to *Leishmania* infection as are Ibizian hounds. But as a group, the Ibizian hound responds more uniformly with a positive DTH reaction. Consequently, we consider the Ibizian hound more *Leishmania* resistant than other canine breeds.

Our second approach as described in chapter 6 was to study several techniques to define immunoprofiles for the four groups mentioned above. The techniques we used were serology, LST, LPA and IFN-γ production. In summary, dogs with clinically patent leishmaniosis are infected animals that have an inefficient immune response to *Leishmania* characterized by the presence of humoral response and the absence of cellular immune response (Abranches *et al*., 1991; Martínez-Moreno *et al*., 1995; Pinelli *et al*., 1994). Asymptomatic dogs, however, constitute a rather heterogeneous group of animals. Our studies suggest that this group includes (1) healthy non-infected animals (seronegative, DTH negative, no production of IFN-γ and absence of specific lymphoproliferative responses); (2) infected dogs which control the parasite by means of a cellular immune response (low or medium titre of specific IgG antibodies, DTH/IFN-γ positive and good lymphoproliferative responses); and (3) infected dogs which, probably, will be symptomatic in the future (variable levels of specific IgG antibodies, low DTH and low production of IFN-γ and absence of specific lymphoproliferative responses). It is necessary to use several techniques to properly classify a single patient into one of the four groups.

Asymptomatic infected dogs can be divided into two groups: dogs that demonstrated a *Leishmani*a-specific cellular immunity, and dogs that failed to have a *Leishmania*-specific cellular immunity and instead showed only humoral immunity. De Luna and coworkers (1999) found that seropositive dogs with the absence of specific cellular immunity were immunodepressed early in the course of infection as demonstrated by a significant reduction in PBMC activation by mitogens. The appearance of resistant dogs reported in the last decade demonstrates that cellular immunity in naturally and experimentally infection is associated with a Th1 like immune response (Cabral *et al*., 1992; Pinelli *et al*., 1995; Pinelli *et al*., 1994). However, the term resistant must be interpreted with caution as the parasites probably remain in such individuals in a state of equilibrium without producing pathology. The persistence of a parasite load could be one mechanism that permits the constant stimulation of memory T-cells leading to protection from reinfection (Aebischer *et al*., 1993) that may be occurs each season to dogs living in endemic areas. T-cell memory would be short-lived in the absence of antigen (Gray & Matzinger, 1991). This would be a situation similar to that seen in humans (Schubach *et al*., 1998a; Schubach *et al*., 1998b) and mice (Aebischer *et al*., 1993; Belkaid *et al*., 2000; de Rossell *et al*., 1992; Nicolas *et al*., 2000), which remain infected but clinically healthy during extended periods of time. The mechanisms leading to the persistence of the parasite are currently being investigated (Bogdan *et al*., 1996; Bogdan & Röllinghoff, 1998; Bogdan & Röllinghoff, 1999). Some authors have demonstrated that *Leishmania* can persist in low nitric oxide producing cells such as fibroblasts (Bogdan *et al*., 2000). In dogs, the *Leishmania* parasite has been described inside fibroblasts in vivo (Ferrer *et al*., 1988a; Hervas-Rodríguez *et al*., 1996).

However, this situation is not necessarily permanent and factors such as immunosupression, for instance, HIV-coinfected patients (Alvar *et al*., 1997), could break the equilibrium and lead to the progression of clinical disease.

Dogs affected by canine leishmaniosis show severe immunological abnormalities. They present a predominantly humoral immune response characterized by the production of high amounts of specific anti-*Leishmania* IgG2 antibodies and a variable production of IgG1 antibodies. The cellular immune response against *Leishmania*, however, is very weak or absent in these patients, as demonstrated by a negative DTH, a low IFN-γ production and a lack of specific proliferative responses of PBMCs. These results are similar to those described by Pinelli and coworkers (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). However, whether a Th2 type immune response *stricto senso* occurs in dogs infected with *L. infantum* remains unknown. The lack of tools to measure canine Th2 type cytokines is a limiting factor. First of all, accurate and easy to use tools for determining canine IL-4 and IL-10 must be developed. Furthermore, dogs appear to be immunodepressed 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 levels of blood $CD4^+$ cells (Moreno *et al*., 1999).

The production of TNF-α by LSA-stimulated PBMCs from sick dogs was elevated and highly variable (chapter 6). Pinelli and coworkers (1994) failed to detect TNF-α activity in supernatants from LSA-stimulated PBMC but detected TNF-α activity in supernatants from Con A-stimulated PBMC. The TNF-α activity was significantly decreased in supernatants from symptomatic compared to asymptomatic or non-infected dogs. They postulated that the results suggest a possible role of TNF-α in resistance against *L. infantum*. In our case, it is difficult to give such a definitive role to the TNF-α activity. TNF-α is a pro-inflammatory cytokine that provides the host a defense against infection (Nacy *et al*., 1991) but which also causes many undesirable effects to the host (Blackwell, 1999). High production of TNF-α could play a role in the protection against *Leishmania* but it could also be responsible of some of the clinical signs and lesions of leishmaniosis such as cachexia, fever, or weight loss as have been reported in humans (Da-Cruz *et al*., 1996; Medeiros *et al*., 1998; Morsy *et al*., 1995; Pearson *et al*., 1992; Ribeiro-de-Jesus *et al*., 1998).

Treatment has a noticeable effect on the immune response of ill dogs. Nevertheless, 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 cellular immunity, and to clinical recovery (Rhalem *et al*., 1999b). Other dogs, however, retain the humoral immune response and fail to develop the cellular immunity. These are the patients with the worst prognosis. Several studies indicate that *Leishmania* may use different strategies to evade induction of macrophage function and to establish infection. Possibly, treatment allows a healing phase to occur that restores protective immunomechanisms such as T cell-mediated immunity (Gazzinelli *et al*., 1998). As has been demonstrated in *Leishmania major* infection in mice (Nabors *et al*., 1995; Nabors & Farrell, 1996), treatment leading to a reduction of the parasite burden might be the inductor of a switch from Th2 to Th1-like immune response. Obviously, many factors influence the evolution of the immune response during treatment, as type and duration of treatment, other coadjuvant therapies,

concomitant infections or diseases, re-infections, and the status of the humoral and cellular immune response at the beginning of the treatment.

In summary, the techniques of serology, LST, LPA and IFN-γ production can discriminate between dogs living in endemic areas thus permitting their classification into one of the four groups: ill, non-infected, resistant and dogs that will develop the disease over a variable time period. All these methods can give accurate information for the diagnosis and prognosis of a single patient.

The main findings regarding the spectrum of immune responses and immunoprofiles are:

1. A whole spectrum of immune responses exists from resistant dogs that present a predominantly cellular response to sick dogs that present a predominantly humoral response.
1. Half of dogs living in endemic area present specific cellular immunity, either alone (40%) or associated with a humoral response (60%). Twenty five percent of dogs present only specific humoral and 25% are non-infected dogs.
2. The Ibizian hound breed responds more consistently with positive LST and variable levels of anti-*Leishmania* IgG antibodies than other breeds. The Ibizian hound can be considered more *Leishmania* resistant than other canine breeds.
3. The techniques of serology, LST, LPA and IFN-γ can distinguish between dogs living in endemic areas thus permitting their classification in one of four groups: ill, non-infected, resistant and dogs that will develop the disease over variable period of time. All these methods can give accurate information for the diagnosis and prognosis of the single patient.

Epidemiology

Prevalence of infection, seroprevalence and prevalence of disease

A given force of infection in a canine leishmaniosis endemic area results in an incidence rate of new cases after each transmission season. Due to the long incubation period and chronic features of the disease, a cross-sectional study reveals the number of infected individuals (prevalence), which represents the cumulative incidence rate of several seasons, minus death and recovery rates. Such information on prevalence is usually easier to obtain than incidence and can provide an indirect evaluation of force of infection in endemic foci (Gradoni, 1999; Toma *et al*., 1999).

Our two studies about prevalence of infection were conducted on the Balearic Island of Mallorca. The results are described in chapters 4 and 5. The first study performed, described in chapter 4, concerns serology and LST in an asymptomatic population. The seroprevalence was 51% (29 out of 56). This result was in the high range compared to other studies performed in the Mediterranean basin (Bettini & Gradoni, 1986) but close to the seroprevalence (49%) reported by Cabral and coworkers (1998) in Portugal.

However, when the rate of infection was calculated using all animals that were seropositive and/or positive in LST, the percentage was higher at 77%.

Once we had the results of the first study, we decided to study a larger population with other methods to complement our initial findings. Our approach was to investigate serology and *Leishmania* parasite DNA on several tissues (bone marrow, conjunctiva and skin) in 100 dogs. The results from our second work are described in chapter 5 and include a prevalence of canine leishmaniosis in Mallorca of 13% and a seroprevalence of 26%. 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) and similar, but slightly higher, than the seroprevalence (14%) previously reported in the Island of Mallorca (Matas & Rovira, 1989). The prevalence of infection, 67%, was calculated by adding all animals that were seropositive and/or positive for PCR in any tissue. The prevalence of infection that we found in Mallorca (67%) was very high and similar to the figure we obtained previously (77%). Thus, our studies confirm that the prevalence of *Leishmania* infection in dogs living in endemic areas has been underestimated.

According to these results, it is important to distinguish different types of prevalence of canine leishmaniosis:

Prevalence of the disease
The rate of dogs showing overt clinical signs of leishmaniosis. This figure underestimates the infection burden in dog populations as reported by our studies and others (Brandonisio *et al*., 1992; Fisa *et al*., 1999).

Seroprevalence
The detection of anti-*Leishmania* antibodies is the simplest and most common method used for the determination of *Leishmania* infection. Seroprevalence can be regarded as an intermediate measure between the prevalence of disease and the prevalence of infection (Gradoni, 1999). Serology can reveal a proportion of asymptomatic carriers, which represents approximately half of all seropositive animals (Fisa *et al*., 1999). A limitation of this method is that there is a serological latency period and antibodies are often not detectable until several months after infection (Dye *et al*., 1993; Gradoni *et al*., 1988). Furthermore, there is evidence that a proportion of seropositive dogs (10-30%) spontaneously convert to seronegative (Acedo-Sánchez *et al*., 1998; Fisa *et al*., 1999; Zaffaroni *et al*., 1999) and that some animals do not develop specific antibodies after infection (Cabral *et al*., 1998; Cardoso *et al*., 1998; Pinelli *et al*., 1994). Variations in seroprevalence as high as ±60% have been detected in the same group of dog depending on the month studied (Acedo-Sánchez *et al*., 1998). Consequently, the seroprevalence rates are not uniform over time. These changes may be due to human intervention, such as mass drug treatment, culling or the displacement of positive dogs (Gradoni *et al*., 1988), or to the natural dynamics of parasite transmission (changes in vectors populations) (Killick-Kendrick, 1990; Killick-Kendrick & Killick-Kendrick, 1999), or to both.

Prevalence of infection
This metric is the rate of dogs harboring *Leishmania*. Until now, three methods have been used to estimate the prevalence of infection in conjuntion with serology: the

demonstration of *Leishmania*-specific cellular immunity, the detection of parasite and the demonstration of leishmanial DNA.

Cabral and coworkers (1998), using LPA and serology, found 65% of asymptomatic dogs (n=49) living in Portugal had evidence of a specific response to *Leishmania*, whereas the seroprevalence was 49% by ELISA and cellular immunity was 40%. Our results, using serology and LST in an asymptomatic dog population in Mallorca are similar to the results mentioned above. In humans, studies carried out in the Mediterranean basin have reported high rates (60%) of *Leishmania* infection in asymptomatic populations by checking cellular responses such as LST (Marty *et al*., 1992) or IFN-γ production (Meller-Melloul *et al*., 1991). Other study showed lower rates (10%) of infection by the detection of humoral responses and leishmanial DNA in human blood donors (Le Fichoux *et al*., 1999).

Parasite culture from bone marrow seems not to be a useful method to detect infection in dogs (Cabral *et al*., 1993; Cardoso *et al*., 1998; Zerpa *et al*., 2000). The polymerase chain reaction (PCR) using different primers has developed to demonstrate the presence of leishmanial DNA in samples of dogs (Ashford *et al*., 1995; Berrahal *et al*., 1996; Mathis & Deplazes, 1995; Reale *et al*., 1999; Roura *et al*., 1999). A survey performed using PCR on skin and conjunctiva samples (n=30) and immunoblotting techniques found that most dogs (80%) living in southern France had been exposed to *Leishmania* (Berrahal *et al*., 1996), whereas seroprevalence was only 6.6%. Our study (chapter 5) found seroprevalence to be 26% and the percentage of positive PCR on several tissues (bone marrow (17%), conjunctiva (32%) and skin (51%)) also demonstrated that *Leishmania* infects the majority of dogs living in endemic areas (63%). Moreover, a parasitological survey of wild red foxes (n=67) in Granada (Spain) demonstrated recently by detection of *Leishmania* DNA in the spleen a rate of infection of 74% (Criado-Fornelio *et al*., 2000). This implies that other mammals living in areas of endemicity can also play an important role in the transmission of the parasite. However, Gradoni, (1999) suggests that there is a risk in overestimating the prevalence of infection if transient, self-limiting infections are accounted for.

In our study (chapter 5), the low percentage of positive bone marrow PCR (17%) suggests that a hematogenous dissemination to bone marrow takes place only in part of the animals, which usually are also seropositive (82%). Zerpa and coworkers (2000) found similar results when they reported 60% positive PCR from bone marrow samples in dogs with strong antibody reaction. Consequently, the detection of *Leishmania* DNA in bone marrow 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 superior diagnostic method compared to serology (Ashford *et al*., 1995).

Half of the dogs studied were positive to parasite DNA detection in the skin. This indicates that the skin is the major tissue reserve of parasites in dogs and that PCR in skin is a sensitive method to detect infection. This finding is in concert with the known 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 sand flies take their blood meal (Killick-Kendrick & Killick-Kendrick, 1999).

In addition to establishing infection, *Leishmania* parasites must replicate to attain sufficiently high levels to persist in vertebrate host tissues in order to favor the

encounter with their Phlebotomine vector (Gazzinelli *et al*., 1998). The capacity of the parasite to establish persistent infection as a means of achieving its transmission, and consequently maintain its life cycle, is a process common to many parasites (Bogdan *et al*., 1996; Bogdan & Röllinghoff, 1999). Recently, some studies in *L. major* infection in mice have re-examined the basic relationship among parasite growth, persistence, dissemination, lesion formation and immunity (Belkaid *et al*., 2000; Kamhawi *et al*., 2000; Nicolas *et al*., 2000). Belkaid and coworkers (2000) have revealed, in a mice natural model of *L. major* infection, two distinct phases in the pathogenesis of cutaneous leishmaniosis. A remarkably silent phase, lasting 4-6 weeks, favoring the amplification of parasites in the dermis without the formation of either a macroscopic or microscopic lesion, followed by the development of a cutaneous lesion that is coincident with the killing of the parasite at the site. The final phase is characterized by the persistence of 100-10.000 parasites, primarily in macrophages, at the site for up to 1-year following resolution in the cutaneous lesions. Nicolas and coworkers (2000) have described, by using a mice natural model that the two sites of multiplication and persistence (over 12 months) of parasites were the site of *L. major* inoculation (skin) and the draining lymph node. At early time points, parasite DNA was also detected in distant tissues, indicating that blood was at least transiently disseminating the parasites. In contrast, *L. major* DNA in liver, spleen or bone marrow remained sporadic. Sustained intracellular growth in the absence of a parasite-driven host response is a hallmark of leishmanial infection and is central to the maintenance of its transmission cycle in nature. These findings about the persistence of parasites at the site of *Leishmania* inoculation are in agreement with our results (chapter 5) about the detection of parasite in the skin.

It would have been interesting to investigate the zymodemes of *L. infantum* that were involved in the dogs we studied. The most widespread zymodeme in the Mediterranean basin is MON-1, which is considered a viscerotropic zymodeme (Martin-Sánchez *et al*., 1995; Martin-Sánchez *et al*., 1994). In humans, the dermotropic zymodemes are MON-33, MON-24, MON-29, MON-183, MON-199 and others (Harrat *et al*., 1996; Jimenez *et al*., 1995). Isolation and identification of other enzymatic variants of the *L. infantum* complex from cases of canine leishmaniosis are rare events (Harrat *et al*., 1996; Martin-Sánchez *et al*., 1994). Nevertheless, three foci have been described in Egypt, Italy and Spain where zymodeme variants (MON-98, MON-72 and MON-199, respectively) were isolated from humans, sand flies and dogs (Gramiccia *et al*., 1992; Martín-Sánchez *et al*., 1999; Shetata *et al*., 1990). At present, very few studies have found different parasite zymodemes isolated from dog skin samples (Martín-Sánchez *et al*., 1999). It is possible that the dermotropic zymodemes in the dog are more common than assumed and further studies should be done to investigate this hypothesis.

An important epidemiological question is whether dogs found positive to the *Leishmania* parasite by the above techniques are also infective to phlebotomine sand flies. This information would provide the best indicator of the burden of infectiousness in a dog population in an endemic area. It was demonstrated that seropositive dogs (symptomatic and asymptomatic), were sources of the parasite for phlebotomine vector sand flies (Guarga *et al*., 2000a; Molina *et al*., 1994). Although, the rate of infected sand flies increased with the appearance and severity of the signs (Molina, 1997). Recently, Guarga and coworkers (2000) have demonstrated a significant association between a decrease in the number of $CD4^+$ T cells and an increase in the infectivity of dogs to the sand flies (Guarga *et al*., 2000b). In human beings, a significant association between a

decrease in the number of $CD4^+$ T lymphocytes and an increase in the infectivity of people coinfected with *Leishmania infantum* and human immunodeficiency virus to the sand flies has also reported (Molina *et al*., 1999). We showed that 54% of healthy dogs living in an endemic area could be considered as asymptomatic carriers of *Leishmania* (chapter 5). Further studies are needed to ascertain the potential of asymptomatic dogs to transmit *Leishmania* to vector sand flies.

We considered the skin to be the most sensitive tissue for detecting infection. However, the usefulness of skin biopsy and *Leishmania* PCR to carry out broad epidemiological studies is not feasible because a skin biopsy is an invasive technique and it is not practical to use when examining large number of dogs. LST is a field tool very useful to detect cellular immunity in dogs much the same as in humans (Jorquera *et al*., 1998; Marty *et al*., 1992) and it is not an invasive method. LST, together with serology, is a good method to carry out epidemiological surveys and estimate the prevalence of infection (Barbosa Santos *et al*., 1998; Cardoso *et al*., 1998; Hermeto *et al*., 1993). However, due to the ease in obtaining sera and performing methodological procedures, serology will probably remain the method of choice for any large scale screening of dogs in endemic areas. Nevertheless, we must realize that we are not detecting all infections. In consequence, the eradication of *Leishmania* infection from an endemic area will likely not be achieved if measures are targeted only at seropositive dogs (Gradoni, 1999). Comparison of the epidemiological sequences obtained from sand flies and mammalian hosts will be crucial for developing hypotheses about the transmission and distribution of *Leishmania spp*. in areas of endemicity. These studies may help to clarify the *Leishmania* transmission from the vertebrate host to the sand fly vector, a complex process still poorly understood.

These studies demonstrate the prevalence of *Leishmania* infection in an endemic area is higher than assumed and that the main tissues reserve in dogs for the parasite is the skin. This information is essential for designing and implementing appropriate control measures that must be addressed when evaluating the efficacy of any treatment or prophylaxis.

The main findings regarding prevalence of *L. infantum* infection, seroprevalence and prevalence of the disease are:

1. The prevalence of *L. infantum* infection in endemic areas is greater than the seroprevalence and the prevalence of the disease.
2. *L. infantum* infects the majority of dogs living in endemic areas.
3. The main tissue reserve for the parasite in dogs is the skin.
4. LST together with serology are the best tools to detect asymptomatic infection.

The integration of data obtained in broad epidemiological and immunological studies (Berrahal *et al*., 1996; Cabral *et al*., 1998), is changing the understanding of canine leishmaniosis. Infection and disease are no longer synonymous and the diagnosis and prognosis rely on multiple sources that allow evaluating both humoral and cellular immunity. Further studies are needed in this area to fill the large gaps in our knowledge of this complex infection. Areas that should be studied include cells, cytokines, genetic

background involved in resistance and susceptibility, intrinsic parasite and sand flies factors that modulate immune responses, etc. Work in these areas could lead to the development of new treatments, immmunotherapies or efficacious vaccines to protect individuals affected by this disease.

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1. *L. infantum* infects the majority of dogs living in an endemic area. Furthermore, the prevalence of *L. infantum* infection in an endemic area is greater than the seroprevalence and the prevalence of the disease.

2. The skin is the main reserve of the parasite in infected dogs without clinical signs.

3. The immune response to *Leishmania* in the dog is highly variable. Some dogs show only humoral mediated immunity, other animals show only cellular mediated immunity, while the majority of dogs show both humoral and cellular mediated immunity.

4. To evaluate and define the immune response of a patient/animal is necessary to combine techniques that assess both humoral and cellular responses. In our work, this was reached by means of serology, LST, LPA and IFN-γ production.

5. The study of immune responses allows the classification of the dogs living in an endemic area into one of the following four groups: non-infected, resistant, dogs that will probably develop the disease, and ill dogs.

6. The humoral immune response in the ill dog consist mainly in a strong production of specific IgG2 while the IgG1 production is highly variable. In asymptomatic, infected animals, the humoral response is polymorphic ranging from background to medium levels of IgG1, IgG2 or total IgG.

7. After long treatment, levels of all immunoglobulins significantly decrease. In responsive-treated animals the decrease of all immunoglobulins is significantly more pronounced than in unresponsive-treated dogs.

8. Genetic backgrounds could be an important factor in the determination of the type of immune response. We demonstrated that the Ibizian hound presents significant higher frequency of positive LST than other breeds. In consequence, the Ibizian hound can be considered more *Leishmania* resistant.

Summary

Leishmania is a parasite found worldwide transmitted by the bite of a sandfly (vector) of the generus *Phlebotomus* or *Lutzomyia* into the skin of mammals (host). The parasite multiplies in the host's macrophages as an intracellular form (amastigot) while the extracellular form (promastigot) multiplies in the vector's intestine. In the Mediterranean basin, the dog is considered the principal host and *L. infantum* is the specie that affects dogs and humans. The seroepidemiological studies carried out in the Mediterranean basin have detected that up to 40% of dogs have been exposed to the parasite. The clinical manifestations of the disease depend on many factors; being the most studied those related to immune system. Studies performed in murine models have provided valuable information about the mechanisms involved in the resistance and the susceptibility to *Leishmania* infection. In mice, the protective immune response against *L. major* is associated with activation of Th1 cells, which produce the cytokines IFN-γ and IL-2. These cytokines are involved in macrophage activation leading to parasite destruction and activation of B cells to secrete the isotype IgG2a. Susceptibility to infection is associated with activation of Th2 cells. These cells produce the cytokines IL-4 and IL-10 that stimulate a polyclonal B lymphocyte response secreting, among others, IgE and the isotype IgG1 and inhibiting some of the protective cellular responses. In dog, disease is related to a predominant humoral immune response while resistance is related to a predominant cellular immune response.

The objective of this thesis was to obtain new knowledge about the immunology and epidemiology of *Leishmania* infection in dogs living in endemic regions.

Chapter 2 describes the expression of IgG, IgG1 and IgG2 specific antibodies to *L. infantum* in a wide population of dogs (ill, treated and asymptomatic). The levels of IgG, IgG1 and IgG2 in asymptomatic dogs were highly variable and lower than those found in ill dogs. In ill dogs, the levels of IgG and IgG2 were very high but the levels of IgG1 were extremely variable. After treatment and followed by clinical improvement, Ill dogs presented a decrease in all immunoglobulins. In asymptomatic dogs, IgG and IgG2 increased during the course of the infection while IgG1 remained the same. In all groups, the correlation between IgG and IgG2 were higher than between IgG and IgG1. Overall results showed a large variation in the IgG1 expression in asymptomatic dogs as well as in symptomatic dogs and a low IgG1 correlation with IgG or IgG2.

Chapter 3 describes the evaluation and comparison of the efficacy of two leishmanins for detection dog *Leishmania* cellular immune response. Leishmanin preparation 1 ($3x10^8$ promastigotes/mL) was superior to leishmanin preparation 2 ($5x10^6$ promastigotes/mL). More positive reactions were obtained at 72h than at 48h suggesting that 72h is the optimum time to take test readings in the dog.

Chapter 4 describes the study of humoral and cellular responses in a population of dogs. Seventy-seven percent of the dogs demonstrated a specific *Leishmania* response either humoral or cellular. Eighty percent of ibizian hounds and 48% of dogs of other breeds presented a cellular response while the humoral response was 48% for ibizian hounds and 56% for dogs of other breeds. The results showed that the rate of infection was higher than assumed previously and that dogs presented a broad range of immune responses to the parasite from resistant to ill dogs. The Ibizian hound manifested a more

uniform cellular response. Consequently, Ibizian hound can be considered more *Leishmania* resistant than other canine breeds.

Chapter 5 describes the study and comparison of the prevalence of *Leishmania* infection, the seroprevalence and the prevalence of canine leishmaniosis in an endemic area. One hundred dogs were used. For each dog, clinical exploration for the presence of clinical signs compatible with leishmaniosis, the titre of anti-*Leishmania* antibodies, and the presence of *Leishmania* DNA by PCR in skin, conjunctiva and bone marrow samples were assessed. The prevalence of the disease was 13% and the seroprevalence was 26%. 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 18%, 32% and 51%, respectively. The prevalence of the infection, 67%, was calculated using all animals that were seropositive and/or positive for PCR in any tissue. The results showed that the majority of dogs living in an endemic area are infected by *Leishmania*.

Chapter 6 describes the study of immunological parameters in the evaluation of dogs infected by *Leishmania* and also defines the immune profiles of this population of dogs. The following parameters were studied: anti-*Leishmania* IgG1, IgG2, total IgG antibodies, LST, LPA, and production of IFN-γ and TNF-α. The majority of infected animals without clinically patent disease showed variable titres of anti-*Leishmania* antibodies ranging from background to medium, a positive LST, a strong *Leishmania* antigenic proliferative response, and a high production of IFN-γ. The remainder showed positive titres of anti-*Leishmania* antibodies with a negative positive LST. Before treatment, ill dogs presented high levels of anti-*Leishmania* antibodies, negative LST, no production of IFN-γ but a production of TNF-α. Clinical recovery was associated with a decrease in the titre of antibodies and an increase of the diameter of the LST. The combination of serology, LST, and measurement of cytokines constitutes a useful, clinically relevant method to evaluate the immune response to *Leishmania* in a single patient.

Finally, in chapter 7, the overall results of these studies are summarized and discussed providing a general view about immunological and epidemiological aspects on *Leishmania* infection in dogs living in endemic regions.

Resum

Leishmania és un paràsit que es troba àmpliament distribuït arreu del món i que es transmet mitjançant la picada de mosquits (vector) del gènere *Phlebotomus* o *Lutzomyia* a la pell de mamífers (hoste). El paràsit es multiplica en els macròfags de l'hoste en la seva forma intracel_lular (amastigot), mentre que la seva forma extracel_lular (promastigot) es multiplica en els intestins del vector. En la conca mediterrània, *L. infantum* és l'espècie que afecta el gos i l'home, tot i que el gos se'n considera l'hospedador principal. Els estudis seroepidemiològics realitzats en la conca mediterrània detecten que un 40% dels gossos han estan en contacte amb el paràsit. Les manifestacions clíniques d'aquesta malaltia depenen de molts factors, essent els més estudiats els relacionats amb el sistema immunitari. Així, els estudis realitzats en els models murins han proporcionat informació valuosa sobre els mecanismes involucrats en la resistència i susceptibilitat a la infecció per *Leishmania*. En el ratolí, la resposta immunitària protectora davant de *L. major* està associada amb l'activació de cèl·lules Th1, les quals produeixen IFN-γ i IL-2. Aquestes citocines estan involucrades en l'activació de macròfags els quals destrueixen el paràsit, i en l'activació de les cèl·lules B que secreten l'isotip IgG2a. La susceptibilitat a la infecció està associada amb l'activació de cèl·lules Th2. Aquestes cèl·lules produeixen IL-4 i IL-10, que estimulen la resposta policlonal de limfòcits B, els quals produeixen, entre d'altres, la IgE i l'isotip IgG1, i inhibeixen algunes de les respostes cel·lulars protectores. En el gos, la malaltia es relaciona amb una predominant resposta immunitària humoral mentre que la resistència es relaciona amb una predominant resposta immunitaria cel·lular.

L'objectiu d'aquesta tesi va ser obtenir nous coneixements sobre la immunologia i epidemiologia de la infecció per *Leishmania* en gossos que viuen en zones endèmiques.

El capítol 2 descriu l'expressió d'anticossos anti-*Leishmania* IgG, IgG1 i IgG2 en una àmplia població de gossos (malalts, tractats i asimptomàtics). Els nivells de IgG, IgG1 i IgG2 en gossos asimptomàtics van ser molt variables i menors que els dels animals malalts. En els animals malalts, els nivells de IgG i IgG2 van ser molt alts però els nivells d' IgG1 molt variables. Després del tractament i recuperació clínica, els animals malalts van presentar una disminució de totes les immunoglobulines. En gossos assimptomàtics, les IgG i IgG2 van augmentar en el transcurs de la infecció, mentre que les IgG1 es van mantenir iguals. En tots els grups, la correlació entre la IgG i la IgG2 va ser més alta que entre la IgG i la IgG1. Els resultats en conjunt van mostrar la gran variació en l'expressió de IgG1 tant en gossos asimptomàtics com en simptomàtics, així com la baixa correlació de la IgG1 amb la IgG o IgG2.

El capítol 3 descriu l'avaluació i comparació de l'eficàcia de dues preparacions de leishmanines en la detecció de la resposta cel·lular davant de *Leishmania* en el gos. La preparació de leishmanina 1 ($3x10^8$ promastigots/mL) va ser superior a la preparació de leishmanina 2 ($5x10^6$ promastigots/mL). Es van obtenir més reaccions positives a les 72h que a les 48h, aquest resultat suggereix que les 72h és el temps òptim per a la lectura del test en el gos.

El capítol 4 mostra l'estudi de la resposta humoral i cel·lular en una població de gossos. El 77% dels gossos va mostrar una resposta immunitària específica davant de *Leishmania*, ja fos humoral o cel·lular. El 80% dels cans eivissencs i el 48% dels gossos

d'altres races van presentar resposta cel·lular, en canvi la resposta humoral va ser d'un 48% en els cans eivissencs i d'un 56% en gossos d'altres races. Els resultats van mostrar que la taxa d'infecció era més alta que la considerada anteriorment, i que els gossos presentaven un ampli ventall de respostes inmunitàries davant del paràsit, des de gossos resistents fins a gossos malalts. El ca eivissenc va manifestar més uniformement resposta cel·lular. En conseqüència, el ca eivissenc pot ser considerat més resistent que altres races canines.

El capítol 5 descriu l'estudi i comparació de la prevalença de la malaltia, la seroprevalença i la prevalença de la infecció en una zona endèmica. Cent gossos van ser examinats clínicament per avaluar la presència de simptomatologia clínica compatible amb leishmaniosi i, en cada gos, es van analitzar els nivells d'anticossos anti-*Leishmania* i la presència d'ADN de *Leishmania* en pell, conjuntiva i moll d'ós. La prevalença de la malaltia i seroprevalença van ser, respectivament, d'un 13% i d'un 26%. En el 63% dels gossos va ser detectat ADN de *Leishmania,* en almenys un dels teixits estudiats. Els resultats de PCR positives en moll d'ós, conjuntiva i pell van ser respectivament, d'un 18%, 32% i 51%. La prevalença de la infecció va ser d'un 67%, calculada a partir dels animals seropositius i/o positius a PCR en algun dels teixits. Els resultats mostren que la majoria de gossos que viuen en zones endèmiques ha estat infectat pel paràsit.

El capítol 6 descriu l'estudi de paràmetres immunològics en l'avaluació de gossos infectats amb *Leishmania*, per definir els perfils inmunològics de la població canina que viu en una zona endèmica. Els paràmetres utilitzats van ser serologia (IgG, IgG1 i IgG2), test intradèrmic amb leishmanina, assaig de proliferació de limfòcits i detecció d'IFN-γ i TNF-α. La majoria dels animals infectats sense simptomatologia clínica presentava nivells variables d'anticossos, des de nuls a mitjos, reacció positiva al test intradèrmic, bona resposta proliferativa al antigen de *Leishmania* i producció d'IFN-γ. La resta va mostrar nivells variables d'anticossos però absència de reacció al test intradèrmic. Abans del tractament, els animals malalts presentaven alts nivells d'anticossos, test intradèrmic negatiu, no producció d'IFN-γ i producció de TNF-α. La millora clínica es va associar amb la disminució dels anticossos i amb l'augment del diàmetre del test intradèrmic. La combinació de la serologia, el test intradèrmic i la medició de citocines constitueixen tècniques útils i d'alta rellevància clínica en l'avaluació de la resposta inmunitària d'un pacient individual.

Finalment, en el capítol 7, els resultats d'aquest estudis són resumits i discutits de forma conjunta, donant una visió general sobre aspectes immunològics i epidemiològics de la infecció per *Leishmania* en gossos que viuen en zones endèmiques.

Resumen

Leishmania es un parásito que se encuentra ampliamente distribuido por todo el mundo y que se transmite por la picada de mosquitos (vector) del género *Phlebotomus* o *Lutzomyia* en la piel de mamíferos (hospedador). El parásito se multiplica en los macrófagos del hospedador en su forma intracelular (amastigote), mientras que su forma extracelular (promastigote) se multiplica en los intestinos del vector. En la cuenca mediterránea, *L. infantum* es la especie que afecta al perro y al hombre, y donde el perro es considerado el hospedador principal. Los estudios seroepidemiológicos realizados en la cuenca mediterránea detectan hasta un 40% de perros que han estado en contacto con el parásito. Las manifestaciones clínicas de esta enfermedad dependen de muchos factores, siendo los más estudiados los relacionados con el sistema inmunitario. Así, los estudios realizados en los modelos murinos han proporcionado información valiosa sobre los mecanismos involucrados en la resistencia y susceptibilidad en la infección por *Leishmania*. En el ratón, la respuesta inmunitaria protectora frente a *L. major* se ha asociado con la activación de células Th1, las cuales producen IFN-γ y IL-2. Estas citocinas están involucradas en la activación de macrófagos, produciendo la destrucción del parásito, y en la activación de las células B para que secreten el isotipo IgG2a. La susceptibilidad a la infección está asociada a la activación de células Th2. Estas células producen IL-4 y IL-10, que estimulan la respuesta policlonal de linfocitos B, produciendo, entre otras, la IgE y el isotipo IgG1, e inhiben algunas de las respuestas celulares protectoras. En el perro, la enfermedad se relaciona con una predominante respuesta inmunitaria humoral, mientras que la resistencia se relaciona con una predominante respuesta inmunitaria celular.

El objetivo de esta tesis fue obtener nuevos conocimientos sobre la inmunología y epidemiología de la infección por *Leishmania* en perros que viven en zonas endémicas.

El capítulo 2 describe la expresión de anticuerpos anti-*Leishmania* IgG, IgG1 y IgG2 en una amplia población de perros (enfermos, tratados y asintomáticos). Los niveles de IgG, IgG1 y IgG2 en perros asintomáticos fueron muy variables y menores que los de los animales enfermos. En los animales enfermos, los niveles de IgG y de IgG2 fueron muy altos, y los de IgG1 muy variables. Después del tratamiento y la mejora clínica, los animales enfermos presentaron una disminución de todas las inmunoglobulinas. En los perros asintomáticos, las IgG y IgG2 aumentaron en el transcurso de la infección, mientras que las IgG1 se mantuvieron iguales. Los resultados en conjunto mostraron la gran variación en la expresión de IgG1 tanto en perros asintomáticos como en sintomáticos así como la baja correlación de la IgG1 con la IgG o IgG2.

El capítulo 3 describe la evaluación y comparación de la eficacia de dos preparaciones de leishmaninas en la detección de la respuesta celular frente a *Leishmania* en el perro. La preparación de leishmanina 1 ($3x10^8$ promastigotes/mL) fue superior a la preparación de leishmanina 2 ($5x10^6$ promastigotes/mL). Se obtuvieron más reacciones positivas a las 72h que a las 48h, sugiriendo este resultado que las 72h es el tiempo óptimo para la lectura del test en el perro.

El capítulo 4 muestra el estudio de la respuesta humoral y celular en una población de perros. El 77% de los perros demostró una respuesta específica frente a *Leishmania*, ya fuera humoral o celular. El 80% de los podencos ibicencos y el 48% de los perros de

otras razas presentaron respuesta celular, en cambio la respuesta humoral fue de un 48% para los podencos y de un 56% para los perros de otras razas. Los resultados mostraron que la tasa de infección era más alta de lo considerado anteriormente y que los perros presentaron un amplio abanico de respuesta inmunitarias frente al parásito, desde perros resistentes hasta perros enfermos. El podenco ibicenco manifestó más uniformemente respuesta celular. En consecuencia, el podenco ibicenco puede ser considerado más resistente que otras razas caninas.

El capítulo 5 describe el estudio y comparación de la prevalencia de la enfermedad, la seroprevalencia y la prevalencia de la infección de una zona endémica. Cien perros fueron examinados clínicamente para evaluar la presencia de sintomatología compatible con leishmaniosis y, en cada perro, se analizaron los niveles de anticuerpos anti-*Leishmania*, la presencia de ADN de *Leishmania* en piel, conjuntiva y médula ósea. La prevalencia de la enfermedad y seroprevalencia fueron, respectivamente, de un 13% y de un 26%. En el 63% de los perros fue detectado ADN de *Leishmania* en al menos uno de los tejidos estudiados. Los resultados de PCR positivos en médula ósea, conjuntiva y piel fueron, respectivamente, de un 18%, 32% y 51%. La prevalencia de la infección fue de un 67%, calculada a partir de los animales seropositivos y/o positivos a PCR en alguno de los tejidos. Los resultados muestran que la mayoría de perros que viven en zonas endémicas ha sido infectado por el parásito.

El capítulo 6 describe el estudio de parámetros inmunológicos en la evaluación de perros infectados con *Leishmania* para definir los perfiles inmunológicos de la población canina que vive en una zona endémica. Los parámetros utilizados fueron la serología (IgG, IgG1, IgG2), el test intradérmico con leishmanina, el ensayo de proliferación de linfocitos y la detección de IFN-γ y TNFα. La mayoría de los animales infectados sin sintomatología clínica presentó niveles variables de anticuerpos, desde nulos a medios, reacción positiva al test intradérmico, buena respuesta proliferativa al antígeno de *Leishmania* y producción de IFN-γ. El resto mostró niveles variables de anticuerpos pero ausencia de reacción al test intradérmico. Antes del tratamiento, los animales enfermos presentaban altos niveles de anticuerpos, test intradérmico negativo, no producción de IFN-γ y producción de TNFα. La mejoría clínica se asoció a la disminución de los anticuerpos y al aumento del diámetro del test intradérmico. La combinación de la serología, test intradérmico y la medición de citocinas constituyen técnicas útiles y de alta relevancia clínica en la evaluación de la respuesta inmunitaria de un paciente individual.

Finalmente, en el capítulo 7, los resultados de estos estudios se resumen y discuten de forma conjunta, dando una visión general sobre aspectos inmunológicos y epidemiológicos de la infección por *Leishmania* en perros que viven en zonas endémicas.