Leishmania infantum and dog: immunological and epidemiological studies about infection and disease

Tesi Doctoral

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Contents

Abbreviations

APC	Antigen-Presenting Cell
a.t.	After treatment
b.t.	Before treatment
BrdU	5-bromo-deoxyuridine
CD #	Cluster of Differentiation # (e.g. CD8)
Con A	Concavalin A
DTH	Delayed Type Hypersenstivity
EDTA	Ethylenediamine-tetra acetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
GRP	Glucose-Regulated Protein
gp	Glycoprotein
H	Histone
HIV	Human Immunodeficiency Virus
Hsp	Heat Shock Protein
IFA	ImmunoFluorescence Assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kD	KiloDalton
KMP	Kinetoplastid Membrane Protein
LPA	Lymphocyte Proliferation Assay
LSA	Leishmanial Soluble Antigen
LST	Leishmanin Skin Test
MDCK	Madin-Darby Canine Kidney cell line
MHC	Major Histocompatibility Complex
ML	Madrid Leishmanin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NNN	Novy-Nicolle-McMeal
NO	Nitric Oxide
OD	Optical Density
P	Acidic ribosomal Protein
PBMC	Peripheral Blood Mononuclear cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PHA	Phytohemagluttinin
PMNC	Polymorphonuclear cell
PPD	Purified Protein Derivative
PSA	Protein Surface Antigen
rK	Kinesin related antigen
RL	Roma Leishmanin
SDS	Sodium dodecyl sulphate
STI	Soybean Tripsin Inhibitor
T	Tween 20
Th	T helper lymphocyte
TCR	T Cell Receptor
TNF	Tumor Necrosis Factor
U	Units
VSV	Vesicular Stomatitis Virus

En aquests moments estic una mica cansada d'escriure davant de l'ordinador. Així que no crec que els meus pensaments sorgeixin amb fluïdesa. Pretenc començar amb aquest estat vulnerable, el pròleg del que serà aquest petit llibre. M'agradaria explicar de forma resumida la meva experiència com a estudiant de doctorat durant aquests quatre anys a la Facultat de Veterinària. En acabar la carrera universitària se'ns barregen dues sensacions: la nostàlgia a causa del final de l'època d'estudiant, i la il·lusió i motivació d'iniciar una nova etapa, la professional. És un moment de canvi, per tant, difícil, però també ple d'entusiasme i energia en el qual has de decidir en quina part de l' àrea veterinària et desenvoluparàs i donaràs el millor de tu. El meu conflicte intern es situava entre fer clínica de petits animals o fer investigació. El finalitzar la carrera, la investigació me la va treure del cap un professor de la Facultat de Veterinària. Així, que seguint els seus consells vaig pujar al "carro" de la clínica de petits animals. La meva experiència en l'hospital de la Facultat de Veterinària com a estudiant, fent les pràctiques de camp, i després com a veterinària interna (uns quants mesos) va ser molt enriquidora i positiva. Considero que vaig aprendre molt tant des del punt de vista professional com personal. Malgrat tot, el meu pas per un cartell anunciant un treball de recerca com a estudiant de doctorat en la leishmaniosi canina just en l'últim moment, va canviar la meva trajectòria. Així que el conflicte va tornar a aparèixer. En aquell moment em vaig haver de decidir entre blanc o negre, no vaig aconseguir trobar cap gris com a solució intermitja. Finalment, vaig optar per l'opció de la investigació, que vaig creure seria molt difícil de realitzar un cop fora de l'àmbit universitari. Així doncs, el febrer de l'any 1997 començava una nova etapa com a estudiant de doctorat en el Departament de Farmacologia i Terapèutica de la Facultat de Veterinària.

Diccionari d'idees

- **Arbre**: Acabava de passar una tempesta, la terra estava mullada, els núvols ocultaven el sol i hi havia poques persones en aquell indret. El fred era evident en aquell mes de febrer. Feia molt vent, la qual cosa va afavorir que caiguessin llavors en aquella terra humida. A la primavera, van començar a aparèixer la verdor i les flors, i entremig d'aquell bosc, tímidament van brotar noves llavors d'arbre. No eren moltes i es podien contar amb els dits de la mà. Creixien amb força, volien arribar a veure el que els esperava allà dalt però mentrestant es divertien en l'harmonia d'aquell bosc del Mediterrani. Hi havia moltes coses que no comprenien, i intentaven resoldre-les, però de vegades no podien, eren llavors encara massa petites per poder comprendre la complexitat d'aquella natura. Observaven els ocells, els escarabats, els homes que caminaven durant el dia i la nit, observaven la lluentor de la lluna, quan n'hi havia, els feia explicar-se contes fantàstics i màgics. Preguntaven coses als arbres, a les plantes, als animals, però no sempre obtenien resposta d'aquells éssers. A l'estiu passaren època de sequera i intentaren trobar amb les seves petites arrels tota l'aigua que podien aconseguir en aquell indret. Passat el temps, es van adonar que ja veien les muntanyes i, més enllà, divisaren el mar. Abans tan sols podien veure aquell subsòl que els rodejava. Van començar a discutir amb els altres arbres que els havien vist créixer. Discutien per tot i de vegades no arribaven a cap conclusió. Ja tenien branques, arrels profundes i endinsades en aquella terra. Alguns dies, les discussions eren amenes, banals i sense contradiccions. Un dia, un ocell es posà en una de les branques. A partir de llavors sempre es posava allà, en els braços d'aquell arbre que el protegia de les inclemències del temps, conversaven i reflexionaven conjuntament. Ja havia vist tot

allò que anhelava quan tan sols era una insignificant llavor. Estava en les alçades i formava part de l'aire, com de la terra, el sol i l'aigua. Tot era molt complexe però intentava, dia rera dia, descobrir alguna cosa nova, perquè l'endemà encara fos tot una mica més complexe. Aquell arbre mil·lenari coneixia aquell bosc a la perfecció però tot i això la naturalesa que el rodejava era més sàvia que ell perquè ho pogués esbrinar tot. Ara, era ell qui feia ombra a aquelles petites llavors, que algun dia arribarien, com ell, a adonar-se de la complexitat del coneixement.

- **Art**: La portada i contraportada d'aquesta tesi mostren pintures (col.lecció de fotogafies pintades del llibre "dog in the dunes" de Barbara Cohen, extretes de www.barbaracohen.com).Volia unir l'art i la ciència d'alguna manera. I s'em va ocórrer aquesta. Possiblement, per a la majoria de la gent aquesta associació de paraules no té ni cap ni peus. Però quan et dediques a la investigació t'adones que ambdues àrees necessiten molta imaginació, innovació i creació d'idees per tirar endavant. Així doncs, la similitud és petita, però hi és. Espero que gaudiu de l'expressió artística i científica d'aquesta tesi.
- **Becaris:** Es diu d'aquelles persones que obtenen una beca. Normalment, joves, ignorants i entusiastes. Es conformen amb poc perquè, a més, se'ls diu que estan en procés de formació. S'encarreguen de la investigació d'aquest país, però durant poc temps (~ 4 anys), perquè la renovació és important per aportar noves idees. T'interessa? Pots trobar els impressos a la pàgina web: www.uab.es.
- **Gos**: Vull dedicar unes ratlles i aquesta tesi al gos. Tota aquesta tesi ha estat emmarcada en l'estudi d'aquest animal. És ben conegut per tothom que el gos és el principal animal de companyia i porta al costat de l'home molts anys, tants que més val no dir quants. El resultat és que el gos té un paper molt important en la nostra societat. El gos no parla però sí que escolta i es comunica, i és una de les millors virtuts que té, de la qual l'home ha tret profit. Espero que algunes de les coses que hem estudiat hagin servit per fer millor la vida d'aquests éssers, així com la dels homes.
- **Investigació:** L'estat espanyol dedica molt pocs diners en aquesta àrea, i així provoca que no es consolidin grups d'investigació. La meva estada a Holanda, em va fer adonar de la precària situació de la investigació espanyola en comparació amb l'holandesa. Allà tenen mitjans econòmics per tenir tècnics, estudiants de doctorat i "postdocs" de manera que aconsegueixen formar grups d'investigació i, per tant, línies d'investigació fortes i sòlides. Espero que aviat arribem a assolir aquest estat en el nostre país perquè així se'ns permeti avançar en el coneixement científic. A més, la investigació ha de ser plural i justa. Els països rics han de dedicar diners i esforços a investigar problemes que afecten als països pobres, encara que aquests problemes no els afectin a ells. Des de la investigació, també es pot ajudar que el món pugui ser, poc a poc, un lloc millor per a tothom.

Ja acabo, espero que aquest pròleg no hagi sigut gaire avorrit i que encara us quedin ganes per continuar amb la resta de fulles. Jo us animo que ho feu. Sort i salut.

Dedico aquest llibre a la meva mare, al Bernat, al Cristian, al Jon, a la Laura, al Rot, i especialment als gossos, perquè sense tots ells segurament no l'hauria escrit mai.

Voldria agrair l'esforç a tota la gent que ha aportat el seu granet de sorra en l'elaboració d'aquesta tesi doctoral, i per tant, també en la meva formació com a investigadora. No ha estat fàcil, ho sé, i per tant voldria repetir-me. GRÀCIES A TOTES I A TOTS: persones, animals i diverses situacions, per haver fet possible la realització d'aquesta tesi.

Faré un llistat per poder mencionar totes les persones que han aportat alguna cosa durant aquest quatre últims anys. Ho faré de forma resumida,

- A la Marga Arboix perquè gràcies a ella, vaig començar i he acabat aquesta tesi. Gràcies pel teu recolzament durant tots aquests anys.
- Als meus directors, Lluís Ferrer i Jordi Alberola, dos homes molt dedicats però també molt ocupats, per a totes les aportacions, idees, crítiques i discussions realitzades durant tots aquests anys i que es resumeixen en aquesta tesi.
- A la gent que forma el grup de leishmaniosi del laboratori de Parasitologia del Departament de Microbiologia i Parasitologia sanitàries de la Universitat de Barcelona: a la Montse Portús, Cristina Riera, Montse Gállego, Roser Fisa pel seu ajut i dedicació en aquesta tesi i en el meu aprenentatge.
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Chapter 1

General introduction

Introduction

The classical definition of infectious disease as a process including infection, an incubation period and disease has evolved recently to the point where infection and disease are not always considered synonymous. In this thesis, we will try to show the difference between infection and disease in dogs affected by *Leishmania infantum* both on immunological and epidemiological findings. The general introduction will be a description of the previous literature about canine leishmaniosis in the Mediterranean basin.

General aspects

Leishmaniosis has a long history. The disease has been present in the Americas for a long period of time as evidenced by the existence of thousand-year old human sculls and designs on pre-Colombian pottery with markings of leishmaniosis. The disease is known to have been present in Africa and India since at least the mid-eighteenth century (Allison, 1993). *Leishmania* infections occur worldwide, with different *Leishmania* species involved in biogeographical regions of the Old and New World, which are responsible for the wide spectrum of clinical illnesses observed in people (Table 1). Today, an estimated 12 million human cases of leishmaniosis exist worldwide, with an estimated number of 1.5-2 million new cases occurring annually: 1-1.5 million cases of cutaneous leishmaniosis and 500.000 cases of visceral leishmaniosis (Desjeux & UNAIDS, 1998). Epidemiologically two different situations occur: (1) The zoonotic form as found in the Mediterranean basin, with the dog as the main source of infection for the female sand fly; and (2) The anthroponotic form as found in East Africa, Bangladesh, India and Nepal where transmission is passed from person to person through the sand fly vector.

In 1903, Leishman and Donovan separately described the protozoan now called *Leishmania donovani* in splenic tissue from human patients in India. In 1908, Nicolle and Comte reported the first dog infection by *Leishmania* in Tunisia. Since these discoveries, the growing body of knowledge about *Leishmania* and its reservoirs has continually expanded improving the understanding of this infection. Today, this parasitic infection in the Mediterranean basin is important not only because of its zoonotic aspects but also leishmaniosis is important to Veterinary Medicine because the dog is considered the main host. In the Mediterranean basin, human leishmaniosis traditionally has affected young children and infants, but with the advent of HIV infection, leishmaniosis is now a common complicating factor in adults infected with HIV or receiving immunosuppressive drugs (Alvar *et al*., 1997; Dedet & Pratlong, 2000; Desjeux, 1999). In the Mediterranean region, dogs are a common pet and the health of these animals is of great concern to dog owners and veterinarians.

Table 1. Clinical forms of human leishmaniosis and associated parasites (Slappendel & Ferrer, 1998)

Form of disease	Old World	New World
Cutaneous	*L. aethiopica* complex	*L. mexicana* complex
	- *L. aethiopica*	- *L. mexicana*
	L. major complex	- *L. venezuelensis*
	- *L. major*	- *L. amazonensis*
	L. tropica complex	- *L. pifanoi*
	- *L. tropica*[d]	- *L. garnhami*
	- *L. killicki*	*L. braziliensis* complex *(viannia)*
		- *L. braziliensis*[a]
		- *L. panamensis*[a]
		- *L. guyanensis*[a]
		- *L. peruviana*
		- *L. lainsoni*[b]
Visceral	*L. donovani* complex	
	- *L. donovani*	- *L. chagasi*[a,c,e]
	- *L. infantum*[c]	

[a] May also cause mucocutaneous leishmaniosis
[b] Not definitively assigned
[c] Main causative agents of canine leishmaniosis
[d] Few cases described in dogs
[e] *L. chagasi=L. infantum*

Etiology

Leishmania is a flagellated protozoan, which belongs to the kingdom *Protista*. The taxonomy of the parasite can be found elsewhere (Chang *et al*., 1985; Cupolillo *et al*., 2000; Lainson & Shaw, 1987). It is not possible to identify the various *Leishmania* species by morphological criteria alone. Currently, classification of *Leishmania* is mainly based on differentiation by biochemical and genetic methods. These methods include DNA peptide mapping, immunologic reactivity to monoclonal antibodies, membrane-shed antigens, and, most important, isoenzyme patterns (grouped in taxonomic units termed zymodemes) (Rioux *et al*., 1990). *Leishmania* species, which share major characters, have been grouped into so-called "complexes". The parasite responsible for canine leishmaniosis in the Mediterranean basin is the *L. infantum* complex, being MON-1 the most frequent zymodeme (Maazoun *et al*., 1981). Enzymatic variations occur, and nine other zymodemes of *L. infantum* have been identified in dogs from the Mediterranean area: MON-11, MON-77, MON-108 (Pratlong *et al*., 1989); MON-98 (Shetata *et al*., 1990); MON-27 (Gramiccia *et al*., 1992); MON-34, MON-37 (Harrat *et al*., 1996); MON-105 (Alvar *et al*., 1997) and MON-199 (Martín-Sánchez *et al*., 1999). Very few cases of *L. tropica* have been reported in dogs: MON-102, MON-113, (Dereure *et al*., 1991a) and MON-76 (Dereure *et al*., 1991b). It is possible that infections by *L. tropica* in dogs are accidental (Dereure *et al*., 1991a).

Epidemiology

Transmission

Sand flies from the genus *Phlebtomus* (Old World) or *Lutzomyia* (New World) are the principal agents of transmission of *Leishmania* in humans and dogs (Killick-Kendrick, 1990). The two vectors in Spain are *Phlebotomus perniciosus* and *P. Ariasi* (Lucientes Curdi *et al*., 1988; Martín-Sánchez *et al*., 1994; Rioux *et al*., 1986). The activity of the adult sand flies is crepuscular and nocturnal (Rioux & Golvan, 1969) from early spring to late autumn (Killick-Kendrick, 1990). Other unusual routes, such as congenital transmission, blood transfusion, direct contact, ingestion and laboratory inoculation have been reported in humans (Alvar, 1994; Blanc & Robert, 1984; Symmers, 1960) and dogs (Mancianti & Sozzi, 1995; Riera & Valladares, 1996).

The *Leishmania* parasite multiplies in the female sand fly as a flagellate form (promastigote). Promastigotes are then inoculated intradermally when the sand fly takes a blood meal. In mammalian hosts, macrophages take up the promastigotes, which then become the rounded, non-flagellate forms called amastigotes. Infected macrophages eventually burst, and amastigotes enter other phagocytic cells of the host (Chang *et al*., 1985).

Distribution

The Mediterranean basin is a diverse biogeographical entity. In different parts of the region, the phlebotomine vectors of *Leishmania* occupy humid, sub-humid and semi-arid niches where endemic foci of canine leishmaniosis have developed (Dereure *et al*., 1999).

The majority of epidemiological studies about the prevalence and incidence of *Leishmania* infection in dog have been based on serologic surveys. The seroprevalence in the Mediterranean basin ranges from 10% to 40% depending on the region (Bettini & Gradoni, 1986). In the literature, serologic surveys can be found from the majority of the Mediterranean countries (Table 2). In contrast, there are fewer studies about the incidence or force of infection. Information on prevalence where a cross-sectional study is needed is usually easier to obtain than incidence (Gradoni, 1999; Toma *et al*., 1999). A serological survey performed in the Priorat, a rural region in southern Catalonia, gave a prevalence of 10.2% and an annual incidence of 5.7% (Fisa *et al*., 1999), results that were quite similar to those found in other rural foci in Southwestern European countries (Abranches *et al*., 1983; Brandonisio *et al*., 1992). Moreover, it is interesting to note the differences in seroprevalence between the beginning and the end of the transmission period. In a rural area of southern Spain, the seroprevalence increased over the transmission period from 12% in April to 19% in October (Acedo-Sánchez *et al*., 1998).

Several studies used other methods to calculate the prevalence of *Leishmania* infection by detecting *Leishmania* DNA in different tissues (Berrahal *et al*., 1996) or by detecting specific-*Leishmania* cellular immunity (Barbosa Santos *et al*., 1998; Cabral *et al*., 1998; Cardoso *et al*., 1998). In France, a survey performed using PCR on the skin and conjunctiva samples and immunoblotting techniques found that most dogs (80%) have been exposed to the parasite (Berrahal *et al*., 1996). Cabral and coworkers (1998), using

lymphocyte proliferation assay (LPA) and serology found 65% of asymptomatic dogs living in Portugal had evidence of specific response to *Leishmania*. Twenty-seven percent of asymptomatic dogs living in Madrid (Spain) had specific cellular immune response when analyzed by a LPA (Fernández-Pérez, 2000). Cardoso and coworkers (1998) reported 47% of asymptomatic dogs living in Portugal had evidence of a specific response to *Leishmania* by means of serology and LST. Similar results (45%) were described in dogs living in Brazil using serology and LST (Barbosa Santos *et al*., 1998). These studies suggest that the rate of infection is much higher than the rates found by serological investigations.

Table 2. Seroprevalence on *Leishmania* infection and leishmanial zymodemes in Mediterranean countries

Country	Seroprevalence (no. of dogs examined)	Reference of seroprevalence	*L. infantum*/zymodemes
Algeria	37.5 % (120)	(Belazzoug, 1987)	MON-1, MON-34, MON-37
Cyprus	10 % (301)	(Deplazes *et al*., 1998)	MON-1
Egypt	-	-	MON-98
France	26.5% (113)	(Neogy *et al*., 1992)	MON-1, MON-108
Greece	22.4% (1638)	(Sideris *et al*., 1999)	MON-1
Israel	14.6 % (213)	(Baneth *et al*., 1998)	-
Italy	26% (16690)	(Zaffaroni *et al*., 1999)	MON-1, MON-27
Lebanon	2 % (150)	(Zahar, 1980)	-
Lybia	1.6% (638)	(Zahar, 1980)	
Malta	27.3% (198)	(Dye *et al*., 1992)	MON-1
Morocco	8.6% (1013)	(Neijar *et al*., 1998)	MON-1, MON-102[a], MON-103[a]
Portugal	0.7%-6.9% (3614)	(Semiao-Santos *et al*., 1995)	MON-1
Spain	10.2% (2110)	(Fisa *et al*., 1999)	MON-1, MON-11, MON-77, MON-105, MON-199
Syrian Arab Republic	-	-	MON-1, MON-76[a]
Tunisia	6% (265)	(Ben said *et al*., 1992)	MON-1
Turkey	3.6% (494)	(Ozensoy *et al*., 1998)	-

[a] *L. tropica*

Clinical aspects of canine leishmaniosis

Clinical signs

Clinical features vary widely as a consequence of the numerous pathogenic mechanisms of the disease process and because of the diversity of immune responses of individual hosts as whether humans or other mammals (Solbach & Laskay, 2000). The main clinical findings in canine leishmaniosis are skin lesions, local or generalized lymphadenopathy, loss of body weight, glomerulonefropathy, ocular lesions, epistaxis,

lameness, and diarrhea (Ciaramella *et al*., 1997; Ferrer, 1992; Koutinas *et al*., 1999; Slappendel, 1988). Skin lesions are the most usual manifestation and several dermatological entities have been described (Ferrer *et al*., 1988b).

Diagnosis

Diagnosis of canine leishmaniosis, due to variety of clinical signs present is very difficult and many diagnostic tests have been developed to aid in making a correct diagnosis. However, it is essential to know the basis of each test, its limitations and its clinical interpretation. Additionally, it is always recommended to use more than one diagnostic test.

There are three categories of diagnostic methods:

(a) Parasitological methods

The oldest method is the detection of amastigotes in stained smears of aspirates of bone marrow or lymph node (Slappendel & Ferrer, 1998). Impression smears can also be made from mucosal (Font *et al*., 1996) or dermal lesions (Strauss-Ayali & Baneth, 2000). Immunocytochemical techniques that detect *Leishmania* in tissue section (normally skin biopsies) are also used (Bourdoiseau *et al*., 1997c; Ferrer *et al*., 1988a; Roura *et al*., 1999a). Parasites from tissues can be cultured in several media. Media most commonly used are Novy-Nicolle-McNeal (NNN), Schneider's *Drosophila* and RPMI 1640 supplemented with 10-30% of fetal bovine sera. However, the culture of the parasite in media is not a routine diagnostic method because of the extended time required (1 month) to determine whether an animal is infected. The main utility of the culture is for the characterization of the isolates (Evans, 1987; Portús, 1987; Portús, 1997).

(b) Serological methods

There is a wide range of serological tests available including immunofluorescence assay (IFA) (Mancianti & Meciani, 1988), direct agglutination assay (Neogy *et al*., 1992), enzyme-linked immunosorbent assay (ELISA) (Soto *et al*., 1998), competitive-ELISA (Rachamim *et al*., 1991), Dot-ELISA (Fisa *et al*., 1997), slide-ELISA (Vercammen *et al*., 1997), immunodifussion assay (Bernadina *et al*., 1997) and western blotting (Aisa *et al*., 1998). The most commonly used are IFA and ELISA methods (Ashford *et al*., 1993; Fisa *et al*., 1997; Mancianti *et al*., 1995; Mancianti *et al*., 1996). In general, good sensitivities and specificities are obtained with these methods which are based mostly on the use of crude antigens. In an animal with compatible clinical signs, high antibody titers support the preliminary diagnosis. However, the presence of antibodies is not synonymous with patent disease (Fisa *et al*., 1999; Lanotte *et al*., 1979).

(c) Molecular methods

The use of a polymerase chain reaction (PCR) for the identification of *Leishmania spp*. DNA in tissues samples from dogs is performed using primers designed against a *Leishmania* sequence of the small-subunit rRNA gene (Mathis & Deplazes, 1995), against the constant region of *Leishmania* kinetoplast DNA (Ashford *et al*., 1995; Ozbel *et al*., 2000; Reale *et al*., 1999; Roura *et al*., 1999b), or against *Leishmania* DNA coding the 51-kD antigen (Berrahal *et al*., 1996). Several tissues have been studied: lymph

node (Mathis & Deplazes, 1995; Ozbel *et al*., 2000; Reale *et al*., 1999), bone marrow aspirates (Ashford *et al*., 1995; Roura *et al*., 1999b), whole blood (Mathis & Deplazes, 1995; Reale *et al*., 1999; Roura *et al*., 1999c), paraffin-embedded skin biopsies (Roura *et al*., 1999a), and frozen skin and eye conjunctiva (Berrahal *et al*., 1996) with varying results. PCR on bone marrow, lymph node, and skin seems to be a sensitive and specific method for the diagnosis of canine leishmaniosis (Mathis & Deplazes, 1995; Reale *et al*., 1999; Roura *et al*., 1999a; Roura *et al*., 1999b). PCR on whole blood seems to be less sensitive than the tissues mentioned above (Mathis & Deplazes, 1995; Reale *et al*., 1999; Roura *et al*., 1999c). PCR on aspirates of lymph node and bone marrow were more sensitive than other parasitological methods such as stained smears or parasite culture (Reale *et al*., 1999; Roura *et al*., 1999b; Zerpa *et al*., 2000). Other molecular methods such as nested-PCR are being developed to improve the diagnosis in cases of doubtful results obtained by conventional PCR (Katakura *et al*., 1998).

Treatment

During the last 50 years, the first choice drugs for treating human and canine leishmaniosis have been pentavalent antimony compounds (Herwaldt & Berman, 1992). In Europe, dogs are commonly treated with meglumine antimoniate (Glucantime, Rhône-Mêrieux, Lyon, France) alone (Riera *et al*., 1999; Valladares *et al*., 1998) or in combination with allopurinol at different regime doses (Alvar *et al*., 1994; Ferrer *et al*., 1995; Slappendel & Ferrer, 1998). Some authors have suggested the use of allopurinol alone (Liste & Gascón, 1995; Vercammen & de Deken, 1996). Other compounds such as amphotericin B (Oliva *et al*., 1995) or aminosidine (Poli *et al*., 1997) have also been used to treat canine leishmaniosis.

Prevention

In endemic areas the prevention of the disease is difficult and currently depends mainly on control of the insect vector, because of the lack of effective prophylactic drugs and vaccines (Dunan *et al*., 1989; Lasri *et al*., 1999). Measures suggested to protect the individual dog include keeping the animal indoors from one hour before sunset to one hour after dawn during the vector season and the use of repellents and insecticides for sand flies (Slappendel & Ferrer, 1998). Deltamethrin collars may protect dogs from bites of sand flies (Halbig *et al*., 2000; Killick-Kendrick *et al*., 1997).

Immune responses to *Leishmania* infection

Leishmania infection can result in either protective immunity or disease in rodents, humans and dogs. Studies in murine models (Liew & O'Donnell, 1993; Solbach & Laskay, 2000), humans (Kemp *et al*., 1996; Mossalayi *et al*., 1999) and dogs (Cabral *et al*., 1992; Pinelli *et al*., 1994b) have demonstrated that protective immunity against *Leishmania* is T cell mediated, while disease susceptibility is associated with the production of antibodies and the absence of cell mediated immunity.

In contrast to the abundant data existing about experimental murine leishmaniosis and human leishmaniosis, little information is available on canine immunology in general and on the immunological basis of *Leishmania* infection in the dog. This situation is

mainly due to the lack of adequate canine immunological tools (Cobbold *et al*., 1994; Cobbold & Metcalfe, 1994).

Humoral immunity

The disease in dogs is a consequence of a predisposition to develop a marked humoral non-protective immune response (Lanotte *et al*., 1979). The increase in serum immunoglobulins (Ig) appears to be mainly due to a polyclonal B cell activation, and their transformation in plasma cells (Martínez-Moreno *et al*., 1993). Only IgG and IgM have been studied in the dog, being the main Ig produced IgG (Martínez-Moreno *et al*., 1995). Ill dogs produce high levels of anti-*Leishmania* IgG antibodies that can be detected by serological techniques. In experimental infection, anti-*Leishmania* IgG antibodies are apparent between 1 to 4 months after challenge (Abranches *et al*., 1991; Martínez-Moreno *et al*., 1995; Nieto *et al*., 1999; Riera *et al*., 1999).

The relative levels of specific-*Leishmania* IgG1 and IgG2 antibodies have been reported to be prognostic indicators of cure and disease, associating IgG1 to the development of the disease and IgG2 to an asymptomatic infection (Bourdoiseau *et al*., 1997a; Deplazes *et al*., 1995).

Treatment of ill dogs induces clinical improvement, often accompanied by a decrease in the specific antibody levels (Lanotte *et al*., 1979; Mancianti & Meciani, 1988; Riera *et al*., 1999). However, in other cases clinical improvement has not been associated with decrease in the titre of specific antibodies (Ferrer *et al*., 1995).

The specific antigenic reactivity of these antibodies has been investigated extensively with the goal of improving the serological methods of diagnosing leishmaniosis, better understanding the host immune response, and identifying antigens for immunoprophylaxis. Researchers have used different strategies to study the antigenic reactivity from western blot using the whole antigen of *Leishmania* promastigotes to the study of recombinant proteins by serological techniques.

Both approaches have shown that the response is pleomorfic. In western blot, dogs with leishmaniosis have antibodies that react to polypeptide fractions of a *Leishmania* antigen with a molecular weight between 12 and 85 kD (Aisa *et al*., 1998). The most specific and sensitive antigen bands recognized by the sera of dogs with clinically patent leishmaniosis differ among investigators probably due to differences in the laboratory procedures (Abranches *et al*., 1991; Aisa *et al*., 1998; Carrera *et al*., 1996; Fernández-Pérez *et al*., 1999; Mancianti *et al*., 1995; Mary *et al*., 1992; Neogy *et al*., 1992; Rolland *et al*., 1994). Aisa and coworkers (1998) and other authors (Mancianti *et al*., 1995; Mary *et al*., 1992) found the highest sensitivity for bands 46, 30, 28, 14, and 12 kD. Proteins of 14 and 18 kD have been identified as nuclear proteins of the parasite (Suffia *et al*., 1995). The presence of antibodies detecting low antigen fractions is a marker of the early phases of the infection (Aisa *et al*., 1998; Berrahal *et al*., 1996). Moreover, the regression of the disease and decreased antibody titers is related to the disappearance of the lower or medium bands while higher molecular weight bands remain (Aisa *et al*., 1998; Riera *et al*., 1999).

Several studies of recombinant proteins focused on which *Leishmania* proteins seroreact with the antibodies of ill dogs. The proteins recognized by antibodies of dogs with clinically patent disease are: P2 (Soto *et al*., 1995a), P0 (Soto *et al*., 1995b), Hsp70 (Nieto *et al*., 1999; Quijada *et al*., 1996), Hsp83 (Angel *et al*., 1996), GRP94 (Larreta *et al*., 2000), rK39 (Badaró *et al*., 1996; Ozensoy *et al*., 1998; Pennica *et al*., 1997), gp63 (Morales *et al*., 1997), KMP-11 (Berberich *et al*., 1997), H2B (Soto *et al*., 1999), gp70 (Rhalem *et al*., 1999a), PSA (Boceta *et al*., 2000). On the other hand, sera from infected dogs without disease —specific-*Leishmania* cell-mediated immunity and low titers of anti-*Leishmania* IgG antibodies using a conventional ELISA (crude antigen)—, did not recognize proteins gp63, gp70 and rK39 by western blot or ELISA (Rhalem *et al*., 1999a). These proteins could serve, as markers of clinical patent disease but not as markers of asymptomatic infections.

Cellular immunity

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 (Cabral *et al*., 1992; Cabral *et al*., 1998; Cardoso *et al*., 1998) and experimentally infected with *Leishmania* (Abranches *et al*., 1991; Pinelli *et al*., 1994b). This result suggests that canine leishmaniosis can display a wide disease spectrum (Cabral *et al*., 1998) similar to that seen in human infection where clinical disease represents one pole and asymptomatic infection the other (Badaró *et al*., 1986).

Dogs with patent disease have high antibody titres, but no leishmanial specific lymphocyte proliferation and no delayed type hypersensitivity (DTH) reaction (Abranches *et al*., 1991; Martínez-Moreno *et al*., 1995; Pinelli *et al*., 1994b). Conversely, asymptomatic infected dogs produce specific lymphocyte proliferation, strong DTH reaction and variable anti-parasite antibody titers (Cabral *et al*., 1992; Cabral *et al*., 1998; Cardoso *et al*., 1998; Pinelli *et al*., 1994b).

Few studies have examined the cellular and humoral response of dogs before and after treatment (Moreno *et al*., 1999; Rhalem *et al*., 1999b). Rhalem and coworkers (1999b) found that dogs presented strong lymphocyte proliferation to *Leishmania* antigen at six months after treatment with pentamidine. On the other hand, Moreno and coworkers (1999) found that five months after therapy with amphotericin B, a lymphocyte proliferative response to *Leishmania* antigen was not evident in the patients. A positive lymphocyte proliferation in treated dogs was detectable only in the first month following treatment.

Lymphocytes populations

Infection of different strains of mice with *L. major* led to the discovery of protective and disease-promoting $CD4^+$ T helper cell sub-populations (Liew, 1989; Müller *et al*., 1989). Following the description of the two functionally distinct $CD4^+$T cell subsets, Th1 and Th2, the characterization of $CD4^+$ subpopulations playing a role in resistance (Th1) or susceptibility (Th2) to infection with *L. major* was possible. Upon antigenic stimulation, Th1 cells produce interferon gamma (IFN-γ) and interleukin-2 (IL-2) and Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (Mosmann & Coffman, 1989). IFN-γ and

IL-2 are involved in macrophage activation, which leads to parasite destruction, and the activation of B cells secreting the isotype IgG2a. IL-4 and IL-10 stimulate a polyclonal B lymphocyte response, which secretes, among others, IgE and the isotype IgG1, and inhibit some of the protective cellular responses. Both subsets may develop from the same T-cell precursor whose differentiation is influenced by the manner and enviroment in which precursors are stimulated. The precursor is a mature, naive $CD4^+$ T lymphocyte that produces mainly IL-2 upon initial encounter with the antigen. The most potent differentiation inducing stimuli are the cytokines themselves. IL-12, produced by activated macrophages, dendritic cells, and B cells is the principal Th1 inducing cytokine. In contrast, the development of Th2 cells from naive precursors is induced by IL-4 (Fig. 1) (Abbas *et al*., 1996; London *et al*., 1998; Sedlik, 1996).

There are few studies concerning the cytokine involvement in *L. infantum* infection in the dog. In experimentally asymptomatic infected animals, analysis of the cytokine secretion pattern of PBMC indicated that protective immunity to *L. infantum* is associated with a Th1-like response. Stimulation of PBMC from asymptomatic animals with parasite antigen resulted in higher production of IL-2, tumor necrosis factor alpha (TNF-α) (Pinelli *et al*., 1994b) and IFN-γ compared to ill dogs (Pinelli *et al*., 1995). PBMC from dogs with clinical patent disease failed to produce IFN-γ (Pinelli *et al*., 1995). These cells secreted IL-2 and TNF-α but in considerably lower amounts than their counterparts from asymptomatic dogs (Pinelli *et al*., 1994b).

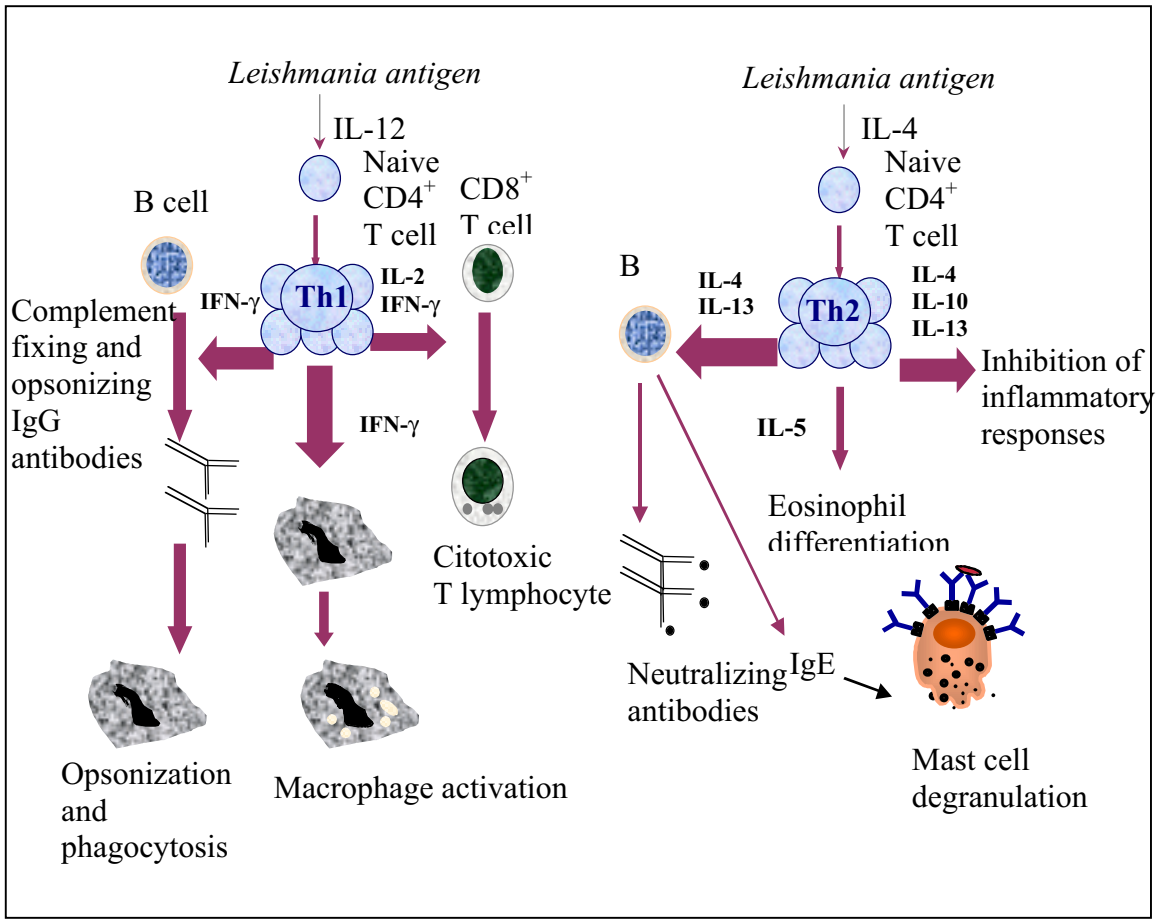
On the other hand, several studies have demonstrated that patent canine leishmaniosis induces changes in lymphoid subpopulations that are associated with the severe depression of T-cell function and with the high levels of *Leishmania* specific serum antibodies observed in ill dogs. A dramatic decrease of $CD4^+$ T-cells in canine leishmaniosis has been described and can be considered an important cause of the diminished cellular immunity (Bourdoiseau *et al*., 1997b; Moreno *et al*., 1999). The lack of a response or a low response to mitogens also reflects the severity of this immunodepression (Cabral *et al*., 1992; De Luna *et al*., 1999; Moreno *et al*., 1999).

Several studies have shown that $CD8^+$ T cells may play a role in protective immunity to leishmaniosis in mice (Bogdan *et al*., 1993; Liew & O'Donnell, 1993), humans (Mary *et al*., 1999) and dog (Pinelli *et al*., 1994a; Pinelli *et al*., 1995). The beneficial effects of $CD8^+$ T cells are probably due to the secretion of IFN-γ, which is required for the activation of macrophages and the suppression of Th2 cells (Chan, 1993; Müller *et al*., 1993). In the dog, $CD8^+$ T cells derived from asymptomatic infected dogs, but not from dogs with clinical patent disease, are capable of producing IFN-γ and can also lyse infected macrophages in a major histocompatibility complex (MHC)-restricted manner (Pinelli *et al*., 1994a; Pinelli *et al*., 1995). On the contrary, an increased proportion of $CD8^+$ T cells with active canine leishmaniosis has been reported (Bourdoiseau *et al*., 1997b; Moreno *et al*., 1999). Nevertheless, the $CD8^+$ T cell population is a heterogeneous group of cell types. $CD8^+TCR\alpha\beta^+$ cell levels as well as the percentage of CD8β chain expressing cells are increased in canine leishmaniosis (Moreno *et al*., 1999).

Contradictory results have been reported concerning B-cells populations in dogs with patent canine leishmaniosis. Bourdoiseau and coworkers (1997b) reported a reduction in the proportion of lymphocytes bearing B cell markers (CD21+/sIg+). Moreno and coworkers (1999) found an increase in the percentage of $sIgG^+$B-cells consistent with

the high levels of *Leishmania*-specific serum antibodies detected and an elevation of TCRγδ+cells. TCRγδ+cells have been associated with the B-cell induced humoral immune response including the secretion of high levels of factors involved in B-cell growth and differentiation (Raziuddin *et al*., 1992).

Fig 1. Effector functions of Th1 and Th2 subsets of $CD4^+$ helper T lymphocytes (Abbas *et al*., 1996)



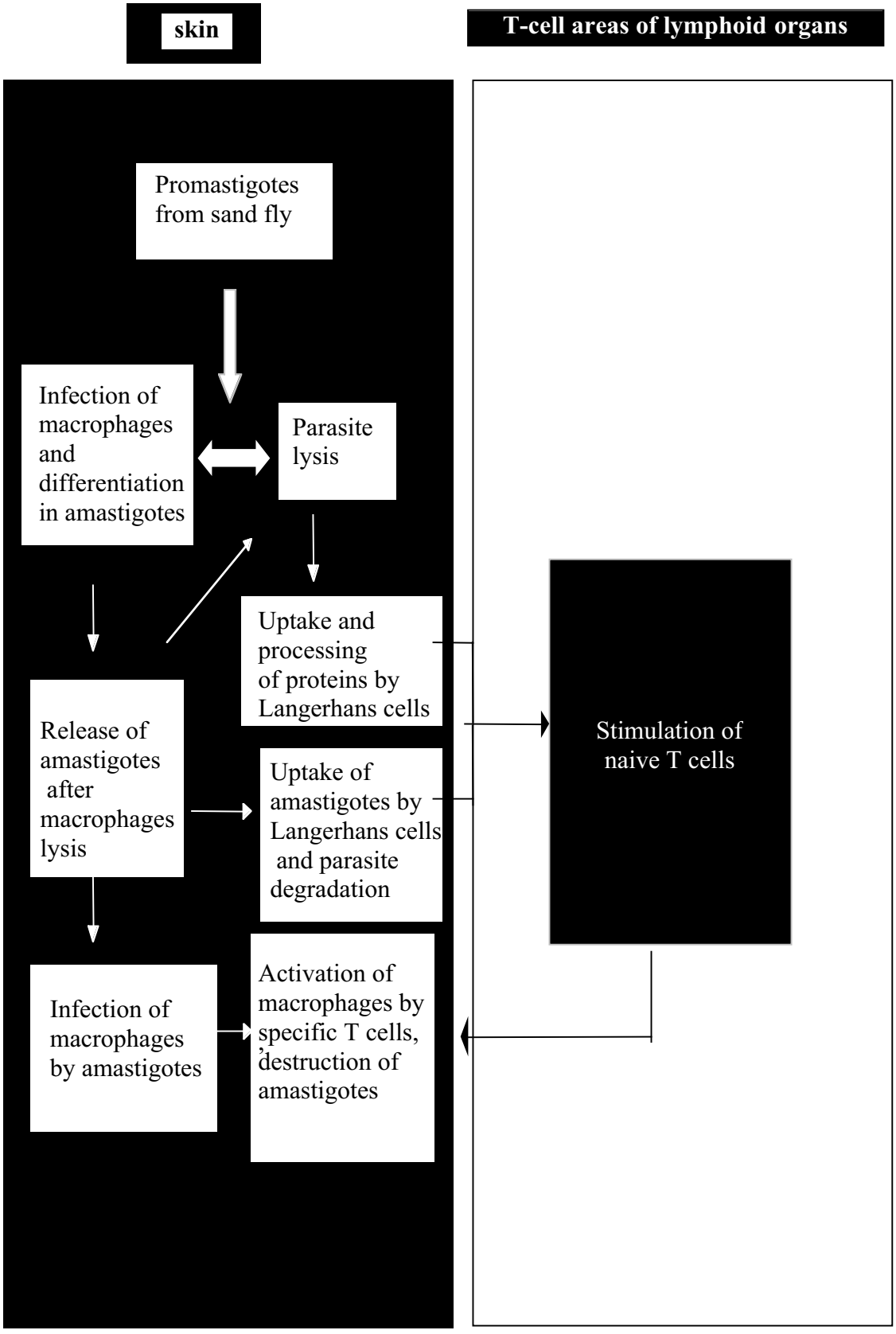
Macrophages, langerhans cells and polymorphonuclear cells (PMNC)

Based on *in vitro* and *in vivo* studies, it is widely accepted that macrophages play a central role in leishmaniosis (Alexander & Russell, 1992; Solbach *et al*., 1991). Lymphokines released by activated T cells regulate the antimicrobial potential of macrophages, which are the final effector cells. Canine *Leishmania* specific-T cell lines that secrete IFN-γ, IL-2 and TNF-α upon stimulation with parasite antigen are able to activate canine macrophage cell lines as evidenced by anti-leishmanial activity, which is mediated by L-arginine-dependent production of nitric oxide (NO) in these cells (Pinelli, 1996; Pinelli *et al*., 2000). Similar results on NO production and anti-leishmanial activity have found using monocyte-derived macrophages from healthy dogs (Panaro *et al*., 1998; Pinelli *et al*., 1995). Moreover, studies using monocyte-derived macrophages from dogs that have undergone successful chemotherapy have shown that these cells present an enhanced anti-leishmanial activity that correlates with the induction of NO synthase (Vouldoukis *et al*., 1996).

Macrophages serve not only as host cells for the parasites, but also as antigen-presenting cells (APC) that mediate the stimulation of specific T cells. T-cell proliferation and induction of effector functions require the recognition of peptide-MHC complexes by the T-cell receptor and interactions between costimulatory molecules on the APC (Bretscher, 1992; Jenkins, 1992). The cellular basis for T-cell unresponsiveness in leishmaniosis is still not fully understood. Recently, it has been reported the decreased proliferation of T-cell lines and IFN-γ production to cognate antigen when canine infected macrophages with *L. infantum* were used as APC. A decreased expression of costimulatory B7 molecules on infected APC was observed, but other surface molecules such as MHC class I and class II, did not change expression upon infection. These data suggest the down-regulation of B7 expression on infected APC as a way for this intracellular pathogen to evade the immune response of the host (Pinelli *et al*., 1999).

The usual site of entry for the *Leishmania* parasite into the mammalian host is the skin. The cutaneous immune response at the early stage of the infection is crucial to the course of the disease (Fig. 2). After deposition of *L. major* promastigotes in the skin by a sand fly, Langerhans cells migrate from the epidermis to the site of infection in the dermis and become active participants in the immune response once a significant parasite load has accumulated locally and substantial numbers of amastigotes have been released into the dermis (probably from macrophages) and, like macrophages, take up parasites (Overath & Aebischer, 1999). A proportion of Langerhans cells then transport parasites from the infected skin to the draining lymph node for presentation to antigen-specific resting T cells. As a result, activated T cells emigrate via the blood into the lesion, where infected macrophages and Langerhans cells that remain in the dermis regulate their effector activity by several mechanisms including cytokine secretion (Moll, 1993; von Stebut *et al*., 2000). Canine Langerhans cells are known to migrate to a regional draining lymph node after antigen uptake (Marchal *et al*., 1995). *L. infantum* parasitized Langerhans cells during canine leishmaniosis. Langerhans cells may be crucial for the initiation and the regulation of the local and general immune responses against *L. infantum* (Marchal *et al*., 1997).

Fig. 2. The *Leishmania* infection cycle (Overath & Aebischer, 1999)



The majority of immunological studies on leishmaniosis have focused on macrophage functions while the interactions between *Leishmania* and circulating phagocytes have not been well elucidated, although these cells can play an important role in carrying parasites from the inoculation site to internal organs (Hill, 1986). The role of PMNC in the immune defence against this parasite is still poorly understood. The percentage of phagocytosis of *L. infantum* promastigotes by PMNC from dogs with patent disease is higher than by PMNC from healthy non-infected dogs. This may be related to the presence of specific opsonizing antibodies in the serum of ill dogs. While PMNC and monocytes of ill dogs do show a lower killing activity in comparison to healthy dogs, treatment does restore parasiticidal activity to these cells (Brandonisio *et al*., 1996). Moreover, granulocytes of healthy dogs exhibit higher O_2 and H_2O_2 production than those of ill dogs. It is well known that *Leishmania* possesses many virulence factors, which impair phagocytic responses (Descoteaux, 1998). The reduced respiratory burst of granulocytes could be one of the factors in the severity of the disease in dogs with canine leishmaniosis (Brandonisio *et al*., 1996). Blood taken from dogs two months after exposure to *Leishmania* seasonal transmission revealed a significant decrease in the production of reactive oxygen intermediates compared with blood samples taken before exposure. These data indicate that immunological changes occur early in *Leishmania* infection in dogs (Vuotto *et al*., 2000).

Aim and outline of the thesis

The aim of the thesis is to gain more insights into the immunological and epidemiological aspects of *Leishmania infantum* infection in dogs living in endemic areas. The objectives of the study were: 1) To investigate the prevalence of *L. infantum* infection in dogs from an endemic region; 2) To evaluate the *Leishmania*-specific humoral and cellular immune responses in dogs living in an endemic area 3) To classify types of *Leishmania* immune responses and 4) To study the frequencies of types of immune responses in the canine population living in an endemic area.

In an effort to reach our goals, several works were developed and are described in chapters 2, 3, 4, 5 and 6. Chapter 2 describes the study of the humoral immune response (specific anti-*Leishmania* total IgG, IgG1 and IgG2 antibodies) from a wide range of canine populations (ill, treated and asymptomatic dogs) in order to clarify the usefulness, role and prognostic value of these Ig's in *Leishmania* infection. In chapter 3, we evaluated and compared the efficacy of two leishmanin preparations to detect dog *Leishmania* cellular-mediated immunity. Next, we looked into the hypothesis that the Ibizian hound was a dog breed genetically resistant to canine leishmaniosis because, more consistently than other breeds, it mounts a succesful cellular immune response to the parasite. Veterinarians practicing in Mallorca have reported very few cases of leishmaniosis in Ibizian hounds. To investigate this hypothesis, we compared the specific-*Leishmania* cellular and humoral immune response in Ibizian to dogs of other breeds from the island of Mallorca. We also investigated the prevalence of *Leishmania* infection in dogs living outdoors in the island of Mallorca using serology and leishmanin skin test. The results are described in chapter 4. Chapter 5 describes the evaluation of the prevalence of *Leishmania* infection, seroprevalence and prevalence of the disease in a canine population living in an endemic area, the island of Mallorca, using serology and detection of parasite DNA in several tissues: bone marrow, conjunctiva and skin. In chapter 6, we defined the immune profiles of these populations

of dogs using several parameters: anti-*Leishmania* IgG1, IgG2 and IgGtotal antibodies, LST, LPA and the production of cytokines. Finally, in chapter 7 these studies are summarized and discussed, resulting in a view on the immunological and epidemiological aspects of *Leishmania* infection in dogs living in endemic areas.

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