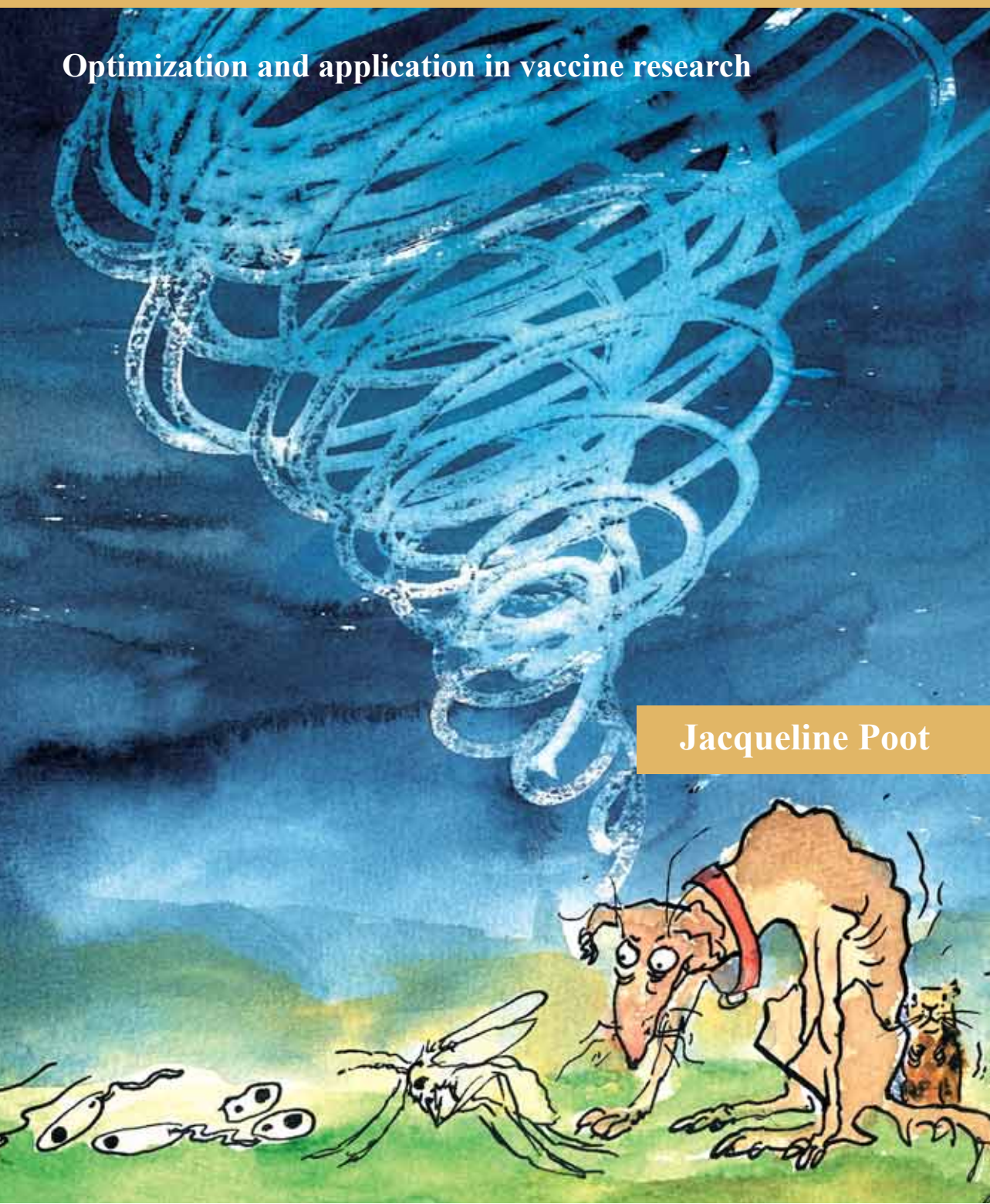
Experimental challenge models for canine leishmaniasis in hamsters and dogs,

Optimization and application in vaccine research



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Experimentele ziektemodellen voor canine leishmaniasis in hamsters en honden, optimalisatie en toepassing in vaccin onderzoek.

(met een samenvatting in het Nederlands)

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CHAPTER 1

Introduction and outline of the thesis

1. The genus Leishmania

Leishmaniasis is a term for a variety of disease manifestations caused by protozoan parasites of the genus *Leishmania*. These parasites can infect vertebrates after transmission by female sand flies of the genus *Phlebotomus* or *Lutzomyia*. In man, *Leishmania* spp. can cause chronic disease involving either the skin (cutaneous form) or internal organs (visceral form). For some species man is the only reservoir, most are however zoonotic with a reservoir in rodents or canines. *Leishmania infantum* and *Leishmania chagasi* are of veterinary importance, as they are not only the cause of zoonotic visceral leishmaniasis in man but also of a severe chronic wasting disease in dogs: canine leishmaniasis.

2. Life cycle

In the vertebrate host, *Leishmania* exists as a tiny (2.5-5.0 to 1.5-2.0 µm), ovoid to round organism, the amastigote, which resides in the phagolysosome of macrophages. Replication of amastigotes can cause host cells to rupture and release parasites capable of invading other macrophages. Parasitized macrophages are mainly detected in the skin and reticulo-lymphatic organs (lymph nodes, spleen, bone marrow, liver) but can be present at other locations. When a sandfly feeds on an infected host, it can ingest parasitized macrophages. Amastigotes released from macrophages in the gut of a sandfly differentiate into the flagellated promastigote stage. Promastigotes attach to the wall of the gut where they multiply and subsequently differentiate into the metacyclic promastigote stage that is infectious to the vertebrate host. By the time the female sandfly takes a new blood meal, the metacyclic promastigotes have affected the feeding mechanism of the sandfly in such a way that they cause regurgitation of midgut content –containing the parasites- into the feeding wound thereby ensuring efficient transmission to a vertebrate host (Rogers et al., 2002). Although most promastigotes will be rapidly eliminated by the host via complement-mediated killing (Dominguez et al., 2002), some are opsonised and taken up by macrophages. These organisms survive and differentiate into the amastigote form that will subsequently multiply and invade new cells. The complete life cycle of leishmania is summarized in Fig. 1.

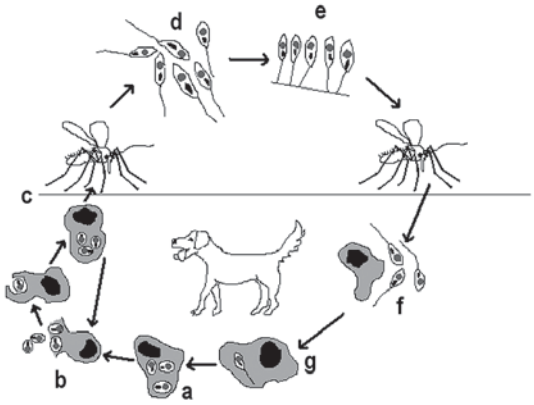

Figure 1. Life cycle of Leishmania.
The letters indicate (a) multiplication of amastigotes in host macrophages, (b) release of parasites and subsequent infection of other macrophages (c) ingestion of infected macrophages by the sand fly, (d) differentiation into and (e) multiplication of promastigotes in the sandfly gut, (f) transmission to the dog, and (g) ingestion by macrophages and differentiation into amastigotes.

3. Canine Leishmaniasis

3.1 geographical distribution

The geographical distribution of different *Leishmania* species is directly dependent on the local presence of the respective specific sandfly vectors. *L. infantum*, the cause of canine leishmaniasis in the "old world" is transmitted by *Phlebotomus spp*. that live mainly in the Mediterranean, the Middle East and North Africa. More specifically; foci of the disease exist in Albania, Algeria, Bosnia-Herzegovina, Chad, Croatia, Cyprus, Egypt, France, Greece, Islamic Republic of Iran, Iraq, Israel, Italy, Jordan, Lebanon, Libyan Arab Jamahiriya, Malta, Morocco, Portugal, Saudi Arabia, Spain, Syrian Arab Republic, Tunisia, Turkey and Yemen. Besides these regions, foci of low endemicity are found in Azerbaijan, Georgia, Kazakhstan, Turkmenia and some provinces of China. Between and even within all of these countries, the prevalence of leishmaniasis varies considerably; seroprevalence in domestic dogs in the Mediterranean basin is reported to range from 1.4% to 48.4% but is on average around 10% (Gradoni, 1999; WHO, 1990). In the "new world" canine leishmaniasis is caused by *L. chagasi*. This species is however indistinguishable from *L. infantum* and is considered to be the same parasite on the basis of genetic data (Mauricio et al., 1999). In the new world the parasite is transmitted by a different vector species: sandflies of the genus *Lutzomyia*. Foci are present in Bolivia, Brazil, Colombia and Venezuela and possibly also in Argentina, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama and Paraguay. Seroprevalence in dogs ranges from 24% to 67% in high endemic clusters (Miles et al., 1999; WHO, 1990).

3.2 Prepatent period

Of the low numbers (1 to 100) of metacyclic promastigotes that are delivered with the bite of a sandfly (Warburg et al., 1986), only few survive and transform into intracellular amastigotes. Therefore, it takes some time for the parasites to multiply and spread from the site of infection. As host factors influence parasite spread and multiplication, it may take between 2 months and many years for symptoms of disease to become apparent (Adler et al., 1934; Slappendel, 1988). The long and variable prepatent period complicates epidemiological and intervention studies in the field as well as experimental challenge models in the laboratory.

3.3 Clinical disease spectrum

Dogs suffering from leishmaniasis show skin problems, weight loss and/or decreased activity. Upon examination by the veterinarian, lymphadenopathy is found in 90% of cases (Ciaramella et al., 1997; Slappendel, 1988). Skin disease is also frequently found (89%) (Slappendel, 1988) and appears as dry exfoliative dermatitis (56%), ulcers (40%), diffuse alopecia (14%) and/or periorbital alopecia (18%) (Ciaramella et al., 1997). Less frequent symptoms (20 - 50% of the cases) include weight loss, anorexia, abnormal locomotion, conjunctivitis, splenomegaly and onychogryphosis. Other symptoms that may be present include (masticatory) muscle atrophy, diarrhea, epistaxis, ocular involvement (keratitis, panophthalmitis), and hyperthermia. In the final stages of the disease, signs of renal failure may develop, such as anorexia, polyuria/ polydipsia and vomiting.

3.4 Pathofysiology

In susceptible animals, the parasite elicits a strong humoral immune response that can even end in uncontrolled polyclonal B-cell activation. The resulting high concentration of circulating immunoglobulin does not protect the dog but rather harms it. Besides the possible occurrence of several types of auto-antibodies, immune complexes may be formed that can precipitate at diverse locations causing specific pathology. In the kidney, precipitated immune complexes may cause glomerulonephritis. This is often found in the advanced stages of the disease and likely to be the ultimate cause of death (Nieto et al., 1992; Slappendel, 1988). Less frequently, immune complexes precipitate in the walls of blood vessels causing vasculitis (Pumarola et al., 1991). Immune complexes and specific auto-antibodies are also suspected to be involved the development of leishmania-associated polymyositis (Vamvakidis et al., 2000). Two theories exist on the etiology of leishmania-associated polyarthritis; the first theory is that immune complexes are deposited in the synovium, leading to synovitis and polyarthritis via a type III hypersensitivity reaction. The second theory is that a granulomatous reaction is caused by the presence of parasites at this location (Ferrer, 1992). Immune complexes may also bind to blood cells thereby decreasing their life span and causing anemia, leucopenia and thrombocytopenia (Miescher et al., 1982). If cryoglobulins are produced in the course of the infection, these may precipitate in the blood vessels of extremities when exposed to low temperatures, causing local ischemic necrosis (Slappendel et al., 1990).

Few investigators have proposed possible causes for the weight loss that is generally associated with leishmaniasis. Local infiltration of infected macrophages in the mucosa of the gut has been proposed (Adler et al., 1934). However, competition for essential amino acids (Keenan et al., 1984) and proteinuria due to glomerulonephritis (Nieto et al., 1992), are also likely to play a role.

The abnormal locomotion that is sometimes observed during infection may be caused by polyarthritis and/or bone lesions (periosteal and intramedullary proliferation) (Agut et al., 2003). Although polymyositis and muscle wasting has been proposed to be another cause of abnormal locomotion, this was not confirmed in a recent study (Vamvakidis et al., 2000).

Skin disorders in dogs suffering from leishmaniasis are diverse, ranging from alopecia and scaling to nodular lesions and ulcers. The presence of parasites in the canine skin generally causes a diffuse non-suppurative dermatitis which results in alopecia and desquamation. Both the nodular and the ulcerative form of skin involvement are rare in dogs. In man, ulceration has been explained as being a consequence of the release of parasites in or near the dermis and the resulting strong inflammatory reaction. The formation of nodules also occurs in cutaneous leishmaniasis in man. Since these lesions contain highly parasitized macrophages it is hypothesized that this indicates a very ineffective host response (Ferrer et al., 1988a).

4. Diagnosis of Canine leishmaniasis

4.1 Clinical diagnosis

Although individual symptoms of leishmaniasis are mostly non-specific, a dog exhibiting a combination of the above mentioned signs is likely to be adequately diagnosed by those that are familiar with the disease. However, depending on the symptoms that are present, leishmaniasis may be difficult to distinguish from infectious diseases like ehrlichiosis, hepatozoonosis or babesiosis. Co-infections with these parasites may also occur. Immune-mediated diseases like polyarthritis, hemolytic anemia or systemic lupus erythematosus, skin diseases like demodicosis or dermatophytosis, endocrine disorders and malignancies like myelo- and lymphoproliferative diseases can also cause similar clinical manifestations.

4.2 Laboratory diagnosis

Due to the rather variable clinical presentation of Leishmaniasis, any clinical suspicion of disease should be confirmed in the laboratory. These tests are either based on identification of the parasite or on serology. The detection of parasites is the 'gold standard' for diagnosis, its sensitivity however is not high.

4.2.1 Parasitological methods

Parasites in clinical samples can be demonstrated either directly or after multiplication in *in vitro* culture. Microscopic examination of Giemsa-stained smears prepared from bone marrow or lymph node aspirates is a classical method with high specificity but low sensitivity, approximately 60%. Examination of histological samples taken from skin, lymph node, spleen or liver is also of use; the histopathological picture however is not specific for leishmaniasis and only the presence of amastigotes can confirm the diagnosis. As the number of amastigotes in tissue samples is often very low, diagnosis can be difficult. The sensitivity of histopathology can be increased by applying immuno-histochemistry using specific antibodies to detect the presence of *Leishmania* in tissue samples (Ferrer et al., 1988b; Sells et al., 1981).

Samples of bone marrow, lymph node and/or spleen can be cultured *in vitro* at allowing the transformation of amastigotes to promastigotes and subsequent multiplication of the latter. As multiplication of parasites occurs in culture, this enhances the chances of parasite detection in clinical samples. Furthermore the method is 100% specific as the identity of promastigotes in culture can not be mistaken. On the other hand, many factors can influence parasite growth and depending on the circumstances cultures may falsely be scored negative. Factors influencing the success of culture methods are: the type of culture medium, the length of the incubation period, the amount of sample inoculated in a given amount of medium and the number and type of samples taken. Still, when optimal conditions are met, the sensitivity of parasite culture may approach 100% (Gradoni, 2002; Mathis et al., 1995).

In more recent years, the use of PCR for parasite detection has gained much attention. Several different primer combinations have been tested, some targeting genomic DNA sequences, others the kinetoplast of which several thousand copies are present in each parasite.

The latter type of PCR test is generally found to be the more sensitive (factor 500 to 5000) (Lachaud et al., 2002b; Lachaud et al., 2002a). Many different types of samples have been used for these tests including bone marrow, lymph node, spleen, skin, peripheral blood and even conjunctival swabs (Ashford et al., 1995; Barrouin-Melo et al., 2004; Reale et al., 1999). In general the specificity as well as sensitivity of the tests is estimated to be close to 100%. In the field, evaluation of PCR tests is hampered by the absence of a real "gold standard". Due to the occurrence of extremely low parasite burdens, non-specific clinical signs and late development of serological responses it is often not clear whether a dog should be designated leishmania positive and it therefore remains uncertain whether a PCR result is true or false positive.

In experimental challenge systems, the parasite burden is widely used as a parameter of disease severity. Several methods have been described for quantification of parasites in biological samples. The method described by Stauber (Stauber, 1958) is based on microscopic examination of Giemsa stained imprints of spleen and/or liver. The parasite burden is expressed in Leishman Donovan Units (LDU), wherein LDU = the number of amastigotes per 1,000 nucleated cells × organ weight (in grams) × 2 × 10^5. A second method was first described by Titus (Titus et al., 1985) and involves the culture of limiting dilutions of spleen or liver tissue in a 96-well format. From the concentration of tissue per millilitre of medium and the last well still containing promastigotes the number of parasites per gram of tissue can be calculated.
The use of Real-Time PCR for quantification of leishmania has recently been reported (Bretagne et al., 2001; Schulz et al., 2003).

4.2.2 Serological methods

Because of the ease of serum sampling and testing, serological methods are widely used for clinical as well as epidemiological purposes. Many different methods have been developed, differing in the type of antigen and/or the detection system used. Antigen may consist of whole killed parasites, total soluble antigen or purified natural or recombinant leishmania antigens. Detection systems used include Direct Agglutination Test (DAT), Immune Fluorescence Assay (IFA) and several modifications of the Enzyme Linked ImmunoSorbent Assay (ELISA). Evaluation revealed relatively high sensitivity (99.5-100%) and specificity (95-100%) without great differences between the different serological assays tested (el Harith et al., 1989; Mancianti et al., 1995; Rachamim et al., 1991; Vercammen et al., 1997).

5. Treatment of canine leishmaniasis

Several classes of drugs are available for treatment of leishmaniasis. Use of these drugs in canine leishmaniasis often has limited success. Although temporary clinical improvement can be achieved, the occurrence of relapses is the rule rather than the exception. Moreover, treatment is usually not associated with parasitological cure and infectivity to the sandfly vector is not completely abrogated.

Meglumine antimoniate (Glucantime®) is widely used for the treatment of leishmania infected dogs. Intramuscular injection of the drug can cause severe local side effects, whereas systemic side effects include gastrointestinal disturbances, muscular pain and joint stiffness.
A serious disadvantage of treatment with antimonials, besides the high cost and toxicity, is the development of resistant strains that pose a threat to both humans and dogs (Gramiccia et al., 1992).

The primary use of Amphotericin B is as an anti-fungal drug, but it is also active against some protozoa. Amphotericin B binds preferably to ergosterol in the leishmania cell membrane but also binds to cholesterol in the host cell membrane, which in part explains its toxic effects. The main side effect in dogs is nephrotoxicity.

Allopurinol is an oral purine analog that is metabolised to nucleotides and incorporated into the parasite RNA. This leads to degradation of the RNA and thus disruption of protein synthesis. The compound has very few side effects in dogs, is relatively cheap and can be administered orally. Clinical remission is achieved in most dogs under continuous treatment, but the parasite remains present (Koutinas et al., 2001).

Hexadecylphosphocholine (miltefosine) accumulates in macrophages and is active against *Leishmania*. It is successfully used as an oral treatment for visceral leishmaniasis in humans but severe side effects in dogs prevent its application against canine leishmaniasis. Structurally closely related substances may prove to be more useful in dogs (Klarhof, 2005).

6. Immune responses against leishmaniasis

6.1 Immunity against cutaneous leishmaniasis

By far the most frequently used model for leishmaniasis is that of *L. major* (causing cutaneous leishmaniasis) in mice. Most inbred mouse strains are resistant to the infection and the local cutaneous lesion heals spontaneously. After healing, animals are clinically healthy and show a strong, long-lasting immunity to the parasite. It was shown that this immunity could be transferred to naïve mice with T- lymphocytes but not by transfer of antibodies or B-lymphocytes (Alexander et al., 1980; Rezai et al., 1980). Further evidence for the importance of cellular responses comes from observations in natural as well as experimental leishmaniasis that resistant individuals show low levels of specific antibodies and a high reactivity in delayed type hypersensitivity tests, whereas this is reversed in cases exhibiting clinical disease (Behforouz et al., 1983; Hale et al., 1981). Together this has led to the view that cell-mediated but not humoral immunity is essential for protection against leishmaniasis.

Both CD4+ and CD8+ T cells have been shown to be important for protection in experimental leishmaniasis (Stern et al., 1988). CD4+ T cells can be divided into two subsets, T helper 1 and 2 cells (Th1 and Th2), that can be distinguished by the cytokines they produce.

In mice the proliferation of Th1 cells is essential for resistance to a *Leishmania* infection, whereas Th2 cells mediate susceptibility. Cytokines like interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) that are produced by Th1 cells, have been shown to play a major role in protective immunity through macrophage activation (Brunda, 1994; Murray et al., 1983; Nacy et al., 1991). *Leishmania* amastigotes reside in phagolysosomes inside macrophages. For the host to be able to control the parasite, infected macrophages need to be activated to induce parasite killing through the production of reactive oxygen intermediates and nitric oxide. Several Th1 type cytokines (IFN-γ, TNF-α) are strong inducers of the macrophage enzyme inducible Nitric Oxide Synthase (iNOS) that produces nitric oxide. In mice that mount a Th2 mediated immune response, characterized by the production of IL-4, IL-10 and IL-13, macrophages are not activated and the parasite is not killed. These animals die of fulminant nonhealing infections (reviewed by: (Sacks et al., 2002)).

6.2 Immunity against visceral leishmaniasis

Models to study the immune response against visceral forms of leishmaniasis are more complicated and thus knowledge is limited. BALB/c mice infected with *L. donovani* or *L. infantum* are often used to study visceral disease as this mouse strain is considered to be susceptible. However, although the infection progresses during the first two weeks after challenge, the parasite is then controlled by the host immune response. In man and dogs however, visceral leishmaniasis manifests itself as a spectrum of diseases ranging from self-healing infections to uncontrolled progressive disease. In order to study progressive disease, a hamster model of infection is used. In this model challenge with *L. donovani* or *L. infantum* yields clinical manifestations similar to those seen in humans and dogs. However, the availability of immunological tools for hamsters is very limited and the usefulness of the model is therefore restricted. In the BALB/c model, IFN-γ and IL-2 were found to be key factors in the development of protective responses. In contrary to the findings in cutaneous leishmaniasis models, IL-4 appears not to be an important factor mediating susceptibility to visceral leishmaniasis. Another Th2 cytokine, IL-10, was found to be related to susceptibility in the BALB/c model as well as in man (reviewed by: (Goto et al., 2004)). In hamsters, the total absence of specific T-cell responses after infection has been noted by several investigators (Rodrigues, Jr. et al., 1998; Vasconcellos et al., 1996). T-cells as well as macrophages have been implicated to mediate this apparent immune suppression.

6.3 Immune responses in leishmania infected dogs

The immune response of *Leishmania* infected dogs has not been thoroughly investigated. Research in this area been hampered not only by the cost and complications associated with the dog infection model, but also by the very limited availability of immunological tools.

Field studies show that in parasitologically confirmed cases of leishmaniasis, *Leishmania* specific antibodies are virtually always detected (el Harith et al., 1989; Mancianti et al., 1995). Although antibody concentration appears to correlate positively with the parasite burden, antibodies probably do not play an important role in either the clearance of the infection or the establishment of immunity to reinfection.

Cellular responses against *Leishmania* have been detected in dogs and this type of immunity appears to be associated with resistance to infection. *Leishmania*-specific lymphocyte proliferation was first detected by Cabral (Cabral et al., 1998) in asymptomatic dogs from an endemic region. Similarly, delayed type hypersensitivity responses were found in asymptomatic dogs (Cardoso et al., 1998). The existence of cellular responses in infected dogs was confirmed by Pinelli who found specific lymphocyte proliferation in experimentally infected asymptomatic animals but not in symptomatic dogs (Pinelli et al., 1994). No specific lymphocyte proliferation was found in dogs exhibiting clinical signs of leishmaniasis either after natural or experimental infection (Martinez-Moreno et al., 1995; Rhalem et al., 1999b). Moreover, treatment of naturally infected symptomatic dogs resulted in clinical improvement which coincided with the occurrence of positive lymphoproliferative responses (Rhalem et al., 1999b).

Indications for a role of Th-1 type cytokines in resistance to infection were found by Pinelli. After stimulation with leishmania antigen, interleukin-2 activity was detected in the supernatant of lymphocytes of infected asymptomatic dogs at significantly higher levels compared with control uninfected or infected symptomatic dogs. Tumor Necrosis Factor-α activity on the other hand, was found to be significantly reduced in symptomatic animals compared to asymptomatic and non-infected control dogs (Pinelli et al., 1994).

In conclusion, from what is known to date it appears that some dogs are able to resist the development of leishmaniasis after infection with the parasite and that this resistance is associated with the development of cellular immune responses that are likely of the Th-1 type.

7. Control strategies for leishmaniasis

7.1 Vector and reservoir control

Control of transmission can be achieved by targeting the vector and/or the host reservoir. Insecticides are an important tool for controlling sandfly vectors; these can be applied inside houses and animals shelters, as well as outdoors on sandfly breeding and resting sites. In addition, individual protective measures like bednets, mosquito coils and deltamethrin-impregnated dog collars (Gavgani et al., 2002) can be of use.

Control of leishmaniasis by controlling reservoir hosts is recommended by WHO. Where man is the reservoir host (*L. donovani, L. tropica*) active case detection, treatment, and measures to prevent reinfection should reduce transmission. For zoonotic visceral leishmaniasis control is directed toward dogs. In Brazil dogs are screened for *Leishmania* specific antibodies and positive dogs are culled. The effect of this control method on the incidence of zoonotic disease is however low; this is in part due to the long interval between testing and culling, and the high incidence of infection in dogs (Courtenay et al., 2002; Reithinger et al., 2004).

Vaccination of dogs is generally thought to be the best option for decreasing both the incidence of canine leishmaniasis and of zoonotic visceral leishmaniasis (Courtenay et al., 2002; Dye, 1996).

7.2 Leishmaniasis vaccines

7.2.1 Live vaccines

In mice and humans, vaccination against cutaneous leishmaniasis with live *L. major* parasites yields a protective immune response. In some endemic areas, individuals infect themselves with virulent *L. major* on body regions where the resulting scar is hidden ("leishmanization"). Treatment is generally not applied after leishmanization as long lasting protective immunity is only achieved when the infection is allowed to run its full course. The vaccination procedure therefore does not differ from a natural infection except for the choice of body region.

In mice, vaccination with attenuated strains of *L. major* has been shown to confer protection against challenge with a virulent strain. The attenuating defect of these lines produced by prolonged *in vitro* cultivation or chemical mutagenesis is not defined, and the vaccine strains may revert to virulence (Marchand et al., 1987). A dihydrofolate reductase-thymidylate synthase null mutant (*dhfr-ts-*) was designed to combine protective capacity with an improved safety profile. This mutant strain was unable to cause disease in both susceptible and immunodeficient mice, and could elicit substantial resistance to subsequent challenge with virulent *L. major* (Titus et al., 1995).

More recently, cysteine peptidase-deficient mutant *L. mexicana* strains were generated using targeted gene deletion. Mice infected with the different mutant strains developed either very slow growing small lesions or no lesions at all. Moreover mice vaccinated with attenuated strains were partially protected from a challenge with wildtype parasites. (Alexander et al., 1998).

Another system of live vaccination is based on the incorporation of a thymidine-kinase gene in the parasite genome. This confers susceptibility to ganciclovir, enabling elimination of the vaccine strain. In mice, vaccination with live mutant *L. major* parasites and subsequent treatment with ganciclovir 4 days later conferred complete protection against subsequent challenge with wild type parasites; treatment after 1 day however resulted in partial protection (Muyombwe et al., 1998).

7.2.2 Whole killed and sub-unit vaccines

Different approaches have been used, or are being used, to develop a leishmaniasis vaccine for use in man. From what is known in the mouse model of infection, a successful *Leishmania* vaccine should probably induce a stable and long-lasting Th1-type cellular immune response. The safety, immunogenicity and efficacy of vaccines consisting of whole killed promastigotes have been tested in the field against Cutaneous *Leishmaniasis* with variable success (Table 1). For these vaccines to be efficacious they need to be combined with a strong (Th1 inducing) adjuvant. In all studies reported thus far, live Bacillus Calmette-Guérin (BCG) has been used as adjuvant.

Table 1: Candidate whole cell vaccines

Antigen	Author	Adj.	Host	Challenge	Result
5 strains	(Mayrink et al., 1985)	BCG	Human	Field (several spp.)	Partial prot.
5 strains	(Armijos et al., 1998)	BCG	Human	Field (several spp.)	Partial prot.
ALM	(Sharifi et al., 1998)	BCG	Human	Field: (*L. major* / *L. tropica)*	No prot.
ALM	(Momeni et al., 1999)	BCG	Human	Field: (*L. major* / *L. tropica*)	No prot.
ALM	(Khalil et al., 2000)	BCG	Human	Field: (*L. donovani*)	No prot.
ALA	(Armijos et al., 2004)	BCG	Human	Field: (*L. viannia*)	No prot.

"5 strains": cocktail of 5 Brazilian Leishmania strains *L. amazonensis, L. major, L. braziliensis*, killed and sonicated (Leishvacin®). ALA: Autoclaved *Leishmania amazonensis* promastigotes (Leishvaccin®) ALM: Autoclaved *Leishmania major* promastigotes. BCG: bacille Calmette-Guérin.

Many vaccination trials using (partially) purified or recombinant proteins have been performed in murine models for leishmaniasis (summarized in Table 2). In most cases powerful adjuvants like recombinant IL-12, *Corynebacterium parvum (Propionibacterium acnes)*, and Freunds Complete Adjuvant (FCA) were used to enhance the immune response. Only few of these vaccine candidates have been tested in dogs, at present none have been tested in humans.

Table 2: Candidate sub-unit vaccines

Antigen	Author	Adjuvant	Host	Challenge	Prot.
Gp46/M-2	(Champsi et al., 1988)	*P. acnes*	Mice: BALB/c C57BL/6	*L. amazonensis*	partial
PSA-2	(Handman et al., 1995)	*P. acnes*	Mice: C3H	*L. major*	partial
rLcr1	(Wilson et al., 1995)	FCA	Mice: BALB/c	*L. chagasi*	partial
P-8	(Soong et al., 1995)	*P. acnes*	Mice: BALB/c	*L. amazonensis*	partial
P-4	(Soong et al., 1995)	*P. acnes*	Mice: BALB/c	*L. amazonensis*	partial
rGp63	(Rivier et al., 1999)	none	Mice: CBA	*L. major*	partial
FML (gp36)	(Paraguai et al., 2001)	saponin	Mice: BALB/c	*L. donovani*	partial
rLACK	(Melby et al., 2001)	rIL-12,	Mice: BALB/c	*L. donovani*	none
rLACK	(Coelho et al., 2003)	rIL-12	Mice: BALB/c	*L. amazonensis*	none
rLeish-111f	(Skeiky et al., 2002)	MPL-SE® rIL-12	Mice: BALB/c	*L. major*	partial
rCP	(Pollock et al., 2003)	rIL-12	Mice: BALB/c	*L. mexicana*	partial
rA2	(Coelho et al., 2003)	rIL-12	Mice: BALB/c	*L. amazonensis*	partial

Gp46/M-2: purified membrane protein of L. amazonensis; rPSA-2: purified membrane protein of L. major; rLcr1: unidentified recombinant protein of L. chagasi; P-8 and P-4: purified amastigote specific antigens of L. pifanoi; rGP63: recombinant major surface antigen of L. major; FML: fucose mannose ligand, glycoprotein complex purified from L. donovani; rLACK: recombinant L. donovani homolog of receptors for activated C kinase; rLeish-111f: recombinant fusion protein of TSA, LmSTI1 and LeIF proteins of L. major; rCP: recombinant cysteine peptidase B of L. mexicana; rA2: amastigote specific antigen of L. donovani; FCA: Freund's complete adjuvant; rIL-12: recombinant murine IL-12; MPL-SE: monophosphoryl lipid A plus squalene; Prot.: induced protection against challenge.

7.2.3 DNA vaccines

DNA vaccines in general have been shown to be able to induce long-lasting cellular immune responses, including the induction of CD8+ T cells. This offers potential advantages over the use of protein-based vaccines (reviewed by: (Gurunathan et al., 2000)). Immune stimulating bacterial-derived unmethylated cytosine-phosphate-guanosine (CpG) motifs were shown to specifically induce Th1 responses and even change a lethal Th2 type response in *L. major* infected BALB/c mice into a curative Th1 response (Zimmermann et al., 1998). These CpG motifs can be added to DNA vaccines to potentiate Th1 responses. Several candidate DNA vaccines have been tested in mouse models of infection with sometimes conflicting results (Table 3).

Table 3: Candidate DNA vaccines

Antigen	Authors	Application	Host	Challenge	Prot.
Gp46	(McMahon-Pratt et al., 1993)	*Vaccinia*	Mice: BALB/c	*L. amazonensis*	partial
Gp63	(Xu et al., 1995)	*Salmonella typhimurium*	Mice: BALB/c	*L. major*	partial
LACK	(Gurunathan et al., 1997)	plasmid	Mice: BALB/c	*L. major*	partial
PSA-2	(Sjolander et al., 1998b; Sjolander et al., 1998a)	plasmid	Mice: BALB/c	*L. major*	partial
PSA-2	(Noormohammadi et al., 2001)	plasmid	Mice: C3H/He BALB/c	*L. major*	none.
CP	(Rafati et al., 2001)	plasmid + rec. protein	Mice: BALB/c	*L. major*	partial
ORFF	(Sukumaran et al., 2003)	plasmid	Mice: BALB/c	*L. donovani*	partial

Gp46: L. amazonensis gene for promastigote surface antigen; Gp63: L. major gene for major surface protein; LACK: L. major gene for homolog of receptors for activated C kinase; PSA-2: L. major gene for parasite surface antigen 2; CP: L. major genes for cysteine peptidases A and B; ORFF: unidentified gene of L. donovani; Prot.: induced protection against challenge.

7.2.4 Other vaccine approaches

Like many blood-feeding arthropods, sandflies have evolved sophisticated methods to circumvent host haemostatic as well as inflammatory/immune mechanisms. For this purpose, sandfly saliva contains molecules with anti-coagulant- and vasodilator properties as well as immune modulators and inflammation inhibitors (reviewed by:(Gillespie et al., 2000; Ribeiro, 1995)). For different parasite-vector combinations it was found that sandfly saliva modifies the host response to *Leishmania* (Lima et al., 1996; Theodos et al., 1991; Warburg et al., 1994). Not only could the infection be enhanced by co-administration of parasites and salivary gland lysate, immunization of naïve mice with saliva –either by injection or by the bite of uninfected sandflies- was shown to partially protect from challenge infection with *L. major* (Kamhawi et al., 2000). A recombinant saliva protein was also shown to confer protection against *L. major* in mice (Valenzuela et al., 2001). In a study in dogs however, the addition of sandfly saliva to an intradermal infection with *L. infantum* promastigotes had no measurable effect on disease severity (Paranhos-Silva et al., 2003).

8. Prevention of leishmaniasis in dogs

As described above, reduction of the canine reservoir of *L. infantum* can reduce the incidence of zoonotic visceral leishmaniasis. However, as many dogs have high emotional or economical value to their owners, prevention of leishmaniasis in dogs is an important goal in itself, regardless of the impact on zoonotic disease. A vaccine is generally regarded the most practical and efficacious means to prevent leishmaniasis in dogs.

8.1 Vector control

The topical application of permethrin, deltamethrin or fenthion can protect dogs from sandfly bites (Reithinger et al., 2001). As mentioned above, an impregnated dog collar (Scalibor® ProtectorBand) that releases deltamethrin over an extended period was shown to provide protection from (> 90%) of sand fly bites for over 6 months (Halbig et al., 2000; Killick-Kendrick et al., 1997). Field trials have shown that the application of the collars can reduce the incidence of canine leishmaniasis (Maroli et al., 2001; Reithinger et al., 2004). Disadvantages of this method include the possibility that a collar is lost, and the need for renewal of the collar after 6 months.

8.2 Vaccination

Only very few candidate vaccines against leishmaniasis have been tested in dogs. Dunan (Dunan et al., 1989) studied the effect of immunization with a partially purified *L. infantum* preparation in seronegative dogs from a holoendemic area. In the first year of the study, the rate of infection in the vaccinated group was significantly higher than in the control group. In the second year this difference disappeared. A more successful vaccination experiment was reported by Mohebali (Mohebali et al., 1998). Dogs were immunized with autoclaved *L. infantum* or *L. major* parasites in combination with live bacille Calmette-Guérin (BCG).

This conferred protection against an experimental challenge infection with virulent *L. infantum* in 7 out of 8 dogs. A vaccine consisting of merthyolated, sound-disrupted *L. braziliensis* promastigotes and BCG was reported to confer protection against experimental challenge with *L. chagasi* in 9 out of 10 dogs (Mayrink et al., 1996). Disappointingly, a field trial performed with this vaccine in Montes Carlos, Brazil, did not result in statistically significant protection of dogs. The incidence rate in the vaccinates (*n*=855) was calculated to be 62.4 per thousand dog-years whereas in the controls (*n*=908) the incidence rate was 68.4 per thousand dog-years (Genaro et al., 1996; Mayrink et al., 1996).

More defined candidate antigens have also been tested: a preparation consisting of a glycoprotein-enriched fraction of *L. donovani* promastigotes (Fucose-Mannose Ligand: FML) was claimed to protect 92% of vaccinated dogs. The published data however, suggest that protection was rather in the range of 50-70% (da Silva et al., 2000).

Four recombinant antigens have been tested in dogs. The first recombinant antigen tested is the *Leishmania* homologue for the receptor of activated C Kinase (LACK). Dogs were given a priming vaccination with plasmid LACK-DNA and a booster injection with recombinant Vaccinia Virus-LACK. Four out of five animals in the vaccinated group were protected from challenge with *L. infantum* (Gonzalo et al., 2002)., The second, chimeric protein Q, consists of 5 antigenic determinants derived from *Leishmania infantum* histone- and ribosomal proteins. Vaccination with this recombinant protein in combination with bcg protected 90% of dogs in a lab trial (Molano et al., 2003). The effect of BCG alone was not studied. Another vaccination-challenge experiment was performed with DNA of *L. infantum* cysteine peptidases A and B followed by a booster with recombinant CPA and CPB protein. Ten out of ten vaccinated dogs were found to be protected from challenge (Rafati et al., 2005). The multi-subunit recombinant protein Leish-111f combined with MPL-SE or Adjuprime® as adjuvant was tested in dogs that were exposed to natural challenge. Protection was not induced by either vaccine preparation (Gradoni et al., 2005).

9. Laboratory models for Leishmania infantum infection

As mentioned before, models for visceral leishmaniasis are problematic. Although mice can be infected with *L. infantum*, an immune response develops and clears the parasite a few weeks after challenge. This is in contrast to the lethal uncontrolled dissemination of the parasite seen in dogs. An alternative model species is the hamster, which is very susceptible to *L. infantum.* The almost complete lack of immunological tools for hamsters is a serious disadvantage and it is unclear to what extent development (or the lack thereof) of immune responses in hamsters and dogs are comparable.

For canine leishmaniasis research the dog is undoubtedly the best model species. Besides this, the dog is also regarded to be a good model for visceral leishmaniasis in humans.

As research in the human field relies on artificial parasite-host models, this area would benefit greatly from developments in the veterinary field. Disadvantages however include cost, limited immunological tools, long incubation period and ethical considerations. These are probably the most important reasons for the limited number of experiments that have been performed in dogs.

9.1 Hamsters

Challenge of hamsters with *L. infantum* is generally performed via intraperitoneal or intracardial injection of relatively high numbers of parasites. The infection is usually successful in all hamsters, but dissemination of the parasite and development of disease symptoms depend on the size of the initial inoculum (Binhazim et al., 1993; Mendez et al., 2001; Requena et al., 2000; Rica-Capela et al., 2003). In comparison to infection with promastigotes, challenge with amastigotes leads to a more rapid increase in parasite burden (Rica-Capela et al., 2003). Clinical signs observed in hamsters in advanced stages of the disease include cachexia, anemia and ascites. Spleen enlargement is a common effect of leishmania infection that was seen from 3 months post infection in hamsters infected with promastigotes (Rica-Capela et al., 2003).

9.2 Experimental infection of dogs

A limited number of publications regarding the experimental infection of dogs with *L. infantum* are available. Small numbers of dogs were used in these experiments (3 to 8 per group) and as a consequence it is difficult to compare them and draw general conclusions about inoculation route, dose or parasite stage. The intravenous route is most commonly used, compared to the intradermal route the former appears more likely to result in the development of clinical disease (Paranhos-Silva et al., 2003; Santos-Gomes et al., 2000). It is generally accepted that the most reproducible and virulent infections are obtained by injection of amastigotes (Abranches et al., 1991; Campino et al., 2000; Hommel et al., 1995). The dose of parasites varies significantly between experiments, ranging from $5 \cdot 10^5$ to $1 \cdot 10^{10}$ per dog (Campino et al., 2000; Nieto et al., 1999). Good results with regard to parasite detection, development of disease and reproducibility were obtained with a model in which dogs are inoculated intravenously with 5 ·10E7 stationary phase culture promastigotes (Riera et al., 1999; Valladares et al., 1998).

Diagnosis of experimental CanL is, in general, mainly relying on serology and parasitology. Although differences in humoral response between experimentally and naturally infected dogs have been reported by some authors (Leandro et al., 2001; Rhalem et al., 1999a), this was contradicted by others (Martinez-Moreno et al., 1995). The outcome with regard to the detection of parasites is very variable between the different experiments reported. This may be caused by differences in infection dose, injection route or parasite strain that can all influence development of the parasite burden. However, differences in sensitivity of the applied parasite detection methods could also be an important factor.

Although most of the above mentioned challenges result in infection of dogs, there are several disadvantages associated with the use of such models in vaccination-challenge experiments.

In contrast to the experimental infections that rely on intravenous injection of high numbers of culture-derived promastigotes, the bite of an infected sandfly delivers a low number of parasites, in combination with vector-derived substances, to the superficial dermis of the dog. The efficacy of candidate vaccines as measured in an experimental challenge model may therefore differ from the protective capacity in the field. In order for candidate leishmaniasis vaccines to be tested properly, it is imperative that an appropriate well-established challenge model is developed. Such a model should resemble the natural infection as closely as possible.

10. Outline of this thesis

As is described above, many vaccine candidates have been tested in mouse models of –cutaneous- leishmaniasis. Several of these vaccines show good potential and should be further evaluated in more appropriate challenge models.
The major goal of the work described in this thesis was to assess the efficacy of candidate vaccines for visceral leishmaniasis in dogs. Both live-attenuated and sub-unit vaccines were tested and this required developing and establishing appropriate (challenge) models in the lab.

The most important test for a candidate vaccine is the evaluation of efficacy in the target animal. As published data on the experimental infection of dogs with *Leishmania infantum* is fragmentary, a pilot challenge study was performed. The experimental challenge model in dogs as it was established in our lab is described in detail in Chapter 2. Due to a number of disadvantages mentioned previously (paragraph models for *Leishmania* infection) studies requiring large numbers of animals are almost impossible to perform in dogs. Therefore we also established a challenge model in hamsters, optimization of the hamster model is described in Chapter 3.

As described above (paragraph live vaccines), the deletion of cysteine peptidase (CP) genes was shown to significantly attenuate *L. mexicana*. Moreover, vaccination of mice with the attenuated strains could confer protection against subsequent challenge with wild type *L. mexicana*. Collaboration with the labs of professors Coombs and Mottram from Glasgow University resulted in the generation of CP-deficient mutants of *L. infantum*. The virulence of a number of clones was established in an *in vitro* model using a canine macrophage-like cell line. Deletion-mutant clones were found to have reduced virulence and this was confirmed *in vivo* in a hamster challenge model of infection. These experiments, as well as the subsequent vaccination of hamsters with attenuated lines are described in Chapters 4 and 5.

Besides the knockout mutants, recombinant cysteine peptidases (rCP's) have also been used as subunit vaccines against *L. mexicana* in the mouse model of infection (see above, Table 2). Subunit vaccines have many advantages over the use of live vaccines, mainly with respect to safety, public health, production and distribution. We therefore tested the used of *L. infantum* rCP's in a vaccination-challenge experiment in dogs; results are described in Chapter 6.

Several authors have reported the use of crude *Leishmania* antigens in canine leishmaniasis vaccines, results ranged from 0% to 90% protection (Chapter 1; prevention of canine leishmaniasis). As leishmania can be cultured relatively easily even in bulk quantities, the combination of crude promastigote antigen with an appropriate adjuvant could be a feasible "first-generation" vaccine. The efficacy of such antigens was therefore tested in hamsters along with *L. infantum* CP's. A number of different adjuvants was tested in combination with the antigens since adjuvants have an important influence on vaccine efficacy. Results of these studies are described in Chapter 7.

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CHAPTER 2

Detailed analysis of an experimental challenge model for *Leishmania infantum* (JPC strain) in dogs.

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Abstract

In this study disease progression after intravenous or subdermal infection of dogs with *Leishmania infantum* JPC strain was monitored. A challenge performed on 14 dogs via the intravenous route with 5×10^7 stationary phase promastigotes of the *L. infantum* JPC strain was 100% successful. During a follow up period of 1.5 years several parameters were evaluated in order to find the most reliable disease markers. Parasite detection by culture and histology were found to be very sensitive (100%). Additionally, regular physical examination, serology and serum γ-globulin levels were found to be useful parameters in the evaluation of disease severity and are recommended for inclusion in vaccination-challenge experiments. Although this intravenous challenge model has practical limitations, the data set confirms it is the best experimental model currently available for vaccine development. Two intravenously infected dogs were treated with corticosteroids for 5 months. This treatment was shown to enhance all aspects of a *Leishmania* infection. Five more dogs were infected by sub-dermal injection of promastigotes mixed with a proteophosphoglycan-matrix (PSG) secreted by *Leishmania* that assists in transmission and infection by sand fly bite. The resulting parasite burdens were low and the animals remained asymptomatic during a two-year follow up period. However, this procedure did result in infection in 80% of the dogs and is appealing for future development as a natural challenge model in vaccine development.

Keywords
Leishmania infantum; Dog; Experimental infection, Immune response, PSG.

1. Introduction

Canine Visceral Leishmaniasis (CanVL) is a major veterinary and public health problem caused by *Leishmania infantum* (syn. *L. chagasi*) in endemic areas of the New World and the Mediterranean basin. In dogs leishmaniasis is a severe, chronic disease that is often fatal when left untreated. Although treatment of dogs will usually result in some clinical improvement, relapses are frequent and in most cases neither achieves parasitological cure nor stops infectivity to the sand fly vector (Baneth and Shaw, 2002). A vaccine, therefore, would be an important tool in the control of visceral leishmaniasis in dogs, and would also dramatically decrease infection pressure of *L. infantum* for humans, since dogs are the main domestic reservoir. In recent years much effort has been put into research on leishmaniasis vaccines, resulting in the discovery of several candidate vaccine antigens including gp63, LeIF, TSA, PSA-2, and LACK (Gradoni, 2001). Evaluation of the vaccine potential of these molecules has thus far been performed mainly in mouse models of infection. However, it is uncertain to what extent mouse models are predictive for protection in dogs. Thus, there is a need for these candidate vaccines to be tested in the target animal under reproducible laboratory conditions.

The ideal experimental *Leishmania* challenge should produce clinical signs and parasites in all animals after a predictable and preferably short prepatent period. Leishmaniasis however is hard to diagnose on the basis of clinical signs alone.. Detection of parasites is also problematic, since they may initially be present in very low numbers. These difficulties are likely to be an important cause of the conflicting results that have been published in reports on experimental canine leishmaniasis, using ostensibly similar challenge route and dose. Many clinical, serological and hematological parameters have been tested by different investigators in order to find those that can be used as an aid in the diagnosis of the disease. Again, results reported by different investigators are very diverse.

Differences between experimental and natural challenge with regard to parasite number, life cycle stage or route of infection may be important when experimental infection is used for the evaluation of candidate vaccines. The intravenous high-dose type of experimental challenge that is used most often may carry the risk of underestimation of vaccine efficacy as the challenge may overwhelm (dose) or avoid (route, life cycle stage) a protective response that is potentially present in the dogs. Challenge with a low dose of parasites via the dermal route may more accurately mimic sand fly bite. During natural transmission sand flies deliver low numbers of metacyclic promastigotes, parasite forms dedicated for infection (Sacks and Kamhawi, 2001), by regurgitation into the skin of a mammalian host (Rogers et al., 2004). Sand flies also secrete saliva to ensure efficient blood feeding. Since the bite of an infected sand fly delivers more than just metacyclic promastigotes to the mammalian host, there may be an opportunity to improve dermal experimental infection models. The effect of adding sand fly saliva to a challenge dose has been tested in the mouse model with results ranging from marked enhancement of infection (Belkaid et al., 1998; Castro-Sousa et al., 2001; Morris et al., 2001; Titus and Ribeiro, 1988; Warburg et al., 1994) to no measurable effect (Belkaid et al.,

1998; Castro-Sousa et al., 2001; Morris et al., 2001; Titus and Ribeiro, 1988; Warburg et al., 1994). In dogs challenged by intradermal injection of metacyclic promastigotes, the addition of sand fly saliva did not enhance infection (Paranhos et al., 1993; Paranhos-Silva et al., 2003). An improved understanding of the transmission mechanism of leishmaniasis has uncovered a new component of the infectious inoculum, a parasite-derived promastigote secretory gel (PSG) generated within the fly (Rogers et al., 2004). PSG was shown to accompany infecting metacyclic promastigotes during fly bite and enhance infection in a mouse model of infection (Rogers et al., 2004), offering an alternative opportunity to improve the experimental dermal challenge model in dogs.

Although data gathered from literature is conflicting, intravenous challenge with a medium-high dose of stationary phase promastigotes appears to be the closest to an ideal experimental challenge. Using this type of challenge we would expect the disease to develop in all animals after a reasonable prepatent period while it has the advantage of using promastigotes. In order to test the robustness of intravenous challenge with the JPC strain in our lab, challenge experiments were performed. Clinical, immunological and parasitological effects observed during a follow up period of maximum 1.5 years after intravenous administration of a medium-high dose of stationary phase promastigotes are described in this paper. Since this was the first time that the JPC challenge strain was used for experimental infection of dogs, a 'positive control' group was included; experimentally infected animals were treated for an extended period with corticosteroids in order to enhance infection. The effects of this treatment on the development of infection are described. Since improvement of the dermal experimental challenge with respect to prepatent period, disease outcome and parasite burden, would lead to an even better model, the effect of administrating promastigotes plus PSG via the dermal route to dogs was tested. This experimental challenge was compared to the 'standard' i.v. challenge.

2. Materials and Methods

2.1. Experimental set-up

Fourteen 6-month-old beagle dogs were challenged via i.v. injection of promastigotes whereas two dogs served as negative (non-infected) controls. Two additional dogs were challenged (i.v.) and subsequently treated with corticosteroids during the first 6 months of the study. Dogs were followed up for 12 to 15 months post infection. Five 6-month-old male beagle dogs received a dermal infection in combination with PSG this group was followed up for 2 years post infection.

2.2. Parasites

Dogs were challenged with *L. infantum* JPC (MCAN/ES/98/LLM-724). This strain was first isolated from a dog in Madrid suffering from leishmaniasis (parasites were kindly provided by Dr. M.I. Jimenez). Parasites were passaged through hamsters in order to retain full virulence. Promastigotes for the challenge infections were expanded at 27ºC in HO-MEM medium (Berens

et al., 1976) supplemented with 20% heat-inactivated Foetal Calf Serum (hiFCS) and allowed to grow to the stationary phase. Promastigotes were subsequently washed twice in Phosphate Buffered Saline (PBS; pH 7.3) and resuspended in PBS to a density of 2.5 x10^7 promastigotes per ml.
Lutzomyia longipalpis sand flies from an established colony were infected by feeding on rabbit blood containing 2x10^6 freshly isolated *L. infantum* JPC spleen amastigotes per ml (Gossage et al., 2003). Eleven days following infection the PSG plug was collected from dissected sand fly guts and prepared according to method of (Rogers et al., 2004). PSG plugs in subdermal infections were used at a ratio of 20 sand fly plugs (approximately 20 μg) per animal.

2.3. Animals and treatments

Beagle dogs, approximately 6 months old, were obtained from Harlan. The animals were housed in pairs in conventional kennels and fed a standard commercial diet (Canex; Hope Farms, the Netherlands) throughout the experiment. Dogs were randomly allocated to the treatment groups.
The corticosteroid treated animals (table 2: no. 15 and 16) received subcutaneous injections with 2 ml of Dexadreson ® (Dexamethasone 2 mg/ml, Intervet, The Netherlands) every Monday, Wednesday and Friday for the first 5 months post infection, then treatment was phased out gradually over a three week period.

Dogs 1 to 16 received an intravenous injection with 5 x10^7 promastigotes in 2 ml of isotonic PBS. Dogs in the PSG group (no. 17 to 21) were given the same challenge dose but subdermally, mixed with 100 μl of PSG suspension just before administration. This injection was placed in the left hind leg (in the region drained by the popliteal lymph node). According to Dutch law, protocols for the animal experiments were evaluated by an ethical committee. The set up was approved by the committee and experiments were performed in accordance with regulations for the use of experimental animals.

2.4. Clinical examination and blood chemistry

During clinical examination the size of prescapular, axilar and popliteal lymph nodes was established (0: normal, 1: slight enlargement 1 or 2 nodes, 2: several nodes severely enlarged), general body condition was assessed (0: normal, 1: decreased, 2: emaciated) and skin/coat condition was scored (0: normal, 1: slight scaling and/or alopecia, 2: severe alopecia and/or lesions). Obvious clinical abnormalities were recorded if present. Serum samples for clinical chemistry were taken at monthly or two monthly intervals. Determination of serum protein content and electrophoresis was performed according to standard procedures at the Dept. of Companion Animal Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands). Haematological values (Packed Cell Volume, total white blood cell count and differentiation) were determined in the same lab.

2.5. Lymphocyte proliferation and cytokine detection

The transcription of interferon gamma (IFNγ) in peripheral blood mononuclear cells (PBMCs) was monitored by quantitative RT-PCR using molecular beacons. Transcription of the household gene Glyceraldehyde 3-Phosphate Dehydrogenase (GPDH) was used to normalize values for IFNγ. This test was performed at monthly intervals. Briefly, RNA was isolated from fresh 8 ml samples of heparinized blood using the RNeasy mini kit (Qiagen, USA). RNA was transcribed to cDNA using a cDNA synthesis kit (Platinum® Quantitative RT-PCR Thermoscript™ One-step system, Life Technologies, USA). Separate Real-Time PCR reactions were subsequently performed for IFNγ and GPDH, two molecular beacons were used to detect the PCR amplicons. Sequences of primers and molecular beacons used are listed in Table 1. Both arms of the beacons were labelled with fluorescein (FAM) and a nonfluorescent quencher (DABCYL). The PCR reaction was performed in an iCycler (BIO-RAD, USA) that measured fluorescence of the samples and of a standard range of template cDNA Real-Time. The iCycler software calculates the template starting quantity for each sample by interpolation of the standard curve. The relative amount of IFNγ transcript was subsequently calculated for each sample (template starting quantity IFNγ / template starting quantity G_3PDH). Transcription of Interleukin 4 (IL-4) was measured essentially as described above, primer and molecular beacon sequences are listed in table 1.

Lymphocyte proliferation assays were performed at monthly intervals for dogs 10-14. PBMCs were isolated from heparinized blood by centrifugation over Ficoll/Hypaque (density 1.077, Pharmacia; Uppsala, Sweden) for 30 min. at 400 x *g*. Cells were washed once in RPMI 1640 medium supplemented with 1% of hiFCS and spun down at 400 x g for 20 min. Cells were resuspended in RPMI 1640 supplemented with 10% Normal Dog Serum, 2 mM of L-glutamine and 50 µg/ml of gentamycine. Cell viability was assessed using the Trypan blue exclusion test and viable cell density was adjusted to 1 x 10^6 cells/ml. Triplicate wells were filled with 50 µl of cell suspension and 50 µl of mitogen/antigen solution. A final concentration of 5 µg of concanavalin A or 1 µg of promastigote freeze-thaw lysate was used. To prepare the lysate, stationary phase promastigotes were washed twice in isotonic PBS and subsequently subjected to 3 freeze-thaw cycles. The resulting suspension was centrifuged (8,000 x g, 15 min.) and the clear supernatant was aliquoted and stored at -70ºC until use. Protein content was determined using a protein assay kit (BCA kit; Pierce, USA).

Table 1: sequence of primers and probes used for quantification of IFNγ, IL-4 and GPDH transcription.

	Forward primer (5'→ 3')	**Reverse primer (5'→ 3')**	**Molecular beacon (5'FAM→ 3'DABCYL)**
IFNγ	CAA AGG AGC ATG GAT ACC	GAC CTG CAG ATC GTT CAC	GCG AGC TAG CAG CAC CAG TAA GAG GGA GGA CGC TCG C
IL-4	ACT GCG GCA GAT CTA TAC	CCA AGA AGT CTT TCA GTG	GCG AGC AAC CTC AGC ATG GCA AAG CTC GC
GPDH	GAG ATC CCG CCA ACA TCA	TCA AGT GAG CCC CAG CCT	GCG AGC ATG CTG GTG CTG AGT ATG TTG TGG AGT GCT CGC

2.6. Detection of Leishmania-specific antibodies in serum

Leishmania crude soluble antigen (LSA) was prepared as follows: end log-phase promastigotes were harvested by centrifugation (15', 4,000 x g) and washed twice in isotonic PBS. The parasites were disrupted by freeze-thawing and subsequently sheared through a 25-gauge needle. Cell debris was spun down (15', 15,000 x g, 4ºC) and the protein content of the supernatant was determined using a BCA assay kit (Pierce, USA). Microtitre plates (Greiner, Germany) were coated overnight at room temperature with 0.05 µg LSA in 0.1 ml coating buffer (50 mM $NaCO_3$-H_2CO_3, pH 9.6). Blocking was performed with 3% skimmed milk powder (Difco™Skim Milk; Beckton Dickinson, USA) in PBS (MPBS), subsequently plates were washed in an ELISA washer (Skan washer, Skatron Instruments, Norway) using 0.01 M PBS. Serum samples were diluted 1:100 in 1% MPBS and diluted serially in the plate (1:1). After incubation for 1 hr at 37ºC, excess antibody was washed off the plates and sheep anti dog-IgG2-HRP (Bethyl, USA) diluted 1:10,000 in 1% MPBS was added. After another 1 hr at 37ºC plates were washed and subsequently incubated with substrate solution (tetramethylbezidine + H_2O_2 in 0.1 M of sodium acetate, pH 5.5). The reaction was stopped by addition of 2N H_2SO_4 and absorbance was read at 450 nm (Titertek® Multiscan Plus; ICN, USA). A negative and positive control serum was included on each plate. The negative control (1:100) was used to calculate the end-point dilution.

For the detection of LSA-specific IgG1, plates were coated with 2.5 µg/ml LSA. The ELISA was performed essentially as described for IgG2. Bound antibodies were detected using goat anti dog IgG1-HRP (Bethyl, USA) diluted 1: 5,000.

End-point dilutions (limiting dilution titres) were calculated using the MultiCalc program (Pharmacia, Finland). The cut-off value for each plate was calculated by the program using AA×factor, where AA was the Average Absorbance for the negative control (n=8) and factor was set to 4.

2.7. Parasite detection

Bone marrow biopsies were taken from the pelvis, lymph node biopsies were taken from the popliteal node. The materials obtained were cultured at 27˚C in HO-MEM medium supplemented with 20% hiFCS and 50 µg/ml gentamycin (Berens et al., 1976) as well as in the biphasic Evans' modified Tobie's medium supplemented with 50 µg/ml gentamycin. Cultures were incubated until positive or for a maximum period of six weeks.

At necropsy, samples of spleen, liver, bone marrow and lymph node were taken aseptically for parasite culturing.

2.8. Histopathology

Samples of spleen, liver, skin and lymph node were taken for histopathology. At microscopic evaluation each sample was given a histological score between 0 and 2 ranging from 'no abnormalities' (0) via 'findings indicative of a *Leishmania* infection' (1) to 'findings characteristic of a *Leishmania* infection' (2).

In spleen and lymph nodes indicative findings were: follicular hyperplasia and/or parasite stages suspected but no clearly evident clusters of lymphocytes and macrophages. In the liver indicative findings were: multifocal mononuclear infiltrates and/or small numbers of foci suspicious of early granulomatous inflammation. In the skin: infiltration with lymphocytes and macrophages.

In spleen, lymph nodes and skin findings characteristic of a *Leishmania* infection were: single infiltrating cells and clusters of lymphocytes and macrophages with occasional amastigotes in the cytoplasm. Characteristic findings in the liver were: granulomatous hepatitis with epitheloid cell granulomas.

Statistical analysis of the histological scores (correlation) was performed using Excell.

2.9. DTH test

L. infantum JPC strain was cultured as described above: stationary phase promastigotes were washed once in isotonic PBS (0.04 M, pH 7.2) and resuspended at the appropriate concentration in PBS-0.5% phenol. For the DTH test performed at 13 weeks p.i. in dogs 5-9 and 17-21 a concentration of $3x10^7$ parasites/ml was used. For dogs 10-14 $8 x10^7$ parasites/ml were used in week 4 whereas $3x10^8$/ml were used in week 13. The $3x10^8$/ml preparation was also used for the DTH test in dogs 17-21 at 65 weeks post infection. The test substance and the control (PBS-0.5% phenol) were injected in the skin of the groin (0.1ml). The injection site was checked at 24, 48 and 72 hrs., if present, the size of induration was determined using the ballpoint method.

3. Results

3.1. Clinical status

Most (79%) of the i.v. challenged dogs developed clinical signs of leishmaniasis during the course of the study although the severity and duration of these clinical abnormalities varied between individual animals. In *Figure 1* the average clinical scores in each of the experimental groups are shown. The first signs of disease became apparent between 15 and 20 weeks post-infection (p.i.) in 10 out of 14 of the i.v. challenged dogs. These signs were mild and mostly involved either (enlarged) lymph nodes or (decreased) body condition or both, and sometimes accompanied by mild conjunctivitis. More serious clinical symptoms became evident between 35 and 40 weeks post-infection in about half of the dogs (6 out of 14). Besides serious weight loss and lymphadenopathy, disorders of skin and coat appeared, while diarrhoea was noted on two occasions (once in dog 23 {control group} at 4 months p.i. and dog 4 was euthanised in week 40 because of acute diarrhoea and wasting that was unresponsive to palliative treatment). Five dogs were oligosymptomatic, showing mild clinical signs from about 20 weeks p.i. until the end of the study. Three dogs remained asymptomatic for the duration of the experiment. The occasional mild clinical signs in these dogs (dogs 3, 5 and 10) could not be attributed to the *Leishmania* infection with certainty. The maximum clinical score (the highest score for each dog that was found at least twice) for individual dogs is shown in Table 2.

Rectal temperature was monitored for dogs 1-4, 22 and 23 (4 infected dogs, 2 non-infected controls). Fever (>40˚C) was not detected during the course of the study (14 months). Significant differences between the control and the challenged groups were not found.

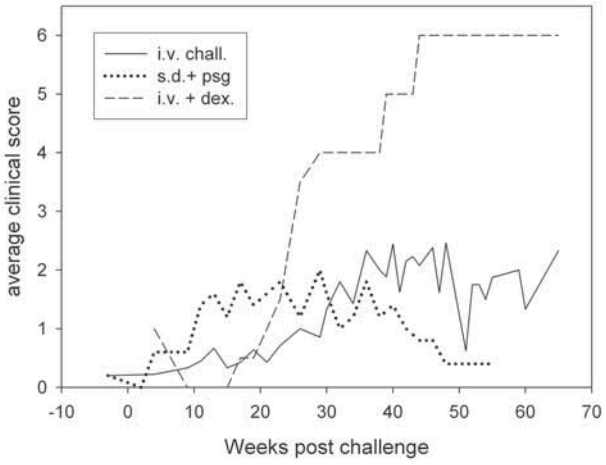

Figure 1: Average Clinical Score per Group.

Comparison of average clinical scores of i.v challenged (i.v. chall.: dogs 1-14), subdermally + PSG challenged (s.d. + psg: dogs 17-21) and i.v. challenged plus dexamethasone treated dogs (i.v. + dex.: dogs 15,16). Clinical score= body condition + skin + lymph node; these three observations are scored between 0 (normal) and 2 (severe abnormalities) the maximum clinical score therefore is 6.

3.2. Parasite detection

At 7 weeks post-infection the first bone marrow and lymph node biopsies were taken. Two out of 10 i.v. challenged animals tested were found parasite positive in culture. The number of parasite positive animals increased during the course of the study resulting in 64% (9 out of 14) positives at 6 months and 82% (9 out of 11) at 1 year post-challenge. The time to first positive biopsy for individual dogs is shown in Table 2. All dogs necropsied were found parasite positive by culture of lymph node, bone marrow, spleen and/or liver. Dogs 6, 8 and 12 were not necropsied; parasites were however detected in biopsies of those dogs. In conclusion, parasites were cultured from 100% of i.v.-challenged dogs.

Table 2: clinical, parasitological, and pathological data for individual animals.

group	Dog no.	Time to +	clin. score	Last neg.	γ-globulin levels (g/l) (normal range: 2-6)							Histopathological score				
					Weeks post infection:							spleen	liver	ln.	skin	Total %
					7	13	20	30	40	50	60					
i.v.	1	18	2	30	2	3	4	6	22	14	14	2	2	2	2	100
	2	54	2	44	4	3	4	3	3	8	20	2	1	2	1	81
	3	67	1	40	3	3	4	2	5	9	6	2	1	0	2	63
	4	18	6	29	3	3	3	19	23			2	2	2	2	100
	5	54	1	54	3	3	3	3				0	1	2	0	38
	6	24	3	41	3	3	4	9				Not performed.				
	7	16	6	19	5	5	9	18				2	2	2	1	88
	8	36	2	55	3	4	7	12				Not performed.				
	9	24	2	21	3	3	4	10				Not performed.				
	10	50	1	47	4		4	5		3		2	1	2	0	63
	11	8	2	36	3		4	20	14	10		2	2	2		100
	12	24	3	23	4		4	6	5	5		2	2	2	0	75
	13	8	6	21	3		4	15	17	14		2	2	2	2	100
	14	15	4	26	3		3	13	16	18		2	2	2	2	100
dex.	15	18	6	19	3	3	16	50	37	46	47	2	1	2	2	88
	16	18	5	15	4	4	16	29				2	2	2	2	100
der.	17	-	2	70	3	3	3	3				0	1	1	0	25
	18	98	2	85	3	4	4	4				2	2	2	0	75
	19	59	3	48	4	5	5	5				2	2	2	2	100
	20	98	2	55	5	4	5	6				2	2	0	0	50
	21	98	1	55	3	4	5	3				0	2	1	0	38
neg.	22	Np	0	65	2	3	3	3	3	2	3	Not performed.				
	23	Np	0	65	2	3	4	3	4	3	5	Not performed.				

Table summarizing individual clinical and parasitological data from dogs after a challenge infection with $5{\cdot}10^7$ promastigotes i.v. (i.v.: dog 1 to 14), i.v. challenge and dexamethasone treatment (dex.: dogs 15, 16), dermal challenge with $5{\cdot}10^7$ promastigotes + psg (der.: dog 17 to 21), or non-infected control dogs (neg.: dogs 22,23). Time to +: time, in weeks, to first sampling (bone marrow or lymph node) that was parasite positive (- = no positive sample; Np= not performed). clin. score: highest clinical score that was reached on two consecutive examinations (clinical score= body condition + skin+ lymph node). Last neg.: time to last negative clinical score (weeks post infection). Histopathological score: samples were scored between 0 (no abnormalities) and 2 (findings characteristic for a leishmania infection). The cumulative score per animal was used to calculate the percentage of the maximum histological score (total%). ln.=lymph nodes.

3.3. Clinical chemistry and haematology

Blood samples from dogs 1-4, 22 and 23 were tested monthly for haematological changes. From 31 weeks post infection PCV values gradually decreased in the challenge group. Between 31 and 54 weeks p.i. PCV values in the challenged dogs ranged from 30 to 51% whereas it ranged from 42 to 57% in the healthy controls. For challenged as well as control dogs the total white blood cell count remained within normal limits throughout the experiment, as did the number of lymphocytes, monocytes, granulocytes and eosinophils. An abnormally high level of lymphocytes was detected in the blood of dog 4 (challenged group) from Day 0 up to week 20.

Increased levels of serum γ-globulin were detected in 11 out of 14 of the i.v. infected dogs within the first year post infection; results are shown in Table 2. Two dogs with normal levels of γ-globulin throughout the study also had low clinical and histological scores and parasites were only found at necropsy (dogs 5 and 10). The third dog with normal γ-globulin levels (no. 12) had medium high clinical scores and was found parasite positive at 24 weeks post-infection. In the negative control dogs, abnormal γ-globulin levels were not found at any time during the study.

3.4. Cellular immunity

Non-specific proliferation of PBLC's in response to stimulation with ConA was positive for all dogs and this did not change after the challenge infection (stimulation index ≥ 2). Stimulation with *Leishmania* soluble antigen however, did not elicit specific proliferation of lymphocytes before or after the challenge infection (stimulation index ≤ 1 ; results not shown).

Transcription of IFN-γ and IL-4 was monitored by quantitative real-time RT-PCR for dogs for dogs 1-9 and 17-23 (IFN-γ) and dogs 5-9 and 17-21 (IL-4) respectively. IFN-γ transcription was found to be similar for negative controls and challenged dogs, but individual variation was large. Toward the end of the follow up period, IFN-γ transcription levels in the i.v. challenged dogs appeared to increase somewhat compared to the healthy controls (figure 2). Interleukin-4 transcription was not detected up to week 23 p.i. but between 25 and 40 weeks p.i. transcripts were detected in both the i.v. challenged and subdermal + PSG groups. Variation was large; transcription of IL-4 was detected in few dogs only and was not consistently present in these dogs. Results are summarized in figure 2.

3.5. Serology

IgG2 titres against crude soluble *Leishmania* antigen (LSA) were detectable in some dogs as soon as two weeks post-infection, titres were found to be positive in all i.v. challenged dogs at around 15 weeks p.i. (figure 3a). A further gradual increase of anti-LSA IgG2 titres was found for most dogs and at 35 weeks post infection 12 out of 14 had very high LSA-specific IgG2 titres. Dogs 10 and 12 only experienced low and transient anti-LSA IgG2 titres. The Delayed Type Hypersensitivity skin-test that was applied in dogs 5-14 (at 13 or at –4 and 13 weeks p.i, respectively.) was found to increase LSA-specific IgG2 titres compared to infected non-DTH tested animals (dogs 1-4). The increase was of medium height and followed by a

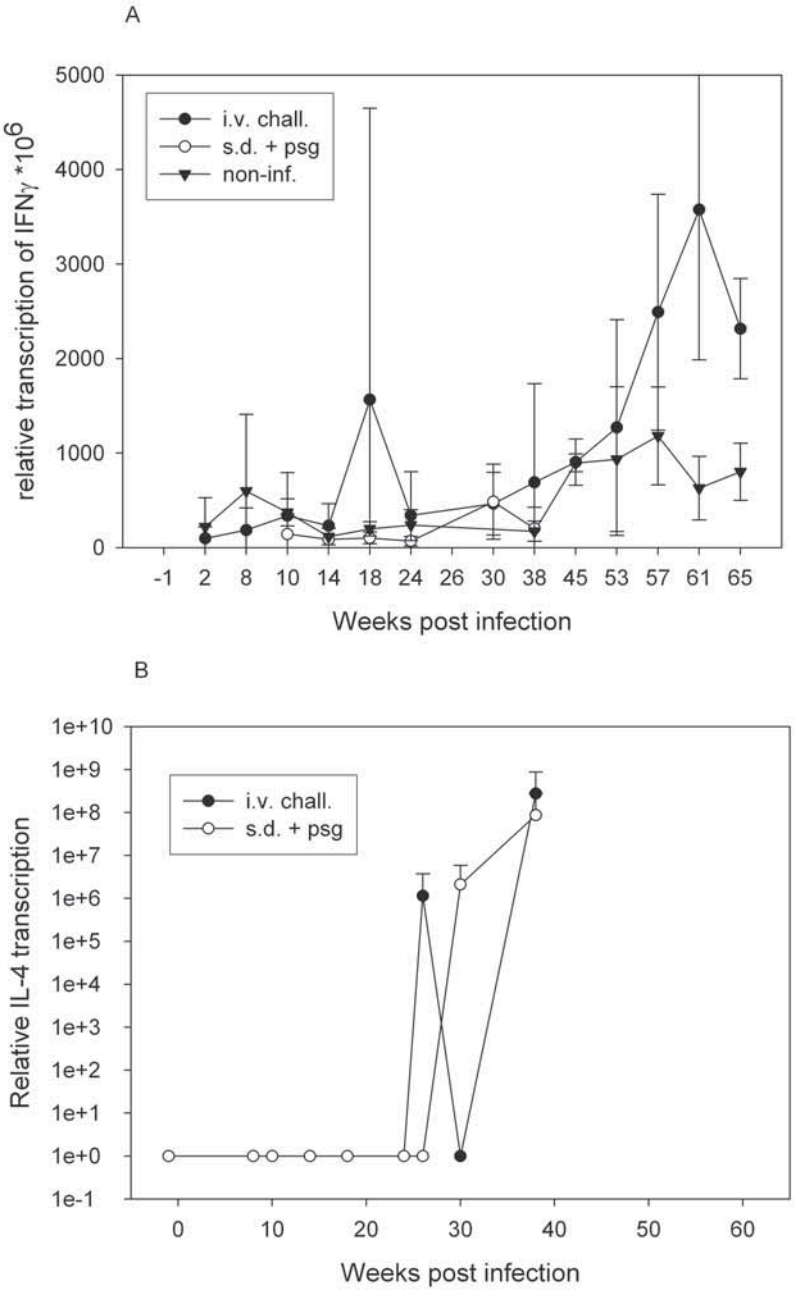

Figure 2: Transcription of IFN-γ and IL-4 in Peripheral Blood Mononuclear Cells.
Panels A and B show respectively the average relative IFNγ and IL-4 transcription levels as measured by Quantitative Real-Time RT PCR in the PBLC's of i.v. challenged (i.v. chall.: dogs 1-14), subdermally + PSG challenged (s.d. + psg: dogs 17-21) and non-infected control dogs (non-inf.: dogs 22,23). The quantity of both interleukins was normalized using the amount of G3PDH transcript in the same sample.

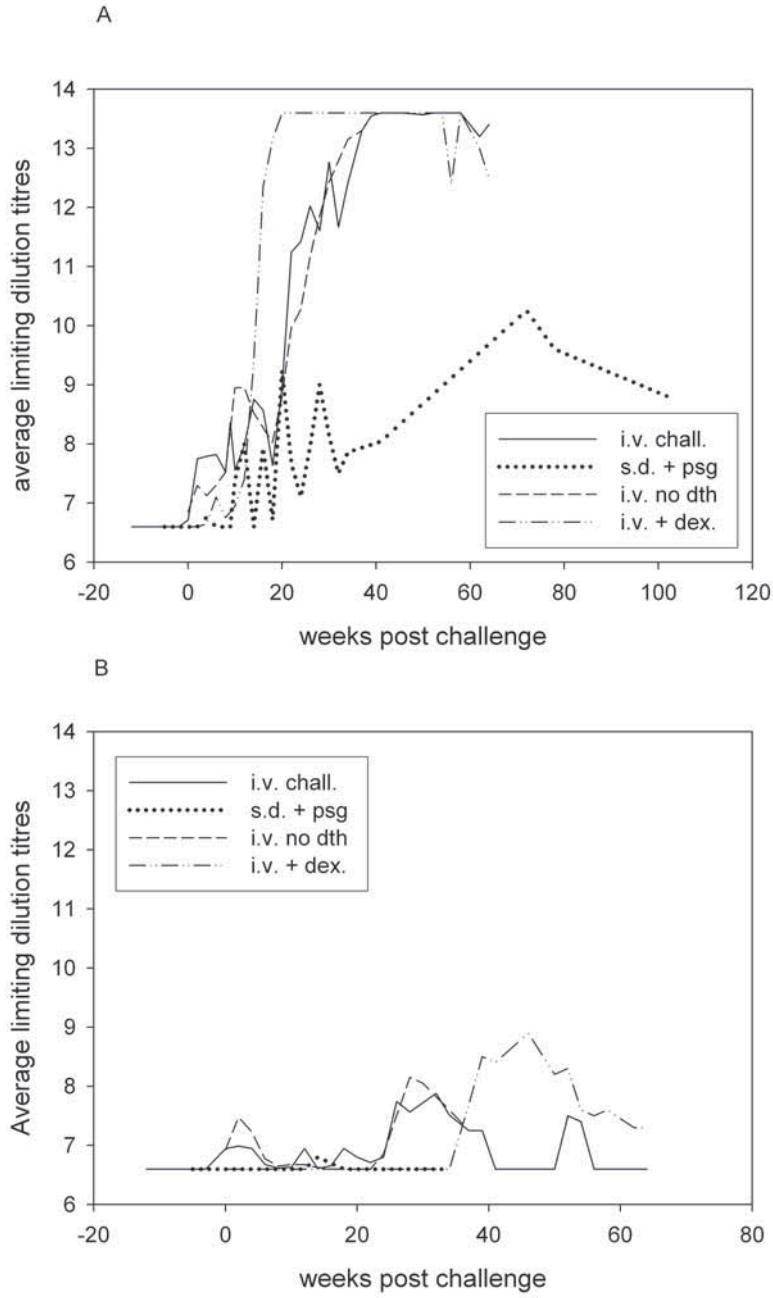

Fig. 3: Antibody titres against crude Leishmania antigens.

Panel A and B show respectively the average Leishmania-specific IgG2 and IgG1 titres that were established using a soluble leishmania antigen ELISA. Positive antibody titres were found in i.v. challenged (i.v. chall.: dogs 5-14), subdermally + PSG challenged (s.d. + psg: dogs 17-21), i.v. challenged - not DTH-tested (i.v. no dth: dogs 1-4) and i.v. challenged + dexamethasone treated dogs (i.v. + dex.: dogs 428, 429). Non-infected control dogs did not develop detectable antibody titres.

decrease in antibody titre of similar magnitude. DTH-testing appeared not to influence the level of antibodies further along in the experiment (after 6 months p.i.). In sera from the negative control dogs, no LSA antibodies were detected throughout the experiment.

LSA-specific IgG1 titres were low overall. In contrast to IgG2, IgG1 titres were not detected in all infected dogs. Brief IgG1-peaks were detected after both DTH tests (at –4 and 13 weeks) in dogs (10-14). Furthermore IgG1 titres were found in dogs 4, 7, 11, 13, 14 and 22; these were all dogs that had high parasite numbers soon after challenge and, except for no. 11, suffered severe clinical disease. Average IgG1 titres per group are shown in figure 3b.

3.6. Necropsy and Histopathology

Eleven of the 14 i.v.-challenged dogs were necropsied, tissue samples from these dogs were examined histologically and given a score, results are shown in Table 2. All dogs that were found parasite positive by culture, were confirmed by histopathology. In samples of spleen and lymph nodes, the highest percentage of abnormalities characteristic for a *Leishmania* infection was found (91%; 10 out of 11 samples). In the liver; 64% characteristic findings and 36% indicative findings, in the skin 50% characteristic, 20% indicative and 30% negative findings. A strong inverse correlation was found between the ranking of the first positive biopsy and the histological score (r^2=0.63, p=0.0001). This suggests that the animals with the highest parasite burdens, that had a positive biopsy first, had the highest histopathology score. Good correlation was also found between the clinical score at the end of the experiment and the histological score (r^2=0.54, p=0.0002).

3.7. DTH test

The DTH test performed on dogs 5-9 and 17-21 (5 i.v.- and 5 s.d.-challenged resp.) at week 13 post infection did not elicit any reaction. A small peak in LSA-IgG2 antibodies was detected after the test in some of the dogs. In dogs 10-14, a DTH test was performed twice; in the first test at 4 weeks pre-challenge no reactivity was recorded but in the second test at 13 weeks post-challenge one dog (no. 11) did develop swelling at the site of injection (5 mm diameter).

3.8. Subdermal challenge + PSG

The average clinical score for the subdermal challenge group was slightly higher than for the intravenous group between 2 and 6 months after challenge. However, the clinical score in the PSG-group decreased somewhat after this time, whereas the average score increased in the i.v.-group (results shown in figure 1). The PSG-challenge group was studied for another year; at the end of the second year the dogs were still showing only mild clinical abnormalities.

Parasites were detected in a biopsy sample from one dog at 59 weeks p.i. (dog no. 19), three more dogs had positive cultures at necropsy (week 96 p.i.) and one dog (no. 17) remained negative throughout the study. Histopathology confirmed the presence of a *Leishmania* infection in 4 out of 5 dogs; for dog no. 17 the result was undecided (table 2).

Abnormal γ-globulin levels were not found in the s.d. challenged dogs (table 2). The level of interferon-γ transcription in the s.d. challenged dogs did not differ from that of the negative control dogs or i.v. challenged dogs. IL-4 transcription was found late in the infection of s.d. challenged dogs (dog 19, 20 and 21 at 30 weeks and dog 21 at 34 weeks) similar to i.v. challenged dogs (Figure 2). Antibodies against soluble *Leishmania* antigen (LSA) were detected, but almost exclusively of the IgG2 isotype. IgG2 titres appeared at around the same time as in the i.v. infected group but levels remained low for the duration of the study for all animals (figure 3a). A DTH-test was performed at week 13 and week 65 post infection: no reactions were detected. Probably as a consequence of the DTH tests; small IgG2 peaks were seen in two dogs (no.17 and 19) at week 17 and one dog (no. 20) at week 20, an increased IgG2 titre was detected in all dogs after the second test as seen in week 74 (figure 3a).

3.9. Corticosteroid treatment

Compared to i.v.-infected non-treated dogs, clinical signs in the corticosteroid treated dogs appeared a few weeks earlier and progressed faster to serious symptoms (figure 1). Dog 16 died in week 26 p.i., the direct cause was probably heart failure (as judged by macroscopic examination and histology) but its death was preceded by rapid worsening of clinical signs.

Both corticosteroid treated animals were found positive at the first biopsy. At necropsy, histopathology confirmed the presence of high numbers of parasites (table 2). The increase in γ-globulin levels started early compared to non-treated infected animals and the levels reached were higher (table 2).

Seroconversion for LSA specific IgG2 was similar to that of the non-treated dogs, although it appeared to occur slightly earlier (12 weeks) and increase slightly more quickly (figure 3). IgG1 titres were found in dog 15, from about 35 weeks onward. Dog 16 had already died by this time.

4. Discussion

All intravenously infected animals were found parasite positive, indicating that the experimental challenge with $5x10^7$ stationary phase promastigotes was 100% successful in establishing a persistent infection. The infection rate is slightly higher than the average rate reported in literature for i.v. challenges. Intravenous challenge of dogs with amastigotes resulted in 87% average infection rate (*n*=30)(Abranches et al., 1991; Binhazim et al., 1993; Campino et al., 2000; Leandro et al., 2001; Oliveira et al., 1993; Rhalem et al., 1999), and challenge with promastigotes resulted in infection of 90% of dogs (*n*=58)(Abranches et al., 1991; Campino et al., 2000; Martinez-Moreno et al., 1995; Molano et al., 2003; Moreno and Alvar, 2002; Nieto et al., 1999; Paranhos-Silva et al., 2003; Riera et al., 1999; Santos-Gomes et al., 2002).} The difference between the (average) reported rate(s) and the 100% infection we found is probably due to a combination of factors: 1) the method of parasite detection: compared to

direct examination of smears, culturing or PCR of lymph node and bone marrow samples is a more sensitive method; 2) the duration of the experiment: some of the dogs in our experiment were first found parasite positive at 1 year p.i. Together, our data indicate that an intravenous challenge with stationary phase promastigotes has a very high (if not 100%) success rate and a dose of 5×10^7 per dog is sufficient. Virulence of the JPC strain was found to be sufficient for use in challenge infections of dogs.

In retrospect, eleven out of fourteen animals (79%) developed clinical leishmaniasis with the first signs appearing within the first 6 months post infection whereas three animals (21%) remained asymptomatic for the whole of the one-year follow up. It is possible that dogs designated asymptomatic at one year p.i. would have developed clinical signs after a more prolonged incubation time; prepatent periods of several years have been reported to occur in naturally infected dogs (Slappendel, 1988). However, in vaccination-challenge experiments, the continuation of experiments for more than one year post infection is undesirable. Others have reported percentages of clinical disease (either oligo- or polysymptomatic) in 0 to 100% of the challenged dogs (Campino et al., 2000; Martinez-Moreno et al., 1995; Paranhos-Silva et al., 2003). Although a similar challenge with promastigotes was used by these authors, and high infection percentages were achieved by all, the difference in percentage of clinical signs is striking. It is very likely that this is due to a difference in the definition of clinical signs indicative for leishmaniasis; a higher percentage of clinical signs is found when subtle changes are also taken into account. Although the appearance of moderately enlarged lymph nodes or a minor change in body condition/muscle volume are not reliable indicators for leishmaniasis in a naturally infected dog that is seen by a veterinarian at an unknown time point after infection, these variables are valuable in an experimental challenge model since they can be monitored longitudinally. Repeated abnormal findings, even though they may be minor, are a sign of disease and therefore most likely due to *Leishmania* infection when found under the controlled circumstances of a challenge experiment.

In order to find disease markers, we tested several methods to detect parameters of cellular and humoral immunity. The lymphocyte proliferation assay could not detect any specific proliferation. A possible explanation for this result is that none of the dogs showed resistance to infection and therefore none of the dogs is expected to have raised specific cellular immunity. These results are in agreement with those of (Martinez-Moreno et al., 1995) for experimentally as well as naturally infected dogs. Previously, (Pinelli et al., 1994) and (Cabral et al., 1992) have detected specific Lymphocyte Proliferation Assay (LPA) responses (exclusively) in asymptomatic dogs. The LPA may be a valuable tool in vaccination-challenge experiments as apparently non-protected dogs do not develop specific responses whereas lymphocytes from naturally infected 'resistant' dogs do respond to Leishmania antigen. Some evidence for this hypothesis was found by (Ramiro et al., 2003); lymphocyte proliferation in vaccinated-protected dogs was found to be much higher than in vaccinated-susceptible dogs.

We found that interferon-γ transcription in peripheral blood leucocytes of infected dogs increased toward the end of the follow up period, consistent with a progressive infection. Interleukin-4 transcripts were also found toward the end of the follow-up period (34 weeks p.i.) but only in a minority of intravenously infected dogs. For IFN-γ, a similar result was found by (Quinnell et al., 2001), who reported increased transcription in the bone marrow of naturally infected dogs. These authors have detected IL-4 transcription occasionally in severely affected animals only. We could not correlate IL-4 transcription with severity of disease in the i.v. group. It is possible the naturally infected animals in the above mentioned study were tested while in a more progressed stage of the disease. (Santos-Gomes et al., 2002) reported a peak in IFN-γ transcription between 8 and 11 months p.i., this is similar to the peak we recorded toward the end of the first year post-infection. Although further research is necessary, IFN-γ and IL-4 appear to be promising candidates for the evaluation of cellular immune responses in dogs.

From our results it is clear that histology is a highly sensitive method of parasite detection and can be used to discriminate between low and high parasite burdens. It is therefore a very useful tool for experimental challenge models. Moreover, some infections were only detected at necropsy; especially in the subcutaneously challenged animals with very low parasite burdens, indicating the importance of performing necropsy and detailed histological studies on all animals in a (vaccination) challenge experiment.

One out of five dogs in the PSG group developed clinical signs of leishmaniasis within the 2 year follow-up period, parasites were detected in four dogs. The subdermal + PSG challenge was therefore not an improvement compared to the intravenous method of infection. As resources precluded a direct test of the disease-enhancing effect of PSG in dogs (i.e. by inclusion of a subdermal challenge control group), we compared the results to those of other studies that used dermal injection of promastigotes without the addition of sand fly-derived substances. Several authors have reported infection of dogs via the dermal route. When these studies are taken together, 14 (30%) out of the 47 dogs that were challenged developed clinical signs of leishmaniasis (Abranches et al., 1991; Killick-Kendrick et al., 1994; Leandro et al., 2001; Paranhos-Silva et al., 2003; Santos-Gomes et al., 2000). It appears therefore that PSG on its own had no obvious effect on establishment of infection and clinical disease in dogs. However, dismissal of the PSG model for canine leishmaniasis is premature at this stage because it is unknown how this model of infection compares with natural challenge of this parasite by sand fly bite. Interestingly, IL-4 transcription was detected in subdermal + PSG challenged dogs, while IL-4 was associated with more severe symptoms in naturally infected dogs (Quinnell et al., 2001). Although IL-4 was not linked with clinical disease in this study, detectable IL-4 was found late in infection both in i.v. and s.d. challenged dogs, suggesting a link with pathology akin to natural infection.

The corticosteroid treatment was shown to enhance all aspects of a *Leishmania* infection: parasite burden, clinical symptoms and serological responses. The enhancement of infection is probably the sum of several effects of dexamethasone treatment: upregulation of mannose

receptor expression on macrophages enhances uptake of the parasite (Chakraborty et al., 2001) while the ability to kill intracellular parasites is reduced due to decreased lymphocyte count. A number of authors have reported a dexamethasone induced shift toward Th2 cytokine secretion: this may also enhance parasite survival and proliferation (Ramirez, 1998). Although corticosteroid treatment makes the challenge more reliable with respect to prepatent period, clinical signs and parasite burden, vaccine-induced immunity in treated animals would most likely be suppressed and it is therefore not useful in vaccination-challenge experiments. This treatment can however be useful if experimentally infected dogs are required for drug testing.

In conclusion, intravenous infection with promastigotes appears to be the most useful challenge model in dogs for the establishment of reliable and rapid infections to facilitate the study of disease pathology, drug efficacy and also vaccine efficacy. For evaluation of the effect of challenge infections, the direct detection of parasites is the most sensitive and reliable parameter.

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CHAPTER 3

Optimization of a *Leishmania infantum* challenge model in hamsters.

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Abstract

In order to optimize the *L. infantum* hamster challenge model and establish the system in our laboratory, hamsters were initially infected with increasing doses (10^2-10^8) of stationary phase promastigotes of *L. infantum* JPC strain. Several routes of infection were tested in order to evaluate the effect on disease progression. Parasite burdens were determined at 1 and 3 months post infection by limiting dilution culturing and histopathology. Infection of hamsters with *L. infantum* JPC strain resulted in dissemination of the parasites, regardless of the infection route (intraperitoneal, subcutaneous or intradermal). Lower challenge doses ($\leq 10^6$) appeared less useful in our model as the parasite burdens were less consistent within the groups and not detectable in some animals. Metacyclic promastigotes did not appear to be more infectious compared to stationary phase promastigotes. Although 10^8 promastigotes is a relatively high dose compared to a natural challenge, it is the lowest dose that resulted in detectable parasite burdens in all animals. In conclusion, our results demonstrate that golden hamster can be infected with L. infantum JPC strain and that for future vaccination-challenge experiments a challenge dose of 10^8 stationary phase promastigotes is most appropriate. Both challenge via the intraperitoneal route for a standard challenge or via the intradermal route for a more cautious approach can be applied.

Introduction

Leishmania infantum is an intracellular parasite that causes zoonotic visceral leishmaniosis and canine leishmaniosis (CanL). The parasite is transmitted by the bite of an infected sandfly and is mainly prevalent in the Mediterranean region and in large parts of South America (*L. chagasi*). Canine leishmaniosis is a severe disease characterized by progressive weight loss, skin problems and, ultimately, renal failure. Treatment is possible, but it is expensive and usually does not result in complete cure. Even after prolonged treatment, relapses are the rule rather then the exception and dogs remain infectious to the vector. Without treatment however, the disease is likely to be fatal. Since dogs are the reservoir species for this parasite, successful control measures in dogs will likely decrease the occurrence of zoonotic disease (Dye, 1996). Possible control measures include vector control, drug treatment, culling and vaccination. Drug treatment and culling are not very effective in reducing the incidence of disease and although vector control can be very effective, a vaccine against CVL remains highly desirable (Dye, 1996).

Continuing research into the biology and biochemistry as well as the genome of *Leishmania* has resulted in the discovery of a range of vaccine candidates; antigens with essential functions, abundant surface proteins or molecules with immunomodulating properties. These molecules, albeit promising in theory, need to be tested *in vivo* in order to establish their true potential. Technically speaking, it would be ideal to evaluate the efficacy of vaccine candidates in the target animal species, under field conditions. This is especially true for (parasitic) infections like leishmaniosis that are caused by vector-borne pathogens and for which it is hard to mimic the natural infection in the laboratory. In reality however, it is not a valid option to perform such studies because of animal welfare, regulations, cost considerations and many more reasons. Therefore, laboratory models need to be used for screening a range of candidate vaccines.

In contrast to dogs, outbred mice are not very susceptible to *L. infantum* and the disease tends to self-cure (Trotter et al., 1980), making this a less appropriate model for CVL. Hamsters are however highly susceptible to VL and experimental infection of golden hamsters (*Mesocricettus auratus*) has long been used as a means of isolating new strains. Nonetheless, few studies have been published about the experimental infection of hamsters with *L. infantum* or on the use of this model in vaccine research.

Since promastigotes are transmitted by the bite of a sandfly, this is the parasite stage preferably used in a model for evaluation of vaccine efficacy. In order to optimize the model and establish the system in our laboratory, hamsters were infected with increasing doses (10^2-10^8) of the JPC strain of *L. infantum*. Several routes of infection were tested in order to evaluate the effect on disease progression. Stationary phase culture, containing both log-phase parasites and the infective –metacyclic- stage, was used for the infection. In comparison, the virulence of purified metacyclic promastigotes was also tested.

Materials and methods

Experimental outline

Nine groups of five hamsters were infected with the JPC strain of *L. infantum* (MCAN/ES/98/LLM-724). Hamsters were infected with different doses, via different infection routes and with different parasite preparations (see table 1). At 1 month post-infection (p.i.) two animals were sacrificed, at 3 months p.i. the remaining three animals were necropsied and samples were taken from blood, bone marrow, spleen, liver, lymphnode and skin. Spleen cells were used for the lymphocyte proliferation assay (LPA). Spleen cells and liver cells were used for detection and quantification of parasites. Serum was used to determine the anti-LSA IgG titres and histopathology was performed on spleen, liver, kidney and skin.

Table 1: Conditions used in hamster infection experiments

group	infection material	dose/hamster	infection route (volume)
10^8 i.p.	Stationary phase promastigotes	10^8	Intraperitoneal (0.5 ml)
10^6 i.p.		10^6	
10^4 i.p.		10^4	
10^2 i.p.		10^2	
10^8 i.d.		10^8	Intradermal (0.1 ml)
10^2 i.d.		10^2	
10^8 s.c.		10^8	Subcutaneous (0.5 ml)
10^4 met.	Metacyclic promastigotes	10^4	Intraperitoneal (0.5 ml)
10^2 met.		10^2	

Table showing details of the challenge infection for each group; parasite population (infection material), dose and infection route.

Parasites and antigens

Amastigotes of *L. infantum* JPC strain (MCAN/ES/98/LLM-724) contained in spleen tissue of infected hamsters were allowed to transform to promastigotes *in vitro* in HO-MEM supplemented with 20% heat-inactivated foetal calf serum (HO-MEM/20) at 27°C. Promastigote cultures were expanded for a maximum of 4 weeks after transformation. When the desired number of parasites was reached cultures were left at 27°C until the stationary phase was reached. The promastigotes were harvested by centrifugation (1,500g, 10', 4°C) and washed once with isotonic Phosphate Buffered Saline (PBS). Parasites were subsequently counted and resuspended in the appropriate volume of PBS. This material was used for infection of hamsters with stationary phase promastigotes.

Metacyclic promastigotes were isolated by density gradient centrifugation according to the method of Spath and Beverly (Spath and Beverley, 2001). Briefly, a suspension of stationary phase promastigotes in PBS was layered carefully over a two-phase Histopaque cushion.

The lower phase at a concentration of 40%, the upper phase 8% w/v Histopaque 1077 (SIGMA, US) in HO-MEM. After centrifugation, the interphase containing the metacyclic parasites was collected and washed once with cold isotonic PBS. Parasites were subsequently counted in a haemocytometer and diluted to the appropriate concentration (see table 1) in PBS.

Promastigote lysate for use in lymphocyte proliferation assay (LPA) and ELISA was prepared from stationary phase promastigotes. Parasites were harvested by centrifugation (15', 4,000 g, 4°C) and washed twice with isotonic PBS. The promastigotes were disrupted by repeated freeze thawing cycles and subsequently sheared through a 25-gauge needle. Cell debris was spun down (15', 15,000g, 4ºC) and the protein content of the supernatant was determined using a BCA assay kit (Pierce, USA). The lysate was stored in aliquots at -70°C until use.

Animals

Six-week-old male *spf* golden hamsters (Harlan, the Netherlands) were divided over the groups as they came to hand. Hamsters were not individually marked, but groups were kept in separate marked cages. Standard commercial feed ("RMH-B 2181", Arie Blok BV, the Netherlands) and water were provided *ad libitum*. After one week of acclimatization, animals were infected with 0.5 ml (i.p., s.c.) or 0.1 ml (i.d.) of parasite suspension (table 1). Blood samples were taken by orbital puncture from all animals at the time of challenge and at day 28 post infection (p.i.), and again from the remaining animals at day 76 p.i.. Serum was collected and stored at -20°C until use.

At one and three months p.i. 2 and 3 hamsters per group were sacrificed respectively. Samples of spleen, liver and bone marrow were removed aseptically and were used for parasite quantification by culturing. Samples of spleen, liver, lymphnode and skin were taken for histopathological analysis.

Lymphocyte Proliferation Assay

Spleen tissue was homogenized in RPMI 1640 + 2% Foetal Calf Serum (FCS), washed once and resuspended in complete RPMI medium (RPMI 1640 (GIBCO BRL) supplemented with 25 mM HEPES, 25 mM $NaHCO_3$, 10% FCS, 2 mM L-glutamine and 50,000 U/ml Penicllin/ streptomycin. Viability was determined by trypan blue and spleen cells were diluted to a concentration of 1 x 10^6/ml. The cell suspension was pipetted into 96 well plates (Nunclon surface, Nunc, Denmark) at 50 µl per well and to each well 50 µl of medium was added containing either 10 µg/ml Con A (Sigma, USA) or 2 µg/ml Promastigote lysate or no additions (all were performed in triplicate). Cells were incubated for 72 hrs at 37ºC 5% CO_2, the last 18 hrs. in the presence of BrdU (BrdU Labelling and Detection Kit III, Roche-Diagnostics, Switzerland). After the incubation period a colorimetric assay was performed according to the manufacturer's instructions. Proliferative responses were expressed as stimulation indices (SI), which represent the ratio of mean absorbance after stimulation to the mean absorbance of non-stimulated controls.

Quantification of parasites

Parasites in the liver and spleen were quantified by culture microtitration according to the method of Buffet et al.(Buffet et al., 1995). Briefly, 100 µl of spleen cell suspension as prepared for the LPA was pipetted in triplicate in the first column of a flat-bottom 96-well micro titre plate (Nunc). This suspension was serially diluted (1:1) up to column twelve. A small piece of liver was weighed and crushed between two microscope slides. The suspension was diluted to a concentration of 100 mg/ml in HO-MEM/20. The cell suspension was serially diluted as described for the spleen cell suspension. After one and two weeks of incubation at 27°C the presence of *Leishmania* promastigotes was monitored by phase-contrast microscopy and for each row the well with the highest dilution that still contained parasites was recorded. From the highest positive dilution the number of parasites in spleen and liver was calculated; triplicates were averaged and the parasitic load per 1g of liver tissue and per 10^6 spleen cells was determined.

Spleen, liver, skin and lymphnode samples were fixed, stained with haematoxylin-eosin and examined microscopically. Findings were summarized in a histological score; minimal and non-specific findings were given value 0, findings indicative –but not characteristic- of a *Leishmania* infection were given value 1 and findings characteristic of a *Leishmania* infection were given value 2. For each animal a score was calculated by adding up values for findings in spleen, liver, skin and lymphnode samples (maximum score: 8), these scores were averaged per group and expressed as a percentage of the maximum score. In spleen and lymphnodes indicative findings were: follicular hyperplasia and/or parasite stages suspected but no clearly evident clusters of lymphocytes and macrophages. In the liver indicative findings were: multifocal mononuclear infiltrates and/or small numbers of foci suspicious of early granulomatous inflammation. In the skin: infiltration with lymphocytes and macrophages. In spleen, lymphnode and skin findings characteristic of a *Leishmania* infection were: single infiltrating cells and clusters of lymphocytes and macrophages with occasional amastigotes in the cytoplasm. Characteristic findings in the liver were: granulomatous hepatitis with epitheloid cell granulomas.

Detection of leishmania-specific antibodies in serum

ELISA plates (medium binding; Greiner, Germany) were coated overnight at room temperature with 0.1µg Promastigote lysate per well in 0.1 ml coating buffer (50 mM $NaCO_3$-H_2CO_3 pH 9.6). Blocking was performed with 1% skimmed milk powder (Difco™Skim Milk; Beckton Dickinson, USA) in phosphate buffered saline (0.1 M, pH 7.4) (MPBS) for 1 h. at 37ºC. Serum samples were diluted 1: 40 in MPBS and serially diluted in the plate (1:1). After incubation for 1 h. at 37ºC plates were washed in an ELISA washer (Skan washer, Skatron Instr., Norway) using 0.01 M PBS. Plates were subsequently incubated for 1 h. at 37ºC with goat anti hamster HRP IgG (H+L) (Kirkegaard & Perry laboratories inc., USA) diluted 1:8,000 in MPBS. After washing, the substrate solution (Tetramethylbenzidine and H_2O_2 in 0.1 M of sodium acetate pH 5.5) was added. Plates were left to develop for 20' at room temperature, the reaction was stopped by addition of 0.05ml 2N H_2SO_4 and absorbance was read at 450 nm (Titertek® Multiscan Plus; ICN, USA). A negative- and positive control serum was included on each plate; the negative control serum was used to calculate the end-point dilution. End-point dilutions (limiting dilution

titres) were calculated using the MultiCalc program (Pharmacia, Finland). Due to the starting dilution (1:40) of the serum, the lowest titre possible was 6.3 whereas the highest titre that could be calculated was 13.3. The cut-off value for positive titres was calculated as the average titre of all day 0 samples added by twice the standard deviation of these titres.

Statistical evaluation

Analysis of Variance (ANOVA) in combination with Duncan's least significant differences test was used for statistical evaluation of results with a probability of $p<0.05$ considered as significant.

Results

In order to establish a challenge model with the JPC strain of *L. infantum* in our laboratory, hamsters were infected with different doses of promastigotes, via different infection routes. At 1 and 3 months after inoculation, parasites were quantified in spleen and liver by culture microtitration and the occurrence of parasite-specific lesions in several organs was monitored by histopathology.

Table 2: parasite quantification

group	Spl. 1 m.	Spl. 3 m.	Avg. spl.	Li. 1 m.	Li. 3 m.	Avg. li.	b.m. 1 m.	b.m. 3 m.	Tot. pos.
10^8 i.p.	2,964	2,271	2,548[(a)]	≥11,200	≥17,204	≥14,803[(c)]	1/2	3/3	5
10^6 i.p.	0	144	87	4	0	1	0/2	1/3	3
10^4 i.p.	0.5	0.3	0.4	0	0	0	0/2	0/3	2
10^2 i.p.	0	0	0	0	0	0	0/2	0/3	0
10^8 i.d.	16	20	19	54	178	128	1/2	1/3	4
10^2 i.d.	0	0	0	0	0	0	0/2	0/3	0
10^8 s.c.	30	1,119	683[(b)]	20	3,413	2,056	0/2	1/3	5
10^4 met.	0	0	0	0	0	0	0/2	1/3	1
10^2 met.	0	0	0	0	0	0	0/2	0/3	0

Table showing the results of parasite quantification in spleen (spl.) and liver (li.) by culture micro titration. Samples were analyzed at 1 (1 m.) and 3 months (3 m.) post challenge and results are shown as the number of parasites per 10^6 spleen cells or per gram of liver tissue. Results for the bone marrow (b.m.) culture are shown as the number of positive cultures as a function of the total number of samples. Average values for spleen (avg. spl.) and liver (avg. li.) were calculated for each group, group averages that differ significantly from each other are indicated by letters (spleen: no letter/[(a)]/[(b)]; liver: no letter/[(c)]). The total number of parasite positive hamsters in each group is shown in the column marked "tot. pos.".

Table 2 shows the results from parasite quantification by limiting dilution culturing. Regardless of the infection route (i.p., i.d., s.c.) the high dose (10^8) of stationary phase parasites was infective to hamsters and resulted in a detectable –and quantifiable- parasite burden at 1 and 3 months p.i.. The intraperitoneal route was more effective and resulted in higher parasite burdens

that were more consistent within the group compared to subcutaneous or intradermal injection of promastigotes. The parasite burden in both spleen and liver was significantly higher in the '10^8 i.p.' group compared to all other groups (Duncan's test, $p<0.05$). In the '10^8 s.c.' group the parasite burden in the spleen was significantly higher that in the other groups –and lower compared to the '10^8 i.p.' group- but the liver burden was not significantly different (table 2).

The lower parasite doses (10^6, 10^4, 10^2) resulted in lower parasite burdens and fewer infected animals; in the group infected with the lowest dose of stationary phase promastigotes (10^2; i.p. or i.d.) no parasites were recovered at all. Although the average burden in the spleen decreased with decreasing challenge doses, differences between these groups were not statistically significant. Infection with purified metacyclic promastigotes resulted in one parasite positive animal at 3 months post infection in the group infected with 10^4 parasites. In the low dose group (10^2) no parasites were detected.

Results of the histopathological examination of liver, spleen, lymphnode and skin samples are summarized in figure 1 as 'histological scores'. Differences between the groups were not statistically significant (ANOVA, $p=0.05$). A trend can however be detected; in the high dose groups (10^8 promastigotes via i.p., i.d. or s.c. route respectively) the histological score was higher compared to the other groups, mainly due to increased scores for liver and spleen. The lymphnode scores were generally high and somewhat less divergent between groups with the lowest score at 40% (10^4 metacylics i.p. group) and the highest score at 90% (10^8 i.d. group). In the skin, changes indicative of a leishmania infection were only found in one animal in the '10^6 i.d.' group.

In the lymphocyte proliferation assay no response was found to promastigote lysate in any of the samples, the average stimulation index per group ranged from 0.82 to 0.96 and differences between the groups were not statistically significant. Addition of mitogen (ConA) to the cells resulted in a proliferation slightly above background level in all groups with average stimulation indices ranging from 1.09 to 1.30; again with no significant differences between the groups (results not shown).

Antibody levels against promastigote lysate were measured in an ELISA. Figure 2 shows the average titre for each group. Hamsters infected with a high dose (10^8) of stationary phase promastigotes via intra peritoneal, subcutaneous or intra dermal routes developed significantly higher titres against the parasite antigen compared to the other groups. For the '10^8 i.p.' and '10^8 i.d.' groups titres were high at 1 month p.i. already; in the '10^8 s.c.' group titres increased slightly slower, reaching levels similar to those of the other two high dose groups at 3 months p.i..
The group that received an intraperitoneal infection with 10^6 stationary phase promastigotes developed a titre at 3 months p.i. although no antibodies were detected at 1 month p.i.. For the remaining groups infected with 10^4 or 10^2 parasites, titres remained below cut off level for the whole of the experiment.

The relatively high background in the '10^2 metacylics i.p.' group at day 0 and at 1 month p.i. was due to unexplained high titres in two animals on the first and one animal on the second sampling date, the average titre however did not exceed the cut-off value.

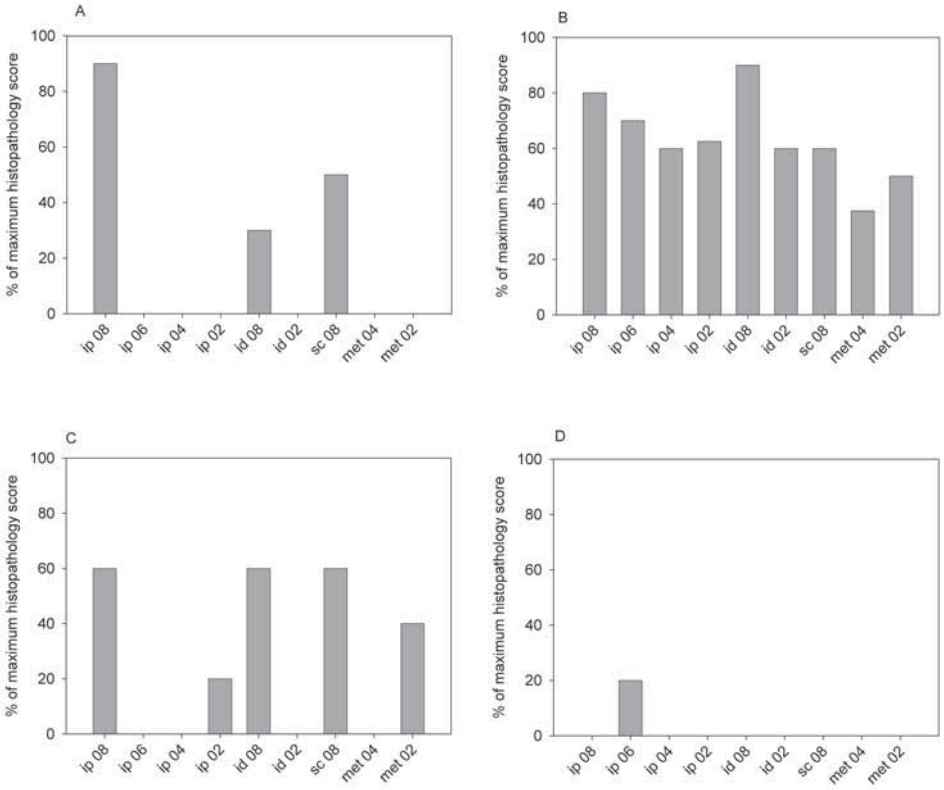

Figure 1; average histopathology scores

Comparison of histopathological changes between the groups. Changes found in samples of liver, lymph node, spleen and skin were scored between 0 and 2; 0=no changes, 1=changes indicative of a leishmania infection, 2= changes characteristic of a leishmania infection. In each group the average score for a tissue type was calculated as the percentage of the maximum score (cumulative score tissue type/no. samples * 2), the resulting values are shown in panels A-D. Labels x-axis: ip 08, ip 06, ip 04, ip 02: groups infected with 10^8-10^2stationary phase promastigotes intraperitoneal; id 08, id 02: groups infected with 10^8 or 10^2 stationary phase promastigotes intradermal; sc 08: group infected with 10^8 stationary phase promastigotes subcutaneous; met 04, met 02: groups infected with 10^4 or 10^2 purified metacyclic promastigotes intraperitoneal.

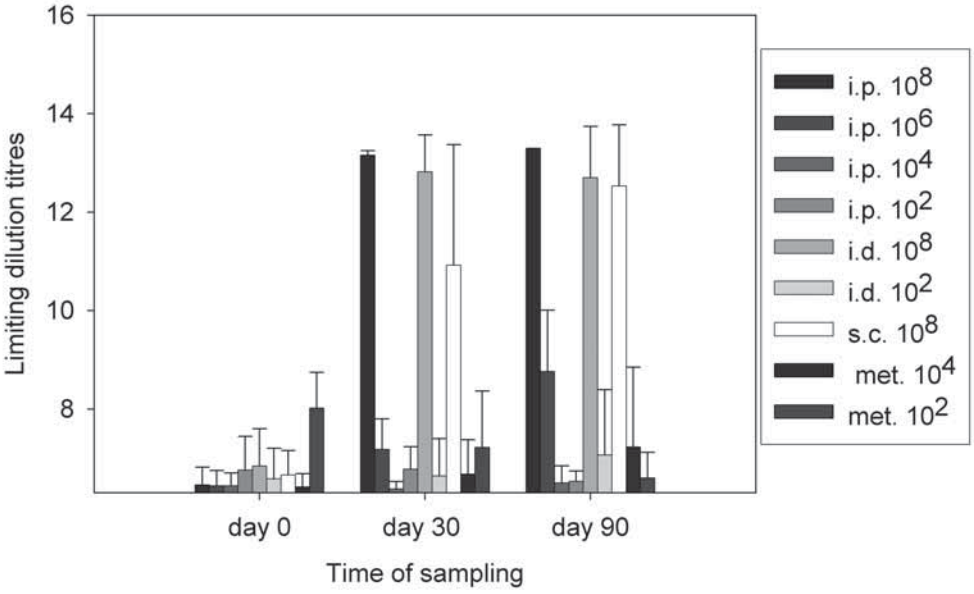

Figure 2; average parasite specific IgG titres

Leishmania-specific IgG titres were established using a soluble leishmania antigen ELISA, titres were averaged for each group. Positive antibody titres were found from day 30 post infection in groups challenged with 10^8 stationary phase promastigotes via intraperitoneal (i.p.), intradermal (i.d.) or subcutaneous (s.c.) routes. Challenge with 10^6 promastigotes i.p. resulted in a low-positive titre at day 90 p.i. but lower challenge doses with either stationary phase or purified metacyclic (met.) promastigotes did not result in detectable antibody titres. Error bars represent the standard deviation. The x-axis crosses the y-axis at 6.3 as this is the lowest titre that could be calculated.

Discussion

It is generally agreed upon that the hamster is a good model for *L. infantum* as both the dissemination of the parasite and the development of disease are similar to what is seen in humans and dogs. However, only few reports exist on the experimental infection of hamsters with *L. infantum*. In the present study we tested different challenge doses of promastigotes via different infection routes in order to determine the optimum dose for vaccine efficacy studies. Challenge of hamsters is usually performed with relatively high doses (10^7-10^8) of parasites, either amastigotes or promastigotes, via intraperitoneal or intracardiac routes (Binhazim et al., 1993; Mendez et al., 2001). Although amastigotes have been found to be more infectious compared to promastigotes (Mendez et al, 2001; Rica-Capela et al., 2003), we decided to use stationary phase promastigotes in our model mainly because under natural circumstances it is the metacyclic promastigote that infects the vertebrate host.

The intra cardiac injection of parasites was not adopted because of animal welfare and technical considerations. However, challenge via the intraperitoneal route is probably less efficacious and it was taken into account that it may require 10 fold higher doses to achieve similar effects through this route (Ott et al., 1967).

In order to minimize variability by host-factors we used hamsters of a single sex and similar age. An effect of gender on *L. donovani* parasite load was shown in a study by (Travi et al., 2002). The relative higher susceptibility of males was shown to be dependent both on suppression of parasite dissemination by estradiol in females and enhancement by testosterone in males. Although differences between weanlings, juveniles and adult hamsters were not large and in part non-significant, it appears that susceptibility to *L. donovani* infection decreases slightly with age (Giannini, 1974). Because of these results and for practical reasons we have chosen to use male juvenile hamsters (between 6 and 8 weeks old at the time of infection) in our study.

In hamsters infected with a high dose (10^8) of stationary phase promastigotes, we found parasites in liver, spleen and bone marrow regardless of the infection route (intra peritoneal, intra dermal or subcutaneous). Due to large individual differences, only the splenic parasite burdens in the '10^8 i.p.' and '10^8 s.c.' groups were significantly different from the groups challenged with lower doses. It appears that the stepwise 100-fold decreases of the challenge dose resulted both in decreases in average parasite burden at 1 and 3 months and in a decrease of the number of animals found parasite positive. Similarly, infection of hamsters with 10-fold dilutions of *L. donovani* amastigotes was shown to result in differences in time to patency and median time to death (Ott et al, 1967). Although parasite burdens were not reported by these authors it is likely that the reported differences resulted from differences in parasite burden. No parasites were found in the cultures from hamsters that received the lowest dose of 10^2 stationary phase promastigotes. However, in some histopathology samples changes indicative of or even specific for a leishmania infection were found and it is therefore well possible that the infection was successful but resulted in a very low parasite burden. Longer post-challenge incubation may have resulted in detectable parasite burdens in the low-dose challenged hamsters as was also shown by (Requena et al., 2000) for a 10^3 intra cardiac challenge dose that resulted in 100% positive cultures only at 1 year post infection.

In our study, hamsters in the '10^8' groups developed a high parasite specific antibody titre after infection. Hamsters infected with 10^6 stationary phase promastigotes i.p. also developed antibody titres, albeit later in the experiment. In the other groups antibodies were not detected. These results appear to be comparable to those reported by (Requena et al, 2000) when taken into account that intra cardiac inoculation was used by these authors. A dose of 10^5 promastigotes of *L. infantum* resulted in detectable antibodies at 1 month post infection whereas serum from hamsters challenged with 10^4 and 10^3 parasites were only positive for anti-Leishmania antibodies at 3 and 4 months p.i. respectively. Although it is not possible to determine a 'threshold' parasite burden from the combined data, the appearance of parasite specific antibodies seems to depend on the size of the parasite burden and can be used to monitor parasite development in hamsters.

However, when using this model for vaccine-efficacy studies it must be taken into account that vaccination may induce antibodies that interfere with this specific test. As a large range of antigens are included in the promastigote lysate that is used for this ELISA, it may detect antibodies induced by a vaccine and in that case titres no longer reflect parasite growth.

It has been suggested that high dose inocula, especially when containing non-infective parasites, "undermine the ability of *leishmania* to initiate infection in a quiescent manner" (Belkaid et al., 1998). According to these authors, non-infective *L. major* promastigotes may be taken up by cells other than macrophages and as a consequence T-cells could become activated. The use of purified metacyclic promastigotes would avoid this activation and more closely mimic the natural infection. Moreover, a dose in the range of what is delivered by the bite of an infected sandfly (100-1,000 promastigotes (Warburg and Schlein, 1986)) should be sufficient for infection of mice with *L. major*. In our experiment challenge with 100 to 10,000 purified metacyclic promastigotes did not result in quantifiable parasite burdens at 1 or 3 months p.i.. Extrapolating from the published information on stationary phase cultures (Mallinson and Coombs, 1989; Sacks et al., 1985) and judging from our own observations we estimate that our stationary phase culture at the time of harvesting contained in the order of 50% metacyclic promastigotes. The doses used for the metacyclic promastigotes should therefore be comparable to $2*10^4$ and $2*10^2$ stationary phase promastigotes. However, both in 'stationary phase' and 'metacyclic' 10^4 and 10^2 groups, parasite burdens were very low or non-detectable. This suggests that if the use of purified metacyclics has doubled the 'effective challenge dose' it was probably not possible to detect a resulting difference in parasite burden. However, it is clear from these results that the use of purified metacyclics did not considerably enhance parasite burden (in the order of 10- to 100-fold) and it is therefore unlikely that presence of non-infective parasites plays an important role in this model as has been suggested for the *L. major* infection model.

Proliferation of lymphocytes after stimulation with antigen was not detected in our experiment, stimulation with mitogen resulted in minimal responses. We have previously optimized the LPA using spleen cells from control hamsters; this resulted in a much higher average stimulation index for ConA of 2.78 (unpublished data). An explanation for the lack of proliferation in this experiment may be immune suppression due to the infection. Several reasons for lack of proliferation have been suggested by different authors. Adherent, macrophage like, cells present in peripheral blood- as well as spleen- mononuclear cell preparations (PBMC and SPMC respectively) of infected hamsters were shown to cause down regulation of lymphoproliferative responses (Dasgupta et al., 1999). Additionally, Rodrigues et al. found a defect in antigen-presenting cells to cause the lack of parasite-specific responses (Rodrigues, Jr. et al., 1992). In a study by Nickol et al. two types of immune suppression were observed; on the one hand a non-specific anergy of lymphocytes resulting in a lack of proliferation after stimulation with a mitogen (conA), on the other hand inhibition of specific responses potentially mediated by a T-cell population (Nickol and Bonventre, 1985). Although in this experiment a general down regulation of lymphocyte proliferative responses can be suspected because of the low response to mitogen, this cannot be concluded as non-infected controls were not included in this study.

In conclusion, our results demonstrate that infection of hamsters with a high dose (10^8) of *L. infantum* JPC strain promastigotes results in dissemination of the parasites, regardless of the infection route. Clinical signs were not observed but parasites were readily detected in cultures of spleen, liver, lymphnode and bone marrow. Although the '10^8' challenge dose is high compared to a natural challenge, it is not excessive for our laboratory model. A 'plateau' does not appear as reduction of the challenge dose has a direct effect on the parasite burden. Thus it should be possible to detect effects on parasite burden and/or dissemination due to vaccination or treatment. Infection with lower doses of promastigotes is possible but longer incubation time (6 months- 1 year) and larger groups have to be considered in order to achieve similar results. For screening of vaccine candidates this is not convenient since the experiments would take too much time. In our model, metacyclic promastigotes do not appear to be more infectious compared to stationary phase promastigotes. The purification of stationary phase promastigotes therefore did not improve the challenge model. Future vaccination-challenge experiments will be performed with a challenge dose of 10^8 stationary phase promastigotes of the JPC strain; via the intraperitoneal route for a standard challenge or via the intradermal route if a more cautious approach is preferable.

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CHAPTER 4

Isolation and characterisation of *Leishmania infantum* CPA-deficient mutants

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Abstract

Visceral leishmaniasis caused by members of the *L. donovani* complex is often fatal in the absence of treatment. Despite its severity the disease has not been studied as much as the related cutaneous leishmaniasis forms. Research has been hampered by the lack of good laboratory models and tools for genetic manipulation. In this study we have characterised a *L. infantum* line (JPCM5) that was isolated from a naturally infected dog and then cloned. We found that the JPCM5 clone qualifies as a good lab model; different stages of the parasite life-cycle could be studied *in vitro,* it is accessible to genetic manipulation and has retained its virulence properties. Furthermore, the *L.infantum* JPCM5 genome has just been fully sequenced.

We have further focused our studies on *LiCPA*, the *L. infantum* homologue to *L. mexicana* Cysteine peptidase A. LiCPA was found to share a high percentage of amino acid identity with CPA genes of other leishmania species. Two independent *LiCPA* deficient mutant clones were generated; the first was recovered after a loss of heterozygocity had occurred while the second clone was generated by classical methods using two virulence markers. In contrast to what has been found previously for *L. mexicana* CPA deficient mutants, both clones were found to have significantly reduced virulence *in vitro* and *in vivo*. Re-expression of only one *LiCPA* allele was sufficient to complement the reduced infectivity of both *LiCPA* deficient mutants for human macrophages which confirms the importance of LiCPA for *L. infantum* virulence. In contrast, *in vivo* experiments did not show any virulence recovery of the re-expressor clone Δ*licpa*C1::CPA compared to the CPA deficient mutant Δ*licpa*C1. Further studies will be necessary to elucidate why the Δ*licpa* mutants have attenuated virulence and why the re-expression of LiCPA produced apparently conflicting results in infection studies of human macrophages and hamsters.

Introduction

Leishmania species are responsible for several pathologies affecting both humans and animals. These parasites are most abundant in developing countries in the Middle East, Asia and South America but also are endemic in the Sub-Mediterranean basin. They have emerged in the past 20 years as the second most important opportunistic agent in AIDS patients (Paredes et al., 2003; WHO, 2000) - after *Pneumocystis carinii* (Alvar et al., 1997). Some cutaneous infections can be resolved and subsequently lead to the development of protection against new infection (Nadim et al., 1983). These results indicate that immunologic approaches could be effective in preventing or curing infections. However, an effective and safe vaccine is yet to be developed. Efforts towards the development of new cures or vaccines have recently been facilitated by the sequencing of *L. major* and *L. infantum* genomes (http://www.sanger.ac.uk/Projects). The latter species is a member of the *L. donovani* complex, which is primarily responsible for life-threatening visceral leishmaniasis. These parasites differ significantly from *L. major* and *L. mexicana.* These species are classified as Old-world and New-world, respectively, based on their distribution. *L. infantum* is present in both Europe and America as it has been imported from its Mediterranean origin to the American continent, where it is named *L. chagasi* (Momen et al., 1993). *L. infantum* can infect humans and dogs, causing zoonotic visceral leishmaniasis in children and immuno-depressed adults as well as canine visceral leishmaniasis (CanL) (Alvar et al., 1997). CanL is a severe disease that can be fatal if left untreated. Treatment is complicated and expensive and generally does not result in compete cure. A vaccine would be an important tool in the prevention of CanL and, since dogs are the reservoir species for this parasite, could reduce the incidence of human visceral leishmaniasis (Dye, 1996).

The sequencing projects for different species of *Leishmania* and other trypanosomatids have led to the identification of many *Leishmania*-specific and several *Leishmania* species-specific genes and proteins that potentially might be exploited for vaccine and drug design (Denton et al., 2004; Martinez-Calvillo et al., 2003; Myler et al., 1999; Vernal et al., 2003). Moreover, additional candidates are likely to be identified as 40% of hypothetical gene products are ascribed a function (Akopyants et al., 2001). However, validation or rejection of a protein as a potential vaccine or chemotherapeutic target requires significant experimental investigations that are time consuming. The question of a protein's conservation and function in other *Leishmania* species should also be a primary consideration, as it is unclear to what extent we can extrapolate the knowledge acquired from one *Leishmania* species to another. This is an important issue as the leishmanias are not all geographically distinct (Grimaldi, Jr. et al., 1989; Hide et al., 1997) and some virulence factors, when used as a vaccine, have been shown to exhibit distinct protective ability between members of *L. donovani* and *L. major* complexes (Campbell et al., 2003; Melby et al., 2001b; Zhang et al., 2003a).

Cysteine peptidases have been characterised as virulence factors in several organisms and, by ourselves, in *L. mexicana* (Coombs et al., 1991; Mottram et al., 1996; Robertson et al., 1996). This last species possesses three families of related enzymes encoded by single (*CPA* and *CPC*) or multicopy (*CPB*) genes (Bart et al., 1995; Mottram et al., 1992). While gene deletions of *LmxCPB,* and to a lesser extent *LmxCPC,* lead to an attenuated phenotype, null-mutants for

LmxCPA exhibited no apparent phenotype (Bart et al., 1997; Mottram et al., 1996; Souza et al., 1994). However, the double null mutant for *LmxCPB* and *LmxCPA* did not induce any lesions in Balb/C mice and the ability of this line to induce an immune response in mice suggested that it has potential as an attenuated live-vaccine (Alexander et al., 1998).

In order to assess their level of conservation in both structure and function, we have characterised cysteine peptidase orthologues in a *L. infantum* line (JPCM5) recently isolated from a naturally infected dog in Spain and then cloned. To validate the usefulness of JPCM5 for study, we confirmed that it has the essential characteristics: it causes visceral leishmaniasis in experimental animals and is accessible to most experimental studies including reproduction of its life cycle *in vitro* and genetic manipulation. The genome of *L. infantum* JPCM5 has been sequenced to 5x coverage (see http://www.genedb.org/genedb/linfantum/). We have now focused a molecular study on *LiCPA*, the *L. infantum* homologue to *LmxCPA*.

Materials and Methods

Parasite lines

L. infantum JPC (MCAN/ES/98/LLM-724) was isolated from the spleen of a naturally infected dog residing in Madrid, Spain in 1998. The parasites were maintained in Novy-MacNeal-Nicolle (NNN) medium (66% (v/v) bacto-agar, 33% (v/v) defibrinated rabbit blood) at 28 °C. The resulting promastigotes were grown in RPMI medium before cloning by limiting dilution in agar blood plates as previously described (Bastein and Wahba, 1989). Five clones were isolated, JPCM1-JPCM5, but all further analyses were carried out with JPCM5 (MCAN/ES/98/LLM-877).

Parasite in-vitro culture

JPC and JPCM5 promastigotes were grown in modified Eagle's medium (designated HOMEM medium (Berens et al., 1976)), RPMI, SDM79 or NNN media with 10% (v/v) heat-inactivated foetal calf serum (FCS) at 28ºC aerobically without agitation (Mottram et al, 1992). Most experiments were carried out in HOMEM medium. Changes between media were accompanied by temporary elevation of FCS concentration to 20% (v/v). Cultures were sub-passaged to 0.5-1x10^6 cells/ml in fresh medium every 3-4 days when the culture had reached late logarithmic phase. The exponential curve regression, the specific growth rate and the doubling time were calculated using Microsoft Excel software from four independent experiments.

Axenic cultures of amastigote-like forms were performed in MAA medium following the protocol previously published with minor modifications (Sereno and Lemesre, 1997). Briefly, promastigotes were grown for 4 to 5 days in HOMEM medium supplemented with 10% (v/v) FCS and 24.5 mM hemin. Then 1 to 5x10^7 parasites were pelleted, washed once in PBS, re-pelleted and re-suspended in 5 ml of MAA2 medium (modified medium 199 with Hank's salts (Gibco BRL), 0.5 % soybean trypto-casein (Promega), 20% (v/v) FCS, 4.8 mM L-glutamine, 24.5 mM hemin, 4 mM $NaHCO_3$ and 25 mM HEPES pH 6.5). The parasites were then incubated at 37°C in the presence of 5% CO_2 for 2 to 3 days before the medium was first changed. Thereafter the medium was changed every 4 to 5 days.

Transformation from amastigotes to promastigotes was performed by transferring 10^6-10^7 amastigotes, which were washed once in PBS, into 10 ml of HOMEM medium supplemented with 20% (v/v) FCS. After incubation at 28ºC under air until more than 90% of the parasites were flagellated (generally after 4 to 5 days), the medium was changed to HOMEM supplemented with 10% (v/v) FCS.

Parasite transfections and selection

Transfections were performed as previously reported [28] using 20 µg of linear DNA. 24 h after the transfections, mutant parasites were selected using one or more antibiotics: 20 µg/ml nourseothricin (Hans Knoll Institute, Germany), 50 µg/ml hygromycin B (Roche, Germany), 10 µg/ml phleomycin (Cayla, France) and/or 20 µg/ml blasticidin (Cayla, France). Clones were either obtained on 0.7% agar-HOMEM plates or in liquid medium, in presence of the appropriate antibiotics, and propagated in HOMEM medium supplemented with the same antibiotics.

Macrophage infections

The U937 human monocyte line, which is non-adherent, was differentiated to macrophages in RPMI+ 5% FCS supplemented with 0.1 ng/ml phorbol 12-myristate 13-acetate (PMA) at 37º C in presence of 5% CO2. The differentiation was effective after 72 h when the cell line became adherent. The cells were washed with RPMI to remove non-adherent cells before adding the JPCM5 parasites at a ratio of 1:20 (macrophages : leishmanias). The infected cultures were incubated at 37º C in the presence of 5% CO2. After 3 hours the cultures were washed to remove free leishmanias that were unable to infect the macrophages.

Samples of infected macrophages were fixed and stained with Giemsa stain at 24, 48, 72 and 96 h post-infection. For each time point, at least 100 macrophages were observed and the number of amastigotes per infected cell was counted. The results were expressed in terms of the % infected macrophages and the number of amastigotes/infected macrophage.

Infections of the canine monocyte-macrophage cell-line DH82 were performed essentially as described by Kiderlen and Kaye (Kiderlen and Kaye, 1990). Briefly adherent and non-adherent DH82 cells were collected in polypropylene round-bottom tubes and stationary phase promastigotes were added at an effector-to-target ratio of 1:8. The cell suspension was incubated for 2 hours at 37ºC with gentle agitation. Remaining extra cellular parasites were washed off by differential centrifugation (220xg, 8 min, 4ºC, four repeats); the resulting cell suspension was seeded into 4-chamber LabTek tissue culture slides (Nunc, Denmark) and incubated for 120 hours. Slides were stained with Giemsa solution (Merck, Germany). For each time point at least 400 macrophages were observed and the number of amastigotes per infected cell was counted; results were expressed as the % infected macrophages and the number of amastigotes/infected macrophage.

Hamster infections

Infections of Golden Syrian hamsters were initiated by intraperitoneal (i.p.) inoculation with 10^7 stationary phase promastigotes in PBS. The hamsters' weight was monitored every week. The hamsters were sacrificed at 3, 6 and 9 months post-infection and the spleens were weighed and sliced into pieces to collect amastigote parasites. These were transformed *in vitro* at 28°C in Homem medium supplemented with 20% FCS to establish promastigote cultures.

Hamsters used for the study of parasite virulence were inoculated i.p. with 10^8 stationary phase promastigotes and sacrificed at 1, 3 and 6 months post-infection. Spleen and liver samples were used for parasite quantification by culture microtitration (Buffet et al., 1995).

PCR amplification

Genomic DNA was prepared from large scale promastigote cultures and extracted using phenol as previously published (Sambrook et al., 1989). PCR was carried out using 50 ng of genomic DNA as template, 100 ng (about 30 pmoles) of each primer, 5% DMSO and 2.5 U Taq polymerase (Perkin-Elmer). Conditions were 1 cycle of 94°C for 5 min then 25 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 2 min followed by 1 cycle of 72°C for 5 min.

LiCPA deletion and re-expression constructs

JPCM5 *CPA* flanking regions were amplified from *L. infantum* DNA with primers OL670-OL671 and OL672-OL673 originally designed to amplify *L. mexicana* sequences and integrated in place of the *L. mexicana* flank sequences in each side of the *DHFR-SAT* cassette of the *Lmxcpb-sat* deletion construct (Mottram et al., 1996a) to generate pGL545. The *LiCPA BLE* (pGL726) and *HYG* (pGL813) deletion constructs were derived from pGL545 by replacing the *Spe*I/*Bam*HI fragment containing the *SAT* resistance gene by the appropriate antibiotic resistance gene extracted from pGL53 and pGL345 (p*Lmxcpb-ble* and p*Lmxcpb-hyg* constructs) respectively. A 1.83 kb DNA fragment corresponding to the *LiCPA* 5' flank and ORF was amplified with primers OL670 and OL1006 in order to generate *Hin*dIII and *Pst*I sites for subcloning. The *LmxCPC* 5' and 3' flank sequences from pGL437 (Brooks et al., 2000) were then replaced by this PCR product and the *LiCPA* 3' flank, amplified as described above, to generate the *LiCPA* re-expression construct pGL793.

Southern blot analysis

For Southern blot analysis, 5 µg *L. infantum* genomic DNA was digested to completion with the appropriate restriction enzymes before being separated and transferred onto membrane according to standard procedures (Sambrook et al, 1989). For wild-type *L. infantum* DNA analysis, the membrane was treated and probed with a non-radioactively labelled PCR product corresponding to the *LiCPA* ORF as described above. Δ*Licpa* mutant blots were probed with radioactively labelled PCR products corresponding to the *LiCPA* ORF 5' or 3' flanks, essentially as published (Denise et al., 2004) except that the membranes, after high stringency washes, were exposed on Phosphorimager plates. The plates were scanned at high resolution on a Typhoon 8600 apparatus (Molecular Dynamics).

Northern blot analysis

Total RNA was extracted from 10^7 to 10^8 parasites using Trizol (Invitrogen, UK) or RNAEasy kit (Qiagen, Germany) according to the manufacturer's instructions. The amount of total RNA extracted was quantified by spectrophotometry and about 5 µg were loaded on 0.8% agarose gel prepared in DEPC-treated 0.5X TBE buffer. After separation, gels were washed once in DEPC-water and transferred onto membrane using to the same procedure as for Southern blot. Hybridization with radioactively labelled probes took place overnight at 65°C. Stringent washes were performed in 0.2x SSC, 0.1% SDS and the membranes were exposed onto Phosphorimager plates. The plates were scanned at high resolution after two days exposure.

RT-PCR

cDNA was prepared from 5 µg of total RNA using the AMV RT module from the GeneRacer kit (Invitrogen, UK). A first RT-PCR was performed on 1 µl of cDNA mixture with 2 units of Thermozyme enzyme (Invitrogen, UK) and 100 ng of first-round primers in 50 µl reaction volume. The resulting PCR mixture was diluted 30 times in water and 1 µl of it was used as template for a nested reaction performed in a 20 µl reaction volume with 1 unit of *Taq*DNA polymerase (ABGene, UK) and 100 ng of nested primers.

The first-round primers used were the Splice Leader primer, (OL618) / OL137 for the 5' amplification and OL67 / GeneRacer Oligo dT primer for the 3' amplification. The primers for the nested PCR were OL618 / OL136 for the the 5' amplification and OL1194 / GeneRace 3′ Nested primer for the 3' amplification.

Oligonucleotides used in this study.

OL67	5'-CAGAACATGCAGACAGCC-3'
OL136	5'-GAGCGGAACCGTAGCACA-3'
OL137	5'-GACAGCGTCCGCAGTGGTG-3'
OL618	5'-AAGTATCAGTTTCTGTACTTTATG-3'
OL670	5'-ATATAAGCTTCTACTGCACCAGGTACTG-3'
OL671	5'-ACGTGTCGACGAGAAGGACGTGACGGGG-3'
OL672	5'-GGTGCCCGGGGCTGTGCACAAACACGACC-3'
OL673	5'-GTCGAGATCTCACTGTCCATGGCACGCCTG-3'
OL1006	5'-CTGCAGCTAGGCCGCTGTCGTCGG-3'
OL1194	5'-GACAGCGTCCGCAGTGGTG-3'

Results

Isolation and characterisation of L. infantum JPCM5

Leishmania parasites were recovered from a naturally infected dog from the Madrid area, Spain. They were characterised as *L. infantum* zymodeme 1 (MON-1) by isoenzyme analysis (Rioux et al., 1990) and transformed into promastigotes *in vitro* before being stored under liquid nitrogen as JPC (MCAN/ES/98/LLM-724). Five independent lines (JPCM1 to JPCM5) were derived from this stock by colony cloning and tested for infectivity in hamsters. Two of the cell lines were recovered from the spleen 15 weeks after infection confirming that they had conserved their infection potential. The clone M5 (JPCM5; MCAN/ES/98/LLM-877) was selected for all further studies.

JPCM5 grew well as promastigotes *in vitro* in many of the media used to grow *Leishmania*, including RPMI, SDM79 and NNN. In HOMEM medium, the parasites had a doubling time of 16.0 ± 2.7 h. After 4 to 5 days in culture, when stationary phase had been reached, slender metacyclic-looking parasites were observed, indicating that metacyclogenesis had occurred. A temporary raising of serum concentration from 10 to 20% (v/v) promoted the recovery rate following adaptation to a different medium, cloning, transformation from, or to, amastigotes and transfection experiments.

Stationary phase promastigotes of JPCM5, grown in HOMEM medium, were transformed *in vitro* into amastigote-like forms using conditions previously described for other *L. infantum* lines (Sereno et al., 1998). The transformation of the entire culture, according to morphological parameters, required 3 to 4 days at 37°C in acidic medium (pH 5.5 for SDM or pH 6.5 for MAA). This was accompanied by changes in protein profile as determined by SDS-PAGE (data not shown). The axenic amastigotes could be maintained, with the help of regular sub-

passages, or transformed back into promastigotes by switching the culture conditions to 28°C in HOMEM medium with 10% (v/v) FCS. It generally required four days for transformation fully back to promastigotes.

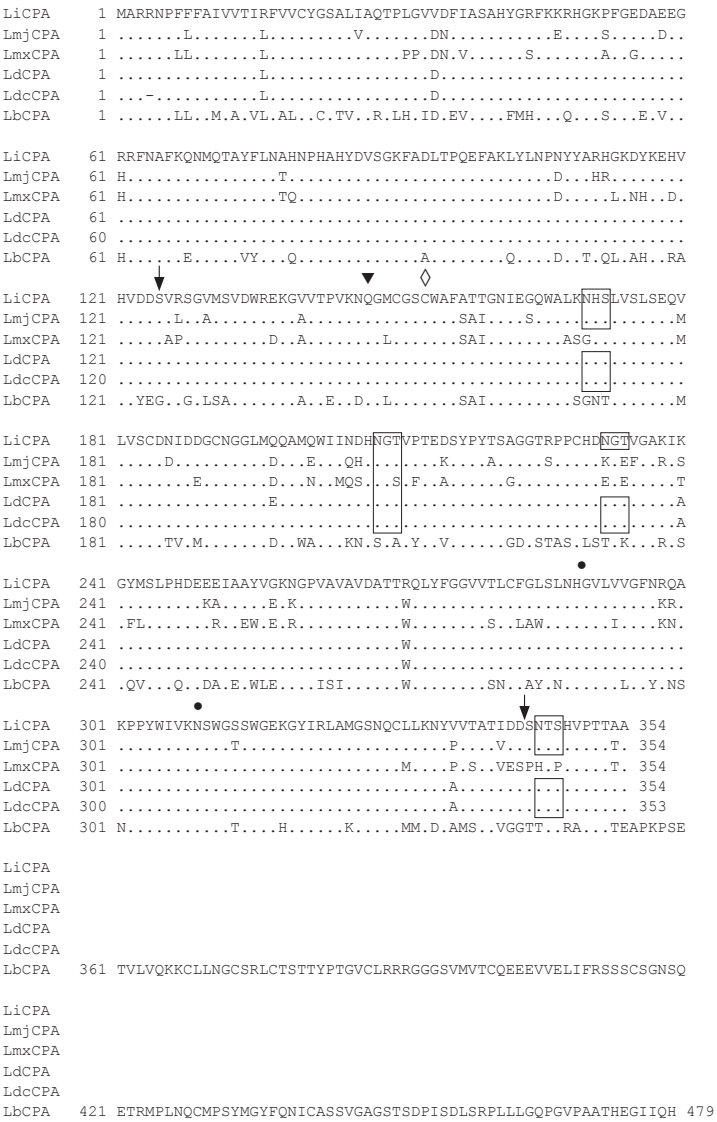
L. infantum JPCM5 were infective to human and dog macrophages (see below) and hamsters. JPCM5 parasites could be recovered from spleen, liver and bone marrow of infected hamsters less than two months post infection. Long-term *in vitro* culture of some *Leishmania* species has been reported to result in loss of virulence (Handman et al., 1983). To test whether this applied to JPCM5, promastigotes were sub-passaged *in vitro* for 8, 17 and 25 weeks (corresponding to about 10, 20 and 30 sub-passages, respectively) before being inoculated into hamsters. In each case, amastigotes were recovered from the spleen after less than three months indicating that the promastigotes had not lost their virulence and were still able to establish an infection. These *in vitro* and *in vivo* results indicated that JPCM5 is accessible to most cell-culture experimentations.

Isolation of the** CPA **cysteine peptidase gene

The complete *CPA* gene of *L. infantum* JPCM5 (*LiCPA*), including the ORF and the 5' and 3' flanks, was amplified by PCR using primers derived from the *L. mexicana* sequence (acc. number X62163, (Mottram et al, 1992)) and sequenced. The *LiCPA* ORF sequence predicted a protein of 354 amino acids corresponding to a size of 38.9 kDa, an isoelectric point of 7.6 and possessing a 10 amino acid C-terminal extension similar to the *L. mexicana* published sequence *(*LmxCPA CAA44094 (Mottram et al, 1992)). Comparison of the deduced amino acid sequence with the translated *CPA* sequences from several *Leishmania* species is shown in Figure 1. LiCPA shared 97.7% amino acid identity with *L. chagasi (*LcCPA), 87.3% with *L. major* (LmjCPA) and 80.3% with *L. mexicana* (LmxCPA) protein sequences, respectively. There were three potential N-glycosylation sites, two in the mature domain (^{208}NGT^{210} and ^{170}NHS^{172}) and one at the beginning of the C-terminal extension (^{345}NTS^{347}); the last two being conserved in all sequences except in *L. mexicana* and *L. braziliensis*. However, amino-acid residues important for the catalysis, ^{153}C, ^{289}H and ^{309}N, as well as the predicted glutamine of the oxyanion hole ^{147}Q, were conserved in all sequences (Mottram et al, 1992). LiCPA did not possess the long C-terminal extension found in LbCPA and was identical in size to CPA from *L. major*, *L. mexicana* and *L. donovani*.

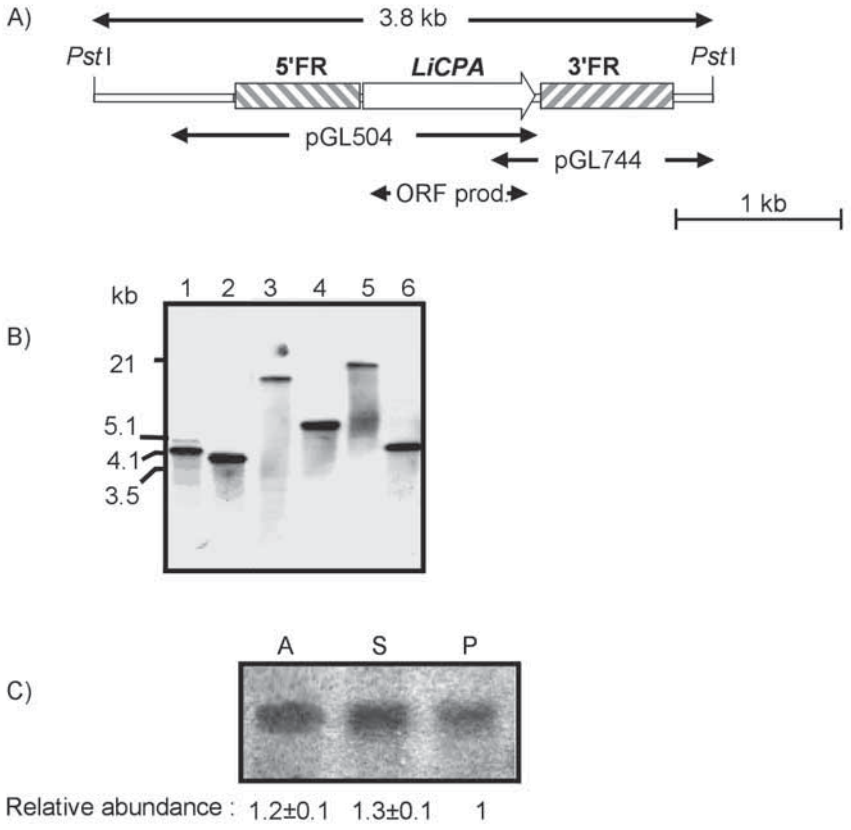
Southern blot analysis showed that *LiCPA* is single-copy (Figure 2, panels A and B) and Northern blotting showed that *LiCPA* is expressed at similar levels in log phase and stationary phase promastigote and amastigote stages (Figure 2C). A *LiCPA* deletion construct containing the *SAT* gene flanked by about 800 bp of *LiCPA* 5' and 3' flank regions was generated and used to transfect JPCM5 parasites. The parasites were selected in 96-well plates with HOMEM medium supplemented with antibiotic. In these conditions, one clone was obtained seven weeks post-transfection. Southern blot analysis (Figure 3A) and PCR analyses (not shown) showed correct integration of the *SAT* construct into the *LiCPA* locus. Moreover, DNA fragments corresponding to the expected remaining wild type allele were absent, suggested that this nourseothricin-resistant clone (named Δ*Licpa*C1) is a null-mutant resulting from loss of heterozygocity.

Figure 1 - Alignment of CPA amino-acid sequences.



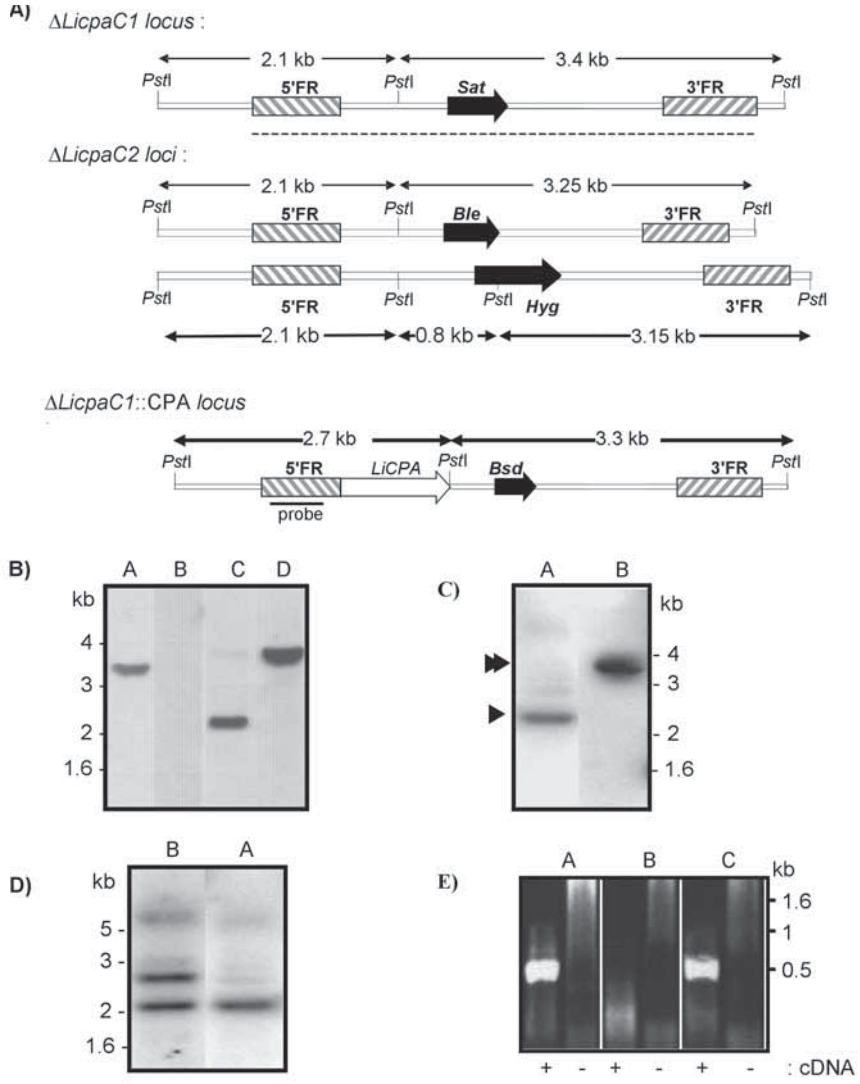
Alignment of deduced amino acid sequence of *LiCPA* (CAD12932) from *L. infantum* JPCM5, with deduced amino acid sequences obtained from GenBank. These included *L. major* (*LmjCPA*, CAC18865.1), *L. mexicana* (*lmxCPA*, CAA44094), *L. donovani (LdCPA,* AAK27384.1), *L. d. chagasi (LdcCPA,* AAC38833) and *L. braziliensis* (*LbCPA*, LbrM19.1180). The dots represent amino acid identity. The arrow shows the predicted cleavage site of pro/mature domains and mature/C-terminal extension, respectively. Amino acid residues important for the catalysis are indicated as follows : active site cysteine C^{153} (◊), the predicted catalytic dyads H^{289} and N^{309} (•) and the predicted glutamine of the oxyanion hole Q^{147} (▼). The potential glycosilation sites are shown in boxes.

Figure 2 : *LiCPA* genomic organisation and expression.



A) Map of *LiCPA* locus with the different subclones analysed indicated. B) Southern blot of *L. infantum* JPCM5 gDNA hybridized with the *LiCPA* PCR product. The gDNA was digested using *Sph*I (lane 1), *Pst*I (lane 2), *Xho*I (lane 3), *Sma*I (lane 4), *Sac*I (lane 5) and *Apa*I (lane 6). C) Northern blot of total RNAs from *L.infantum JPCM5* log phase promastigotes (P), stationary phase promastigotes (S) and axenic amastigotes (A) hybridized with the *LiCPA* ORF PCR product. After washing, the membrane was exposed on a phosphor-activated screen and the relative abundance of *LiCPA* mRNA in each lane was determined (n=2).

Figure 3 : Generation of ∆*Licpa* null-mutants.



A) *Pst*I restriction map of ∆*Licpa*C1, ∆*Licpa*C2 and ∆*Licpa*C1::CPA loci. The size of the restriction fragments expected is indicated. The dotted line underneath represents the extent of the *LiCPA* deletion construct. 5'FR and 3'FR indicate the 5' and 3' *LiCPA* flank sequence, respectively. B) Southern blot analysis of the ∆*Licpa*C1 mutant gDNA digested with *Pst*I and hybridized with the following probes : *LiCPA* 5'FR (lane A), ORF (lane B) and 3'FR (lane C). Lane D presents wild-type gDNA hybridized with the 3'FR probe. C) Southern blot analysis of the ∆*Licpa*C2 mutant (lane A) and wild-type (lane B) gDNA hybridized with the *LiCPA* 5'FR probe. The single and double arrowheads indicate the deleted and the wild-type alleles, respectively. D) Southern blot analysis of the ∆*Licpa*C1 (lane A) and ∆*Licpa*C1::*CPA* (lane B) re-expresser mutant gDNA digested with *Pst*I and hybridized with a 5'FR probe. E) RT-PCR analysis of wild-type (lane A), ∆*Licpa*C1 (lane B) and ∆*Licpa*C1::*CPA* (lane C) parasites. Nested PCR were carried out in presence (+) or in absence (-) of cDNA using the primer pairs OL136/Splice Leader primer as indicated in "Materials and methods".

A second transfection was performed on JPCM5 promastigotes with a second deletion construct derived from the previous one by replacement of the *SAT* marker by the *BLE* gene. The transfection and selection steps were performed as described above, except that 10 µg/ml of phleomycin was used in place of nourseothricin. Four clones grew onto agar plates after two weeks. PCR and Southern blot analysis demonstrated that these four clones were Δ*Licpa* heterozygote mutants having correctly integrated the *BLE* construct into one *LiCPA* allele (data not shown). One of these (clone 3047) was selected and re-transfected with a third *CPA* knockout construct, pGL813, carrying the *HYG* gene. A total of 16 clones were obtained from four 96-well plates after two weeks of selection in the presence of both phleomycin and hygromycin. Southern analysis of one of these clones, called Δ*Licpa*C2, confirmed that it was indeed a hygromycin- and bleomycin-resistant Δ*cpa* null mutant (Figure 3B). The *LiCPA* gene was re-introduced into the CPA locus in both Δ*Licpa* mutants. The re-integration construct, pGL793, contained the *LiCPA* ORF surrounded by its 5' flank and the 3' *DHFR* sequences, and the blasticidin resistance gene enclosed between 5' and 3' *DHFR* flank sequences. Both Δ*Licpa* mutants were transfected with the re-integration cassette and selection achieved in the presence of 10 µg/ml of blasticidin. Several clones were isolated from each transfection (the ones characterised in this study were named Δ*Licpa*C1*::CPA* and Δ*Licpa*C2*::CPA*) and the correct replacement of previously integrated deletion cassettes was confirmed by PCR and Southern blot (Figure 3C). *LiCPA* re-expression was demonstrated by RT-PCR (Figure 3D), similar results were obtained with the Δ*Licpa*C2 mutant and the corresponding re-expresser Δ*Licpa*C2*::CPA* (data not shown).

The Δ*Licpa*C1 and Δ*Licpa*C2 mutants were grown as promastigotes in HOMEM medium. In these conditions, they exhibited no apparent growth reduction compared to wild type JPCM5. They were also both able to transform into axenic amastigotes. In order to assess whether the mutants lacking CPA were virulent, their infectivity toward human macrophages was investigated. In comparison with JPCM5, the Δ*Licpa*C1 and Δ*Licpa*C2 mutants had a reduced ability to infect human macrophages (Figure 4A) and a reduced number of amastigotes/infected macrophage (Figure 4B). The infectivity was restored to wild type levels by re-expression of *CPA* in the Δ*Licpa*C2 mutant, but Δ*Licpa*C1*::CPA* had a virulence phenotype midway between the Δ*Licpa*C1 mutant and wild type parasites. Some cell lines were also tested for their ability to infect macrophages of dogs (Figure 4C and D). No statistically significant differences (ANOVA; $p \leq 0.05$) were observed between wild type, Δ*Licpa*C1 or Δ*Licpa*C1*::CPA* with respect to percentage of infected macrophages or the number of amastigotes per infected macrophage.

The virulence of Δ*Licpa*C1 was found to be significantly less than wild type In a hamster infection model (Figure 5); parasites were not detected in the liver and only very low levels were detected in the spleen, with the number of parasites decreasing between 1 and 6 months post infection. The overall parasite burden for these clones was between 10^2 and 10^4 fold lower compared to JPCM5 at 1 and 6 months post infection, respectively. The virulence of Δ*Licpa*C1*::CPA* was also compared *in vivo* to JPCM5. Low numbers of parasites were found in the spleen of Δ*Licpa*C1*::CPA* infected hamsters 3 months post-infection, whereas no parasites were detected in spleen or liver of hamsters at other time points, indicating the virulence was not restored in the re-expresser clones.

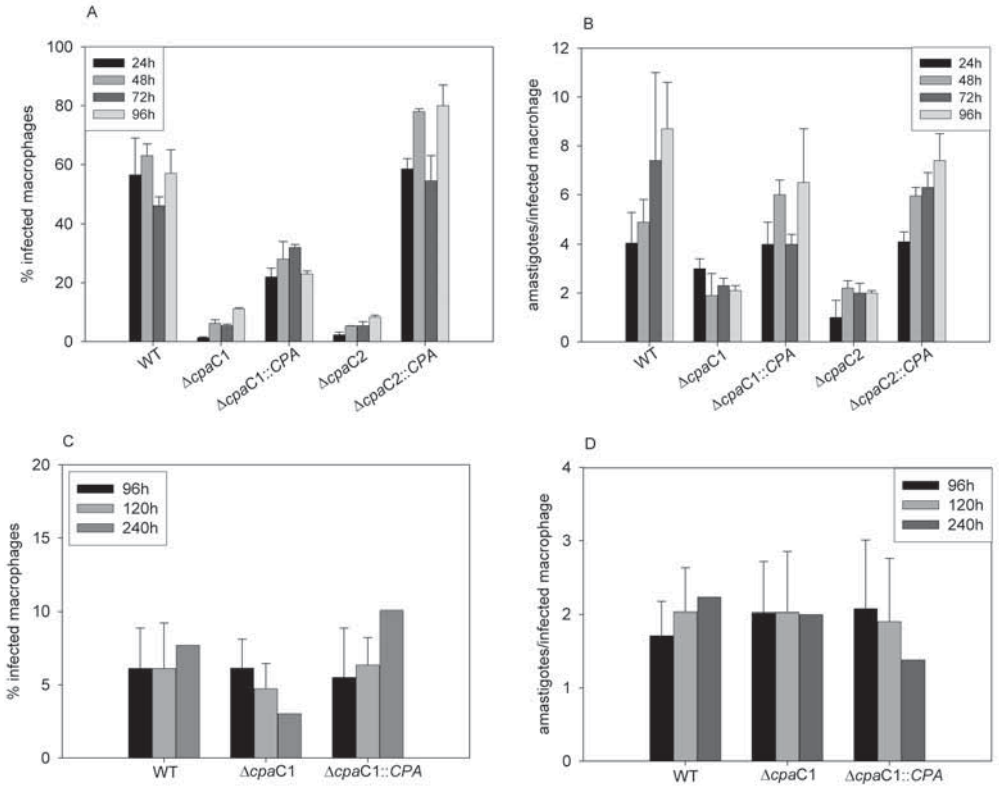


Figure 4 : *LiCPA* mutant infectivity *in vitro*

Human monocyte cells (U937) were infected with wildtype (WT), CPA null mutants (Δ*Licpa*C1, Δ*Licpa*C2) or re-expresser clones (Δ*Licpa*C1::*CPA*, Δ*Licpa*C2::*CPA*) of *L. infantum*. Cell cultures were monitored for four days; results are presented as the percentage of infected macrophages and the number of amastigotes per infected cell in panels A and B respectively. Similar experiments were performed in a canine monocyte cell line (DH82) using WT, Δ*Licpa*C1 and Δ*Licpa*C1::*CPA L.infantum* clones. Cell cultures were monitored for five to ten days after infection; results are presented in panels C and D. Depicted values at 96 and 120 hrs incubation are the mean of four independent experiments and error bars indicate the standard deviation. The data for the ten day incubation period is derived from one experiment, hence the absence of a standard deviation.

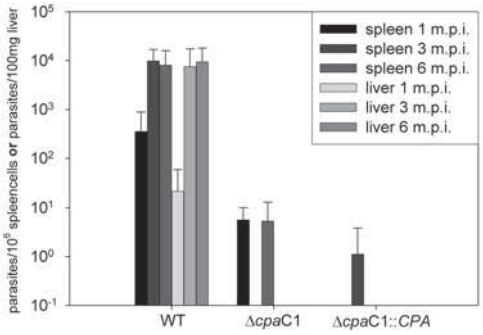


Figure 5: *LiCPA* mutant infectivity *in vivo*
Hamsters were infected with wild type (WT), CPA null mutant (Δ*Licpa*C1) or re-expresser clone (Δ*Licpa*C1::*CPA*) of *L. infantum*. The parasite burden in spleen and liver was determined by limiting dilution culturing at one, three and six months post infection (1, 3 or 6 m.p.i.). Results are shown as the average parasite burden per group; error bars indicate the standard deviation.

Discussion

While other forms of the disease are self-healing, and generally not life-threatening, visceral leishmaniasis is often lethal in the absence of treatment (Melby, 2002). However, research has been focussed on cutaneous and muco-cutaneous leishmaniasis and not so much on visceral forms of the disease. This has been enforced by the availability of well developed mouse models for cutaneous leishmaniasis using *L. major* and *L. mexicana* enabling both *in vitro* and *in vivo* studies. Moreover, these model strains can be easily transfected using well-established procedures and their complete life-cycle can be reproduced axenically. The main model for visceral leishmaniasis is *L. donovani,* that was, until recently, only accessible to laboratory manipulation in the promastigote form, a condition which carries the risk of artificial reduction of infectivity (De and Roy, 1999; Debrabant et al., 2004; Dey et al., 2002). *L. infantum* could represent an alternative model. It mostly infects canine species and its importance increases due to the fact that it is present on both sides of the Atlantic. Moreover, axenic culture of amastigote forms had been previously reported (El Fadili et al., 2002; Larreta et al., 2004; Sereno et al., 1998).
In order to ensure the isolation of a fully virulent strain, *Leishmania* parasites were collected from a naturally infected dog of the Mediterranean basin where the canine population has a high prevalence of infection. This high prevalence also means several different populations of parasites could have infected the animal. Five individual parasites were therefore isolated and their clonal population was checked for virulence. Hamsters were chosen as the host model since mice were found to be uninfected (data not shown) while dog experiments were unsuitable as they were deemed too expensive and time consuming (Melby et al., 2001a; Poot et al., 2005). Only two of the cell lines were recovered from the spleen after 15 weeks confirming that the original population was indeed heterogeneous.

In the cloning process, we showed that most parasites were able to transform *in-vitro* from the non-flagellated intracellular amastigotes to the free-swimming promastigote forms. These can easily be propagated in well-defined medium supplemented with pre-tested foetal

calf serum. We mostly cultivated the promastigote parasites in relatively rich medium which promoted good growth rate and recovery. We found that the parasites could be adapted from one media to another by progressively replacing the media; raising the foetal calf serum concentration appeared to be helpful during this step. Similarly we also raised it temporarily when promastigotes were transformed axenically in amastigotes. Despite these precautions, we observed that a certain proportion of parasites did not transform although the exact ratio was difficult to determine. We hypothesized that they corresponded to "early-log" phase parasites as they were more prevalent when inoculums came from dividing logarithmic rather than stationary phase cultures. They were not a problem for our study as they generally disappeared from our amastigote culture following the first subpassage, most probably by lysis. Similarly, the axenic amastigotes transformed back to promastigotes even after having been maintained for long periods in MAA medium at 37°C. Contrary to what has been previously observed with *L. mexicana,* the transformed parasites showed similar infectivity to hamsters as JPCM5 promastigotes (Denise et al., 2003). Host infection begins with macrophage invasion. As this phenomenon occurs relatively quickly, it can only be studied *in-vitro*. Thus it was of particular importance to be able to infect macrophages and JPCM5 was found suitable for canine and human macrophage experiments. While canine macrophages could be considered a more natural model, studying human macrophages is relevant as *L. infantum* is also found in people even if they harbour generally no clinical signs and maintain the parasitaemia to very low level, except when immuno-depressed. No attempt to transmit these parasites through sandflies has been performed although preliminary results indicate that JPCM5 promastigotes were maintained in fly guts 7 days after a single infected blood meal (data not shown). Taken together, these results indicate that, in appropriate conditions, the different stages of the parasite life-cycle could be studied *in-vitro* using the JPCM5 clone. Animal experiments indicated no significant differences in infectivity between parasites that had been maintained as promastigotes for 5 up to 30 sub-passages. This last point is of particular importance as genetic manipulations often require several weeks of maintenance in culture for the transfection, selection and cloning processes. Again, JPCM5 qualifies as a good lab model as we report that it is accessible to genetic manipulation. Furthermore, its genome has just been fully sequenced (http://www.genedb.org/genedb/linfantum/).

In this study we used four selection markers (nourseothricin, hygromycin, phleomycin and blasticidin). Additionally we established that the two others markers available in *Leishmania* (puromycin and neomycin) can also be used in JPCM5 promastigotes (data not shown). We successfully disrupted and then re-integrated the locus coding for LiCPA, a cathepsin-L like proteinase. Our laboratory demonstrated that cysteine proteinases are potent virulence factors in *L. mexicana* but we have been unable to associate an attenuated phenotype to Δ*Lmxcpa,* a mutant lacking the LiCPA homolog (Frame et al., 2000). This disqualified LmxCPA as a major virulence determinant in *L. mexicana* but does not imply that counterparts in other species are not of importance. Virulence determinants are not always conserved between species; for example the *L. donovani* major antigen A2 was shown to not be expressed in *L. major* (Zhang et al., 2003b). Furthermore, we have previously shown that the double null-mutant Δ*Lmxcpa* Δ*Lmxcpb* exhibited a more marked attenuated phenotype compared to the Δ*Lmxcpb*

mutant (Alexander et al, 1998) indicating that LmxCPA is playing a role, although minor, in *L. mexicana* virulence. No such evidence has been accumulated in *L. infantum*. CPA enzymes from all *Leishmania* species share the active site residues and are all encoded by a single copy locus. As expected, LiCPA was found closer to CPA from *L. chagasi* than from *L. major* or *L. mexicana* (97.7 vs 87.3 and 80.5% identity, respectively). Interestingly, LiCPA did not exhibit the strong amastigote specific expression of LmxCPA, LmjCPA and LdcCPA although it was slightly more expressed in stationary phase promastigotes, a culture phase enriched in infectious metacyclic parasites. Only the *L. braziliensis* CPA enzyme possesses a long C-terminal extension of 120 amino-acids. Without this extension, LbCPA and LiCPA share 64.8% of their amino-acid. Intringuily, when translated in the same frame, the 3' flank from *LiCPA*, *LmxCPA* and *LmjCPA* encode a peptide with high homology to the LbCPA C-terminal extension (63.7% amino-acid identity between the C-terminal extension of LbCPA and LiCPA, data not shown). This will need further investigation.

Two independent Δ*Licpa* mutants have been generated. The first mutant, Δ*LicpaC1,* was the result of a loss of heterozygocity, an event that has been reported to occur naturally in response to an increase of drug concentration, resulting in duplication of the allele carrying the antibiotic resistance gene and leading to higher expression of a gene product essential for parasite survival (Gueiros-Filho and Beverley, 1996). This is evidence that *LiCPA* is non-essential to promastigotes *in vitro*, and also indicates that the concentration of nourseothricin used (20 µg/ml), which was inferred from *L. mexicana* experiments, was higher than necessary to select *L. infantum* JPCM5 promastigotes. Indeed we have subsequently determined that a concentration of 15 µg/ml was sufficient. In any case, it could not have been foreseen that such a small increase in nourseothricin concentration was sufficient to initiate a loss of heterozygocity, that is normally observed in presence of strong selection pressure, but this drug is stable in culture medium and a single clone was obtained from this experiment indicating that the incidence of this event had been very low. The second mutant, Δ*LicpaC2,* was generated classically using two independent markers and the selecting concentrations were found to be identical to the ones used in *L. mexicana* (Mottram et al, 1996). For both mutants, molecular analysis confirmed that the expected gene replacements had taken place. This signifies that except for the nature of the antibiotic resistance genes, the Δ*Licpa* mutants could be considered genetically indistinguishable at the level of the deleted *LiCPA* locus. Indeed both mutants exhibited an attenuated phenotype, *in vitro,* in human macrophages, and, *in vivo,* in hamster infections. This represents a strong indication that LiCPA plays a more important role in *L. infantum* virulence than was extrapolated from the *L. mexicana* studies. These findings were apparently not corroborated by dog macrophage experiments where Δ*Licpa*C1 was found as infective as wild-type parasites. However, the overall level of infection encountered – almost 12 times less infected dog macrophages than infected human macrophages with JPCM5 wild-type parasites – may be too low to reflect a possible attenuated phenotype for Δ*Licpa*C1. On the other hand, the number of amastigotes per macrophage was not significantly different between wild-type and mutant parasites which seems to indicate that LiCPA is not required for *L. infantum* maintenance in dog macrophages. This would be in agreement with the expression pattern exhibited with no high expression in amastigote parasites.

The finding that re-expression of only one *LiCPA* gene was sufficient in itself to complement the reduced infectivity of both *Licpa* deficient mutants for human macrophages confirm the importance of LiCPA for *L. infantum* virulence. Δ*Licpa*C1::CPA was only half as virulent as Δ*Licpa*C2::CPA but this may indicate a lower level of LiCPA re-expression in the first cell lines. Alternatively, we could not exclude that the loss of heterozygocity had not been accompanied by other re-arrangements destined to help the parasites to adapt to the higher drug concentration (Dujardin et al., 2000) and consequently that Δ*Licpa*C1 and Δ*Licpa*C1::CPA were less fit than Δ*Licpa*C2 and Δ*Licpa*C2::CPA to infect macrophages; investigating this will require more sensitive methods as the level of macrophages infected observed with the deficient mutants were very low. In contrast, *in-vivo* experiments did not show any virulence recovery of Δ*Licpa*C1::CPA compare to Δ*Licpa*C1. One explanation is that the Δ*licpa*C1::CPA clone expresses only one LiCPA gene compared to two for the wild-type parasites. Also there is dissimilarity between the *LiCPA* genetic context in these two cell lines; the 5' *LiCPA* flank sequence of both clones was identical, however the nature of the 3' flank was different in that the re-expressers possessed the *L. major* 3' DHFR flank sequence downstream of the *LiCPA* gene. This sequence is classically used in *Leishmania* constructs to stabilize the mRNA, leading to higher levels of expression (Beetham et al., 1997; Brooks et al., 2001). The exact nature of these key signals is still cryptic and the re-expresser lines may provide a tool to investigate them.

Further studies will be necessary to elucidate why the Δ*Licpa* mutants have attenuated virulence. As indicated earlier, we have not yet determined if LiCPA is playing a part in the maturation process of other proteases like does LmxCPA for LmxCPB in *L. mexicana*. If this is the case, we would have to consider that no alternative way of processing these enzymes exists in *L. infantum*, explaining the attenuated phenotype of the mutants. In support of this hypothesis, the high homology level between CPA proteins from *L. infantum* and from *L. mexicana,* and in general from all *Leishmania* species, together with the conservation of the four residues implicated in the catalysis suggests that these enzymes have related substrates and could have a similar mode of action. The zymogram indicated no activity toward gelatin for both enzymes (data not shown) rendering the detection of LiCPA activity difficult. Investigation of LiCPA localisation will require raising specific antisera as antibodies against LmxCPA very poorly recognized LiCPA on western-blot (data not shown). Genomic comparison of *L. infantum* and *L. major* may also provide clues to further explain the results reported here and to understand the virulence determinants in *L. infantum*.

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CHAPTER 5

Virulence and protective potential of several Cysteine peptidase knockout strains of Leishmania infantum in hamsters

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Summary

Few vaccines have been successfully developed against parasitic diseases. Among those that have been commercialised, many contain live –attentuated- parasites. The complex nature of most parasites and of host-parasite interactions may well be an important cause for the relative success of live vaccines compared to whole killed- or subunit vaccines. In order to explore the possibility of using this approach for a canine leishmaniosis vaccine, specific knock-out strains of *Leishmania infantum* were produced. Cysteine peptidases (CP's) are virulence factors of *Leishmania* parasites and thought to be involved in various processes that are of vital importance to the parasite, including immune modulation. CP knockout strains of *Leishmania infantum* have been generated and were tested in this study for virulence and protective potential in hamsters. The CPA knockouts as well as a number of CPB/cpb heterozygote clones were all found to have strongly reduced virulence in hamsters. Since the heterozygote strains were expected to have a higher residual virulence than what was found, the exact mechanism behind the attenuation of the mutant strains is uncertain and needs further investigation. Vaccination with attenuated clones did not induce protection against a wild type challenge infection in our model.

Introduction

Canine leishmaniosis is a devastating parasitic disease caused by *Leishmania infantum* in the Mediterranean region and by *L. chagasi* in large parts of South America. Treatment of infected dogs is difficult and expensive and most dogs are not cured. Moreover, *L. infantum (L. chagasi*) can cause zoonotic visceral leishmaniosis in children and immune compromised people. Infected animals can therefore be a threat to public health as they serve as parasite reservoirs. A preventive vaccine for dogs would be a valuable tool, not only protecting dogs but potentially also reducing the incidence of zoonotic visceral leishmaniasis (Dye, 1996; Tesh, 1995).

Vaccines against parasitic diseases are not very numerous. Among those that have been developed successfully are many that contain live –attentuated- parasites (e.g.; *Eimeria*, *Toxoplasma*, *Dictyocaulus*). The complex nature of most parasites and the complexity of host-parasite interactions may well be an important cause for the relative success of live vaccines compared to whole killed- or subunit vaccines. The vaccine strain parasites can migrate, invade host cells and sometimes even multiply in the host thus providing full stimulation of the immune response. In order to explore the possibility of using this approach for a canine leishmaniosis vaccine, specific knock-out strains of *Leishmania infantum* were produced.

Cysteine peptidases (CP's) are virulence factors of *Leishmania* parasites. The Cathepsin L-like enzymes CPA and CPB are thought to be involved in various processes that are of vital importance to protozoan parasites. This includes immune modulating activities such as the induction of IL-4 production, inhibition of IL-12 production by macrophages and degradation of MHC class II molecules in the parasitophorous vacuole (Mottram et al., 2004). Because of these functions, the *Leishmania* CP's are not only potential drug targets but also of interest for vaccine develoment.

Sequential knockout of two alleles of both Cysteine peptidase A and Cysteine peptidase B, produced single and double null mutants of *L. mexicana*. Subsequent tests in mice revealed that the knockout strains exhibited a virulence phenotype, The CPA knockout (Δ*cpa*) retaining most virulence followed by the CPB knockout (Δ*cpb*). The double knockout (Δ*cpa/cpb*) was found to be the most attenuated strain, not causing any lesion development in mice. Moreover, vaccination with the double knockout (Δ*cpa/cpb*) induced a shift of the immune response in mice toward a protective Th1-type and provided partial protection against subsequent challenge with wild type *L. mexicana* (Alexander et al., 1998).

In accordance with the work in *L. mexicana*, we set out to generate and test a *L. infantum* (Δ*cpa/cpb*) double null mutant strain. Despite numerous approaches, the double knockout has not been created today and it appears that the CPB gene is essential in *L. infantum* (unpublished data). However, two CPA knockouts and a number of CPB heterozygotes were created. The virulence of these clones was determined in hamsters and the protective potential of some of the attenuated strains was subsequently evaluated in a vaccination-challenge study in hamsters results of which are reported here.

Materials and methods

Experimental setup

Experiment A was designed to evaluate the virulence of ten CP-mutant clones of *L. infantum*. Ten groups of six hamsters each were infected with different knockout strains of *L. infantum* (group 1-10). One group of twelve hamsters was infected with the wild type clone (group 11) (table 1). One, three and six months after infection animals were sacrificed and samples were taken for detection and quantification of parasites and for histopathology.

Table 1: groups and treatments

Exp.	Group	Infection / vaccination	Dose, route	Challenge
A	1	CPB/Δcpb::hyg-tk	10^8, i.p.	-
A	2	CPB/Δcpb::hyg-tk	10^8, i.p.	-
A	3	CPB/Δcpb::hyg-tk	10^8, i.p.	-
A	4	CPB/Δcpb::hyg-tk	10^8, i.p.	-
A	5	CPB/Δcpb::hyg-tk	10^8, i.p.	-
A	6	CPB/Δcpb::hyg	10^8, i.p.	-
A	7	CPB/Δcpb	10^8, i.p.	-
A	8	CPA/Δcpa::ble	10^8, i.p.	-
A	9	Δcpa::ble/Δcpa::hyg	10^8, i.p.	-
A	10	Δcpa::sat/Δcpa::sat	10^8, i.p.	-
A	11	JPC M5 (wild type)	10^8, i.p.	-
B	12	CPB/Δcpb	10^8, i.d.	10^8, i.p.
B	13	Δcpa::sat/Δcpa::sat	10^8, i.d.	10^8, i.p.
B	14	Δcpa::ble/Δcpa::hyg	10^8, i.d.	10^8, i.p.
B	15	Δcpa::sat//CPB::bleo3'/Δcpb::hyg	10^8, i.d.	10^8, i.p.
B	16	JPC M5 (wild type)	10^8, i.d.	10^8, i.p.
B	17	-	10^8, i.d.	10^8, i.p.

Table showing the experimental setup; Exp.: experiment A or B. Group: group number. Infection / vaccination: L. infantum clone used to infect group for evaluation of virulence in experiment A or for vaccination in experiment B. Dose, route: number of parasites and route of injection used to infect (exp. A) or vaccinate (exp. B) animals. Challenge: number of parasites and route of infection used to challenge animals in experiment B. I.p.: intra peritoneal, i.d.: intra dermal.

In order to investigate the protective potential of a number of attenuated *L. infantum* CP mutants a second experiment was performed (exp. B). Six groups of thirteen hamsters each were used; four groups were vaccinated with different attenuated lines whereas two groups served as controls. Groups 12-15 received an intradermal vaccination with live attenuated parasites. Group 16 received a control "vaccination" with live virulent parasites whereas group 17 was not vaccinated (table 1). At one month post vaccination two animals from each group were sacrificed and samples were taken to check for the presence of –vaccine strain- parasites. The remaining animals were challenged with wild type *L. infantum* promastigotes. At one month post challenge five animals from each group were sacrificed, three more hamsters were sacrificed at both three and six months; samples were taken to determine parasite dissemination.

Animals

Six-week-old male *spf* golden hamsters (Harlan, the Netherlands) were used in the experiments. Animals were divided over the groups as they came to hand. Hamsters were not individually marked but different groups were kept in separate marked cages. Standard commercial feed ("RMH-B 2181", Arie Blok BV, the Netherlands) and water were provided *ad libitum*. Hamsters were allowed to acclimatize for one week before the start of the experiment.

Parasites

L. infantum CP mutants were generated by targeted gene disruption. Seven CPB heterozygotes clones were tested: one clone (*CPB/*Δ*cpb*) without residual antibiotic resistance, five clones with a *hyg-Tk* disruption cassette (*CPB/*Δ*cpb::hyg-Tk*), one clone with a *hyg* disruption cassette (*CPB/*Δ*cpb::Hyg*). Three CPA mutants were tested; two knockouts (Δ*cpa::sat/*Δ*cpa::sat* and Δ*cpa::ble/*Δ*cpa::hyg*) and one heterozygote (*CPA/*Δ*cpa::ble*). One clone was tested that was a Δ*cpa* knockout with one completely and one partially (3' end) disrupted CPB allele (Δ*cpa:: sat//CPB::bleo3'/*Δ*cpb::hyg*).

All clones were expanded *in vitro* in HO-MEM medium supplemented with 20% fetal calf serum (HO-MEM/20) at 27°C, where applicable antibiotics (bleomycin 10 µg/ml, hygromycin 50 µg/ml and/or nourseothricin 15-20 µg/ml) were added to maintain selection pressure. For the preparation of infection material cultures were maintained until the stationary phase was reached. Parasites were then harvested (1500 x g, 10 min, 4ºC) and washed once with isotonic phosphate buffered saline (PBS: 0.04 M, pH 7.3). Parasites were counted and resuspended in isotonic PBS at the appropriate concentration and 0.5 ml (intraperitoneal) or 0.1 ml (intradermal) of the suspension was used to infect hamsters within one hour of preparation.

Necropsy

In experiment A two hamsters per group were sacrificed at one, three and six months post infection. In experiment B two hamsters per group were sacrificed at one month post vaccination, five hamsters at one month post challenge and three hamsters both at three and six months post challenge. In all cases spleen, liver, bone marrow, lymph node and skin were removed aseptically for further analysis by (limiting dilution) culturing and/or histopathology.

Quantification of parasites

Parasites in the liver and spleen were quantified by culture microtitration according to the method of Buffet et al.(Buffet et al., 1995). Briefly, 100 µl of spleen cell suspension as prepared for the lymphocyte proliferation assay was pipetted in triplicate in the first column of a flat-bottom 96-well micro titre plate (Nunclon surface, Nunc, Denmark). This suspension was serially diluted (1:1) up to column twelve. A small piece of liver was weighed and crushed between two microscope slides. The suspension was diluted to a concentration of 100 mg/ml in HO-MEM/20. The cell suspension was serially diluted as described for the spleen cell suspension. After two and three weeks of incubation at 27°C the presence of *Leishmania* promastigotes was monitored by phase-contrast microscopy and for each row the well with the highest dilution that still contained parasites was recorded.

From the highest positive dilution the number of parasites in spleen and liver was calculated; triplicates were averaged and the parasitic load per 1g of liver tissue and per 10^6 spleen cells was determined.

Lymphocyte proliferation assay

Spleen tissue was homogenized through gauze into RPMI 1640 + 2% Foetal Calf Serum (FCS), washed once and resuspended in complete RPMI medium (RPMI 1640 (GIBCO BRL) supplemented with 25 mM HEPES, 25 mM $NaHCO_3$, 10% FCS, 2 mM L-glutamine and 50,000 U/ml Penicllin/streptomycin. Viable cell count was determined using trypan blue and spleen cells were diluted to a concentration of 1 x 10^6/ml. The cell suspension was pipetted into 96 well plates (Nunclon surface, Nunc, Denmark) at 50 µl per well and to each well 50 µl of medium was added containing either 10 µg/ml Con A (Sigma, USA) or 2 µg/ml Promastigote lysate or no additions (all were performed in triplicate). Cells were incubated for 72 hrs at 37ºC 5% CO_2, the last 18 hrs. in the presence of BrdU (BrdU Labelling and Detection Kit III, Roche-Diagnostics, Switzerland). After the incubation period a colorimetric assay was performed according to the manufacturer's instructions. Proliferative responses were expressed as stimulation indices (SI), which represent the ratio of mean absorbance after stimulation to the mean absorbance of non-stimulated controls.

LSA ELISA

Leishmania crude soluble antigen (LSA) was prepared as follows: end log-phase promastigotes were harvested by centrifugation (15', 4,000 x g) and washed twice in isotonic PBS. The parasites were disrupted by freeze-thawing and subsequently sheared through a 25-gauge needle. Cell debris was spun down (15', 15,000 x g, 4ºC) and the protein content of the supernatant was determined using a BCA assay kit (Pierce, USA). Microtitre plates (Greiner, Germany) were coated overnight at room temperature with 0.1 µg LSA in 0.1 ml coating buffer (50 mM $NaCO_3$-H_2CO_3, pH 9.6). Blocking was performed with 1% skimmed milk powder (Difco™Skim Milk; Beckton Dickinson, USA) in PBS (MPBS), subsequently plates were washed in an ELISA washer (Skan washer, Skatron Instruments, Norway) using 0.01 M PBS. Serum samples were diluted 1:40 in 1% MPBS and diluted serially in the plate (1:1). After incubation for 1 h at 37ºC, excess antibody was washed off the plates and goat anti hamster IgG-HRP (Kirkegaard & Perry Laboratories, U.S.) diluted 1:1,000 in 1% MPBS was added. After another 1 h at 37ºC plates were washed and subsequently incubated for 30' at room temperature with substrate solution (tetramethylbezidine + H_2O_2 in 0.1 M of sodium acetate, pH 5.5). The reaction was stopped by addition of 2 N H_2SO_4 and absorbance was read at 450 nm (Titertek® Multiscan Plus; ICN, U.S.). A negative and positive control serum was included on each plate. The 8-fold negative control (1:40) was used to calculate 2 log end-point dilutions. A cut off value was calculated as follows; the average titre of all negative samples (t=0) added by twice the standard deviation of these samples.

DTH testing

At three weeks post vaccination (day 21), hamsters in experiment B (groups 12 to 17) were tested for Delayed Type Hypersensitivity responses against leishmania antigen. Animals received intradermal injections with parasite antigen (1 x 10^6 promastigotes in 100 µl 0.5% phenol/PBS) and control (100 µl 0.5% phenol/PBS) on a shaven piece of skin on the back. After 48 and 72 hrs the appearance and size of reactions was recorded.

Histopathology

Spleen, liver, skin and lymph node samples were examined histologically and findings were summarized in a histological score; minimal and non-specific findings were given value 0, findings indicative –but not characteristic- of a Leishmania infection were given value 1 and findings characteristic of a Leishmania infection were given value 2. For each animal a score was calculated by adding up values for findings in spleen, liver, skin and lymph node samples (maximum score: 8), these scores were averaged per group and expressed as a percentage of the maximum score. In spleen and lymph nodes indicative findings were: follicular hyperplasia and/or parasite stages suspected but no clearly evident clusters of lymphocytes and macrophages. In the liver indicative findings were: multifocal mononuclear infiltrates and/or small numbers of foci suspicious of early granulomatous inflammation. In the skin: infiltration with lymphocytes and macrophages. In spleen, lymph nodes and skin findings characteristic of a *Leishmania* infection were: single infiltrating cells and clusters of lymphocytes and macrophages with occasional amastigotes in the cytoplasm. Characteristic findings in the liver were: granulomatous hepatitis with epitheloid cell granulomas.

Results

Parasite quantification

Experiment A was designed to investigate the relative virulence of several CP mutant strains of *L. infantum*. The number of parasites in spleen and liver was determined by limiting dilution culturing in samples taken at one, three and six months post infection (p.i.). Figure 1 shows the average parasite burdens in spleen and liver for the different strains. In the wild type JPC M5 infected group, parasites were found in the spleen at the first sampling one month p.i.. The parasite burden in this group increased thereafter, reaching an average of 10,000 parasites/10^6 spleen cells at six months p.i.. Parasites were found at one month p.i. in some of the other groups (*CPB/*Δ*cpb::hyg, CPB/*Δ*cpb, CPA/*Δ*cpa::ble,* Δ*cpa::ble/*Δ*cpa::hyg*), for groups 7 and 8 (*CPB/*Δ*cpb, CPA/*Δ*cpa::ble*) spleen parasite numbers were even significantly higher than all other groups. However, the spleen parasite burden in these groups decreased between one and six months p.i. in contrast to the wild type controls. Maximum parasite density in the CP knockout infected groups was also lower compared to the wild type infected group and for most groups (7 out of 10) spleens were parasite negative by six months p.i.. Average spleen parasite burdens were very low in the *CPB/*Δ*cpb::Hyg-Tk* groups and the Δ*cpa::sat/*Δ*cpa::sat* group throughout the experiment.

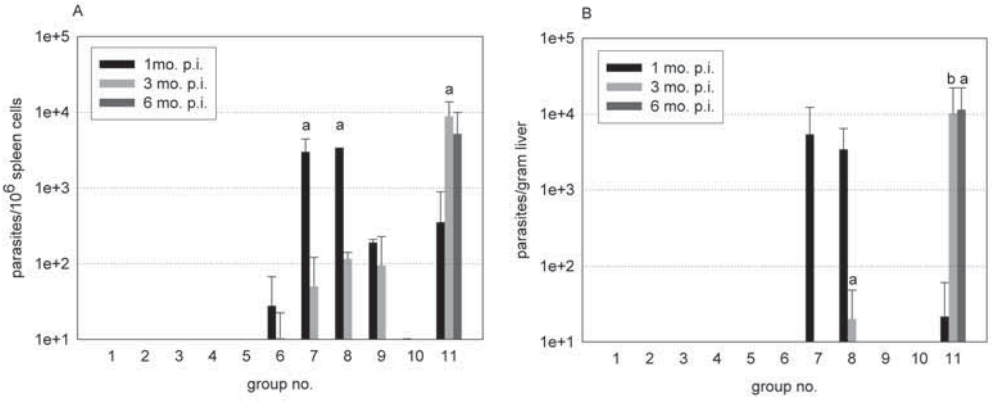

Figure 1: average parasite burdens in experiment A

Graph showing the average parasite burden as determined by limiting dilution culturing in spleen (panel A, parasites/10^6 spleen cells) and liver (panel B, parasites/ gram liver) of hamsters infected with different CP mutants. Samples were taken at one month (1 mo. p.i.), three months (3 mo. p.i.) and six months (6 mo. p.i.) post infection. Bars represent the standard deviation, letters indicate significant differences between the groups ($p<0.05$).

The average liver parasite burden of hamsters infected with wild type parasites increased steeply and reached values above the detection limit of the test by three months p.i.. With the exception of the heterozygote clones *CPB/Δcpb* (group 7) and *CPA/Δcpa::ble* (group 8), knockout parasites were not detected in the liver. For the two groups mentioned above initial parasite burdens at one month p.i. were high but the difference with the wild type infected group was not significant. Thereafter liver parasite burdens in group 7 and 8 decreased steeply leaving livers from both groups parasite negative at six months p.i.. The parasite burden in the wild type infected group was therefore significantly higher than all other groups at 3 and 6 months p.i..

In four of the CPB heterozygote groups (groups 1, 3, 4 and 5) bone marrow samples remained parasite negative throughout the study. In the remaining CP mutant infected groups (groups 2, 6, 7, 8, 9 and 10) one or two samples were positive at one month and/or three months p.i. but at six months p.i. the bone marrow was parasite negative for all of these groups. The wild type infected hamsters had positive bone marrow samples throughout the study; 3 out of 4 at one and three months p.i., 2 out of 4 at 6 months p.i.

In experiment B, parasite burdens in liver and spleen were determined after vaccination, as well as at one, three and six months after wild type challenge. Results are summarized in figure 2. At one month post vaccination, parasites were only detected in the spleen of the wild type JPCM5 vaccinated group ($p<0.05$). After the challenge infection, parasites appeared in the spleen in all groups from one month p.i.. Spleen parasite burdens did not differ significantly between the groups at any point after challenge. Parasites appeared in the liver at one month post challenge in all groups and the burden remained high thereafter, significant differences between the groups were not detected.

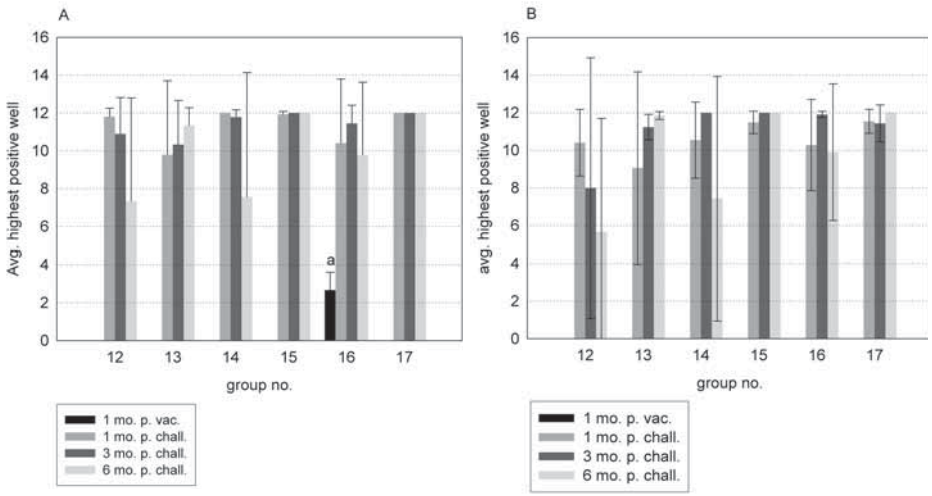

Figure 2: average parasite burdens in experiment B

Graph showing the average parasite burden as determined by limiting dilution culturing in the spleen (panel A) and liver (panel B) of hamsters vaccinated with different live –CP mutant- L. infantum clones and challenged with the wild type JPC strain. Results are depicted as the average highest positive well. Samples were taken at one month post vaccination (1 mo. p. vac.) and at one month (1 mo. p.chall.), three months (3 mo. p.chall.) and six months (6 mo. p.chall.) post challenge. Bars represent the standard deviation, letters indicate significant differences between the groups (p<0.05).

Bone marrow cultures in experiment B first became parasite positive at one month post challenge, in all groups. All bone marrow samples were positive at three months p.i. but at six months p.i. one sample was negative both in the Δ*cpa::ble/*Δ*cpa::hyg* and wild type vaccinated groups and two were negative in the *CPB/*Δ*cpb* group. The average parasite burdens in spleen, liver and bone marrow appeared to decrease somewhat between three and six months post infection in the *CPB/*Δ*cpb*, Δ*cpa::ble/*Δ*cpa::hyg* and wild type vaccinated groups whereas the parasite burden in the non-vaccinated group remained very high throughout, this difference was however not statistically significant.

Histology

Histological findings are summarized in figure 3 for experiment A and B. For each animal a total score was calculated by adding up values for findings in spleen, liver, skin and lymph node samples (maximum score: 8). For each group and for each time point the percentage of the maximum score was calculated.

At one month post infection abnormalities in experiment A were mainly found in groups 7, 8 and 9 (*CPB/*Δ*cpb, CPA/*Δ*cpa::ble* and Δ*cpa::ble/*Δ*cpa::hyg* respectively) these scores were significantly higher compared to the other groups. This had changed somewhat at three months p.i. when scores in the Wild type infected group were significantly higher than all other groups although scores in groups 7 and 9 were also still significantly higher compared to the other groups. At six month p.i. the scores in the Wild type group were still significantly higher compared to all other groups.

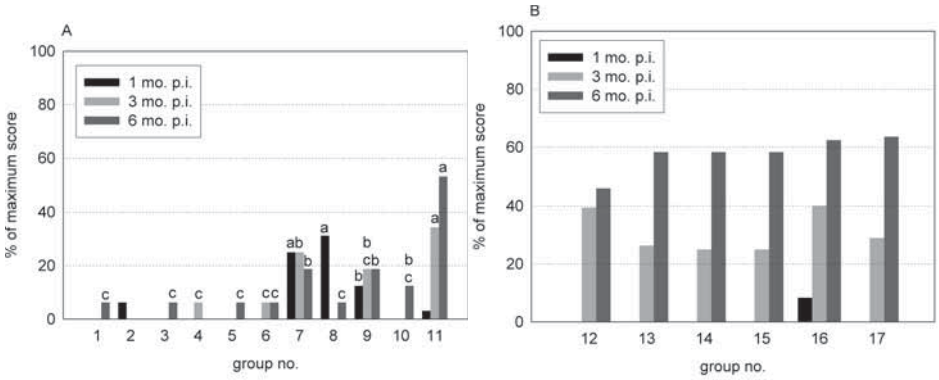

Figure 3: Average histopathology scores

Graph showing the average histopathology scores for groups in experiment A (panel A) and experiment B (panel B) plotted as percentage of the maximum score. Hamsters in experiment A were infected with different CP mutant clones and samples were taken at one month (1 mo. p.i.), three months (3 mo. p.i.) and six months (6 mo. p.i.) post infection. Hamsters in experiment B were vaccinated with different live –CP mutant- L. infantum clones and challenged with the wild type JPC strain. Samples were taken at one month post vaccination (1 mo. p. vac.) and at one month (1 mo. p.chall.), three months (3 mo. p.chall.) and six months (6 mo. p.chall.) post challenge. Letters indicate significant differences between the groups ($p<0.05$).

Except for the appearance of some abnormalities at 1 month post vaccination in the wild-type vaccinated group, no significant differences in histological scores were detected between the groups at any time in experiment B. The development of histological scores from 1 to 6 months post challenge was remarkably similar between vaccinated and control groups.

LSA ELISA

Figure 4 shows the average antibody titres against soluble *Leishmania* antigen for each group at different time points before and after challenge and/or vaccination. At one month p.i. in experiment A, seven groups (4, 5, 6, 7, 9, 10 and 11) had developed antibody titres above the cut off value, indicating parasite growth in these animals. By three months p.i., titres in two of the groups (4 and 9) that were positive at 1 month had dropped back to below cut off value whereas titres in three groups (2, 3 and 8) that were previously negative were now above cut off value. In summary, antibody titres in all groups increased between day 0 and 3 months p.i. but in some groups antibody levels already decreased somewhat between one and three months p.i.. The antibody levels in most groups dropped further after 3 months causing titres to decrease to below cut off values by 6 months p.i.. In the wild type infected group however, titres remained well above cut off and hardly decreased between 1 and 6 months p.i..

Titres in experiment B were overall very similar between the groups. A striking difference however between the non-vaccinated control group and the remainder of the groups is the absence of *Leishmania* specific antibody in the former at one month post vaccination.

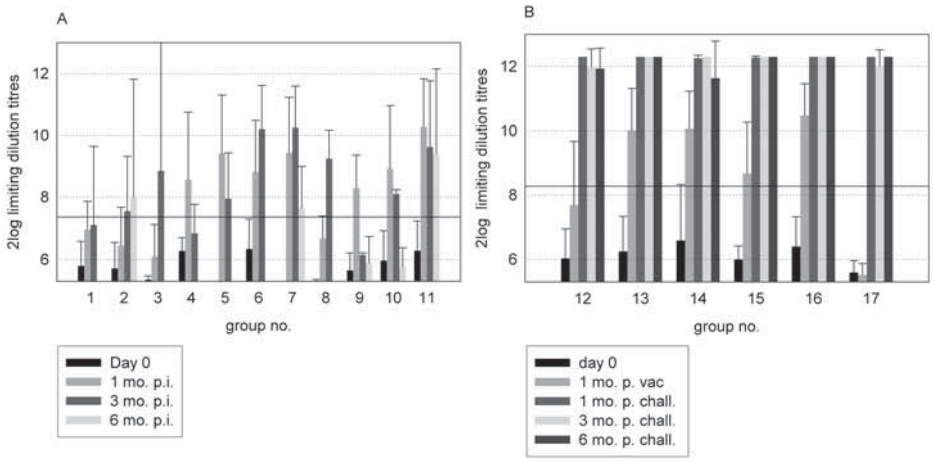

Figure 4: Leishmania specific antibody titres

Graph showing the average Leishmania-specific antibody titres in experiment A (panel A) and experiment B (panel B) plotted as 2 log limiting dilution titres. Hamsters in experiment A were infected with different CP mutant clones and samples were taken at the start of the experiment (Day 0) and at one month (1 mo. p.i.), three months (3 mo. p.i.) and six months (6 mo. p.i.) post infection. Hamsters in experiment B were vaccinated with different live –CP mutant- L. infantum clones and challenged with the wild type JPC strain. Samples were taken at the start of the experiment (Day 0), at one month post vaccination (1 mo. p. vac.) and at one month (1 mo. p.chall.), three months (3 mo. p.chall.) and six months (6 mo. p.chall.) post challenge. Bars represent the standard deviation, solid lines represent the cut off value above which titres are considered positive.

The average titre in group 12 (*CPB/Δcpb*) was still below cut off at one month post vaccination although the antibody level did appear to have increased compared to *t=0* and was higher compared to the non-vaccinated control group.

Lymphocyte proliferation assay

Results of the lymphocyte proliferation assay that was performed at one and three months post infection in experiment A are shown in figure 5 as stimulation indices. In all groups the proliferation of lymphocytes after stimulation with the mitogen (conA) was positive (>1) at one month post infection. At this time incubation with Leishmania antigen did not result in substantial cell proliferation. At three months p.i. incubation with mitogen resulted in minor cell proliferation only; in all groups average stimulation indices were just above 1. Incubation with Leishmania antigen again did not induce cell proliferation. Unfortunately, cells from non-infected controls were not included in the study and therefore failure of the test at three months p.i. can not be excluded

Delayed type hypersensitivity

No reactions were detected in any of the hamsters after dermal injection of "leishmanin" antigen.

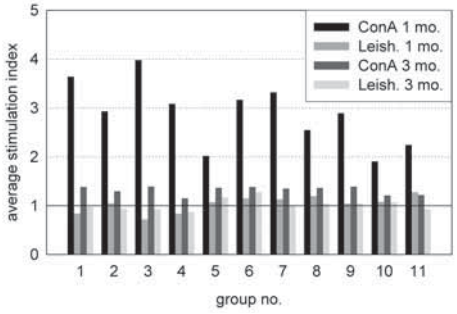

Figure 5: Lymphocyte proliferation
Graph showing the results of the lymphocyte proliferation assay performed in experiment A, plotted as average stimulation indices. Hamsters in experiment A were infected with different CP mutant clones and samples for LPA were taken at one and three months after infection (p.i.). ConA 1 mo., conA 3 mo.; cells taken at one month and three months post infection respectively, incubated with the mitogen concanavilin A. Leish. 1 mo. and Leish. 3 mo.; cells taken at one and three months p.i. respectively, incubated with Leishmania antigen. A stimulation index higher than 1(solid line) means that cell proliferation in the sample was higher than for non-stimulated control cells.

Discussion

In *Leishmania mexicana* mutants lacking cytsteine peptidase genes were found to have reduced virulence in mice (Alexander et al., 1998). In the study by Alexander *et al.* a Δ*cpa* mutant produced lesions in mice that were almost four times smaller compared to the wild type. The Δ*cpb* and Δ*cpa*/Δ*cpb* mutants tested were found to have a strong virulence phenotype with Δ*cpb* producing very small lesions (over 600 times smaller compared to wild type) and Δ*cpa*/Δ*cpb* producing no lesions at all. Moreover, vaccination with the Δ*cpa*/Δ*cpb* mutant resulted in partial protection against subsequent wild type *L. mexicana* challenge. Extensive efforts to create a double Cysteine peptidase knockout (Δ*cpa*/Δ*cpb*) in *Leishmania infantum* did not lead to the desired result. However, two Δ*cpa* mutants, a *CPA*/Δ*cpa* heterozygote, several *CPB*/Δ*cpb* heterzygote clones and a Δ*cpa*//*CPB 3'*/Δ*cpb* mutant were generated. The virulence phenotype of these clones was investigated and additionally the protective potential of some of them was evaluated.

The *L. infantum CPB*/Δ*cpb* heterzygote clones that have incorporated the *Hyg*-*Tk* cassette (exp. A; groups 1-5) were found to have strongly reduced virulence in hamsters. In these groups very few parasites were detected in the limiting dilution assay of spleen tissue and in cultures of bone marrow, whereas liver cultures remained negative. Strikingly, the remaining two *CPB*/Δ*cpb* heterzygote clones, *CPB*/Δ*cpb*::*hyg* and *CPB*:Δ*cpb* (group 6 and 7 respectively) appear to be somewhat less attenuated. Both groups have higher average parasite burdens in the spleen compared to the *CPB*/Δ*cpb*::*hyg*-*Tk* heterozygotes. Moreover, in group 7 (*CPB*/Δ*cpb)* the liver parasite burden, the number of positive bone marrow samples and the histopathology score were also higher. The strong virulence phenotype of the *CPB*/Δ*cpb* heterzygotes is surprising as we would expect the remaining intact CPB allele to be able to retain some virulence. Although the loss of one allele can in some cases cause changes in the phenotype, other possible explanations

include loss of virulence due to prolonged culturing in the course of the genetic manipulation or an unknown effect due to insertion of the recombination cassette. The attenuating effect of prolonged culturing was tested on the parent JPC M5 clone (Jimenez et al., 2006); in vitro culturing of promastigotes for up to 25 weeks induced no apparent effect on infectivity for hamsters. It therefore seems unlikely that prolonged culturing was the cause of reduced virulence in the CP mutants. Whether or not the presence of the recombination cassette was causing (part of) the phenotype effect remains uncertain. However, the small but apparent difference between the *CPB/*Δ*cpb::hyg-Tk* and the two other *CPB/*Δ*cpb* heterzygotes is intriguing and it even appears that the *CPB/*Δ*cpb* clone is less attenuated then the *CPB/*Δ*cpb::hyg* clone possibly indicating that the size of the –residual- insertion is of influence on the virulence phenotype.

Three different CPA mutants were tested for virulence, one heterozygote (*CPA/*Δ*cpa::ble*) and two knockout mutants (Δ*cpa::ble/*Δ*cpa::hyg* and Δ*cpa::sat/*Δ*cpa::sat*). Virulence in the *CPA/*Δ*cpa* heterozygote was affected unexpectedly strong. Although there is a remaining functional allele in this clone, the parasite burden in spleen and liver decreased rapidly after 1 month p.i. and became almost negative at six months p.i.. For both Δ*cpa* mutants the attenuation was expected to be in the same order as what was found for the *L.mexicana* Δ*cpa* knockouts (4 times smaller lesion). However, the Δ*cpa::ble/*Δ*cpa::hyg* clone was found to have strongly reduced virulence; the parasite burden in the spleen at 1 and 3 months p.i. was apparent but low whereas no parasites were detected in the liver at any time. For the Δ*cpa::sat/*Δ*cpa::sat* mutant the effects were even more dramatic; parasite burdens in spleen and liver were below the detection limit throughout the study although some samples (spleen, liver, bone marrow) did become parasite positive indicating that infection was at least possible. A possible reason for the strong attenuation of the Δ*cpa::sat/*Δ*cpa::sat* mutant may be that this clone was cultured *in vitro* for an extraordinary long period. It was recovered after one round of transfection and subsequent culturing under high selective pressure and had lost the second CPA allele by homologous recombination. This can however not explain the strong virulence phenotype of the Δ*cpa::ble/*Δ*cpa::hyg* mutant nor the *CPA/*Δ*cpa::ble* heterozygote since neither of these was cultured for a prolonged period. Again, either the loss of the CPA alleles or an unknown effect of the disruption cassette or perhaps a combination of both must have caused the observed attenuation.

Vaccination of hamsters with attenuated strains of *L. infantum* had no protective effect. The conclusion from experiment A, that the CP mutants are all less virulent than the wild type JPC M5, was confirmed in this experiment. Before the challenge with wild type JPC M5, no parasites were recovered from groups vaccinated with CP mutants. In experiment A however, *CPB/*Δ*cpb* and Δ*cpa::ble/*Δ*cpa::hyg* were readily detected at one month p.i.. The difference in parasite burden between experiments A and B is likely due to the route of infection; intra peritoneal injection was used in experiment A whereas intradermal injection was used for the vaccination in experiment B. We have previously shown that the intraperitoneal route infection is more efficient in hamsters although infection is possible via both routes (chapter 3).

We have previously reported that *Leishmania* specific antibody titres reflect the size of the parasite burden in hamsters (chapter 3). With this is mind, we can conclude from the increase in antibody titre in the groups infected with one of the *CPB/*Δ*cpb::hyg-Tk* heterozygotes or the Δ*cpa::sat/*Δ*cpa::sat* mutant that although parasite burdens were very low or non-detectable, some parasite development did take place. Average antibody titres in all CP mutant infected groups dropped to values near or below the cut off value by the sixth month p.i. indicating the disappearance of parasites as was also seen by culturing and histopathology. In the vaccinated groups (exp. B) titres increased between t=0 and 1 month post vaccination, again indicating development of –vaccine- parasites although the parasites could not be detected at the time. In contrast, antibodies were not detected in the non vaccinated controls until after the challenge infection. The challenge infection induced a strong increase in antibody titres in all groups, corresponding with the rapid increase in parasite burden and histopathology scores.

Lymphocyte proliferation was tested for all groups in experiment A, at one and three months p.i.. At one month p.i. cell proliferation was detected after addition of a mitogen, but specific antigen did not elicit detectable responses. This indicates a lack of cellular responses toward *Leishmania* antigen in all groups including those with low or non-detectable parasite burdens. At three months post infection reaction to neither mitogen nor antigen was detected in any of the groups. We have previously observed a practically identical effect after challenge with both high and low doses of the wild type JPC strain (chapter 3); at one month p.i. cells responded to mitogen but not to antigen, at three months p.i. neither mitogen nor antigen elicited a reaction, moreover, no differences were found between the groups infected with different challenge doses. The results of these two experiments seem to point toward a general down regulation of lymphoproliferative responses caused by the *Leishmania* infection as was previously observed in a study by (Nickol et al., 1985). These authors reported a non-specific anergy of lymphocytes resulting in a lack of proliferation after stimulation with a mitogen (conA).

In conclusion, all CP mutants tested exhibited a strong virulence phenotype when considering the parasite burden and histological damage. The effect of the CP mutants on cellular immune responses appeared to be similar to what is observed after infection with the wild type strain. The exact mechanism behind the attenuation of the mutant strains is uncertain and needs further investigation. Vaccination with attenuated CP mutants did not induce protection in hamsters.

Acknowledgements

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CHAPTER 6

Vaccination with a preparation based on recombinant Cysteine peptidases and canine IL-12 does not protect dogs from infection with *Leishmania infantum*.

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Abstract

Cysteine peptidases (CP's) have been implicated in various processes central to the pathogenicity of *Leishmania* parasites, and are thought to be key factors in the host-parasite interaction. In order to fully evaluate the potential of the CP's as vaccine candidates, studies in natural host species are required. In the study we report here, recombinant *L. infantum* CP's CPA and CPB were used to vaccinate dogs. In order to induce an appropriate response against the antigens, recombinant canine IL-12 was added as an adjuvant either by itself or in combination with Quil A. After vaccination, dogs were given an intravenous challenge with promastigotes of *L. infantum* JPC strain. In both vaccinated groups (CP's with IL-12 or CP's with IL-12 and Quil A) CP-specific antibodies were detected after vaccination, indicating that there was a reaction to the vaccine. However, all dogs were found parasite-positive and all developed some degree of clinical leishmaniosis. The observed lack of efficacy of the candidate vaccines could be due, completely or in part, to a number of factors associated with the vaccine antigen, the adjuvant or host-parasite interactions. When compared to results from other studies, it seems less likely that the molecular conformation of the rCP's or rIL-12 caused this lack of efficacy. More plausible explanations are the dose and timing of the IL-12 application and the potentially different effects IL-12 induces as an adjuvant in either the murine or the canine leishmaniosis model.

Keywords: Leishmania infantum, Cysteine Peptidases, vaccination, canine, IL-12

Introduction

Leishmania infantum (syn. *L. chagasi*) is the causative agent of both canine leishmaniasis and zoonotic visceral leishmaniasis in children and immune-compromised adults. In humans as well as in dogs disease symptoms are severe and can be fatal if left untreated. The parasite is transmitted by the bite of an infected sandfly and dogs serve as the most important reservoir of the parasite. Canine leishmaniasis (CanL) occurs in rural as well as (peri-)urban areas both in the New World and the Old World including Mediterranean basin. In recent years, several groups have reported increased incidence of CanL and the occurrence of new endemic areas in the Mediterranean area; environmental as well as demographic changes have been implicated as causes for the increased geographic distribution (Baldi et al., 2004; Capelli et al., 2004).

CanL is a serious veterinary and public health problem, with seroprevalences reaching over 40% in some regions (Baldi et al, 2004; Cardoso et al., 1998; Fisa et al., 1999). Although treatment of dogs with leishmaniasis will often achieve clinical remission, relapses are reported to occur frequently and, moreover, animals remain infectious to the vector (Baneth and Shaw, 2002; Ginel et al., 1998). A vaccine for dogs would be an invaluable tool in the prevention of canine leishmaniasis. Moreover, protection of dogs could potentially decrease the incidence of zoonotic visceral leishmaniasis in humans (Dye, 1996; Tesh, 1995).

Cysteine peptidases (CP's) are thought to be involved in various processes that are of vital importance to protozoan parasites, including facilitating infections in mammalian hosts (McKerrow, 1989). The cathepsin L-like CP's CPA and CPB of *Leishmania* are virulence factors that are involved in immune modulating activities such as the induction of IL-4 production, inhibition of IL-12 production by macrophages and degradation of MHC class II molecules in the parasitophorous vacuole (Mottram et al., 2004). These *Leishmania* CP's are therefore considered to be potential drug targets as well as vaccine candidates (McKerrow, 1989). Experiments with CP knockout mutants of *L. mexicana* have shown that deletion of the CPA and CPB genes (Δ*cpa/cpb*) not only significantly reduces virulence, but also induces a shift of the immune response in mice toward a protective Th1-type (Alexander et al., 1998). Recombinant CPB protein (rCPB) of *L. mexicana* was found to induce a Th2-type of response in mice, which led to exacerbation of the disease. However, in combination with an appropriate adjuvant, recombinant interleukin 12 (rIL-12), vaccination of mice with rCPB induced a Th1 response and partial protection against a challenge infection with wild type *L. mexicana* (Aebischer et al., 2000; Pollock et al., 2003). Moreover, protection against a challenge infection with *L. major* was found after vaccination with purified native CP (Rafati et al., 2000) and after vaccination with plasmids containing *CPA* and *CPB* genes (Rafati et al., 2001).

In order to further evaluate the potential of the CP's as vaccine candidates, studies in natural host species are required. In the study we report here, recombinant *L. infantum* CPA and CPB were used to vaccinate dogs. In attempts to induce an appropriate response against the antigens, recombinant canine IL-12 was added as an adjuvant either by itself or in combination with the saponin Quil A as this could potentially enhance the effect of rIL-12 (Hancock et al., 2000; Silla et al., 1999).

Materials and Methods

Cultivation of L. infantum

L. infantum JPC (MCAN/ES/98/LLM-724) strain was first isolated from a dog in Madrid suffering from leishmaniasis (parasites were kindly provided by Dr. M.I. Jimenez). Parasites were passaged through hamsters in order to retain full virulence. Promastigotes for the challenge infections were cultured from amastigotes in hamster spleens at 27ºC in HO-MEM medium (Berens et al., 1976) supplemented with 20% heat-inactivated Foetal Calf Serum (hiFCS). Expanded cultures were allowed to grow to the stationary phase, promastigotes were washed twice in Phosphate Buffered Saline (PBS; pH 7.3) and resuspended in PBS to a density of 2.5 $x10^7$ promastigotes per ml.

Preparation of cysteine peptidase antigen

CPA and *CPB* open reading frames were cloned from *L. infantum* (JPCM5, MON-1) into the pQE-30 expression vector (Qiagen, U.K.). Recombinant proteins were expressed in *Escherichia coli* M15(pREP4) cells, predicted protein sequences are shown in table 1.

After induction with 1mM IPTG for 4 hours, cells were harvested by centrifugation (6000 g, 20 min, 4 ºC), resuspended in sucrose buffer (50 mM TrisHCl, 5 mM EDTA, 5% sucrose; pH 8.0) and disrupted by two cycles of freeze-thawing and subsequent sonication (6 cycles of 30 seconds at maximum output and 30 seconds rest). Inclusion bodies, containing the recombinant protein, were harvested from the cell slurry by centrifugation (8000 g, 20 min., 4 ºC) and dissolved in an equal volume of 8 M urea buffer (10 mM TrisHCl, 100 mM NaCl, 50 mM NaH_2PO_4, 8 M urea; pH 8.0). Nickel affinity purification was performed using Talon beads according to the manufacturer's instructions (BD biosciences, US). Recombinant protein was eluted from the Talon beads in elution buffer (0.1 M EDTA, 1% SDS; pH 8.0). A BCA protein assay (Pierce, USA) was performed to establish protein concentration and the purity of the sample was assessed on SDS-PAGE and western-blot. Both recombinant proteins were recognised by α-HIS antibodies (RGS·HIS™, Qiagen). rCPB was further recognised by polyclonal rabbit serum raised against *L. infantum* soluble antigens and by serum from *L. infantum* infected dogs. rCPA was not recognised clearly by the above mentioned sera but was detected by serum raised against *L. mexicana* CPA.

Recombinant canine IL-12

Recombinant single-chain canine Interleukin-12 (rIL-12; sequence is shown in table 1) was produced in insect cells using a baculovirus system essentially as described before (McMonagle et al., 2001). The recombinant protein was purified from the culture supernatant by Ni-affinity purification according to the manufacturers' protocol (Talon beads, BD biosciences, US) and subsequent dialysis against PBS. The purity was checked using SDS-PAGE and by Western blotting, protein content of the sample was determined according to the method of Lowry (Lowry et al., 1951). Bioactivity of the purified rIL-12 was determined in a bioassay using equine lymphoblasts as previously described (McMonagle et al, 2001). The test was considered valid if the proliferation of cells after addition of the standard was well above the proliferation of cells in medium alone.

Table 1: sequences of recombinant proteins

Product	Aminoacid sequence
rCPA	MRGSHHHHHHGSVCYGSALIAQTPLGVDDFIASAHYGRFKKRHGKPFGEDAEEGRRFNAFKQNMQTAYFLNAHNPHAHYDVSGKFADLTPQEFAKLYLNPNYYARHGKDYKEHVHVDDSVRSGVMSVDWREKGVVTPVKNQGMCGSCWAFATTGNIEGQWALKNHSLVSLSEQVLVSCDNIDDGCNGGLMQQAMQWIINDHNGTVPTEDSYPYTSAGGTRPPCRDNGTVGAKIKGYMSLPHDEEEIAAYVGKNGPVAVAVDATTWQLYFGGVVTLCFGLSLNHGVLVVGFNRQAKPPYWIVKNSWGSSWGEKGYIRLAMGSNQCLLKNYVVTATIDDSNTSHVPTTTA
rCPB	MRGSHHHHHHGSACAPARAIYVGTPAAALFEEFKRTYRRAYGTLAEEQQRLANFERNLELMREHQARNPHARFGITKFFDLSEAEFAARYLNGAAYFAAAKQHAGQHYRKARADLSAVPDAVDWREKGAVTPVKNQGACGSCWAFSAVGNIESQWARAGHGLVSLSEQQLVSCDDKDNGCNGGLMLQAFEWLLRHMYGIVFTEKSYPYTSGNGDVAECLNSSKLVPGAQIDGYVMIPSNETVMAAWLAENGPIAIAVDASSFMSYQSGVLTSCAGDALNHGVLLVGYNKTGGVPYWVIKNSWGEDWGEKGYVRVVMGLNACLLSEYPVSAHVPRSLTPGPGTESEERAPKRVTVEQMMCTDMYCREGCKKSLLTANVCYKNGGGGSSMTKCGPQKVLMCSYSNPHCFGPGLCLETPDGKCAPYFLGSIMNTCQYT
rIL-12	MDPQQLVISWFSLVLLASPLMAIWEEKDVYVVELDWHPDAPGEMVVLTCHTPEEDDITWTSAQSSEVLGSGKTLIQVKEFGDAGQYTCHKGGKVLSRSLLLIHKKEDGIWSTDILKEQKESKNIFLKCEAKNYSGRFTCWWLTAISTDLKFSVKSSRGFSDPQGVTCGAVTLAERVRVDNRDYKKYTVECQEGSACPSAEESLPIEVVVDAIHKLKYENYTSFFIRDIIKFDPPTNLQLKPLKNSRHVEVSWEYPDTWSTPHSYFSLTFCQAQGKNNREKKDRLCVDKTSAKVVCHKDAKIRVQARDRYYSSSWSDWASSCSGGGGSGGGGSGAGGGSRSLPTASPSPGIFQCLNHSQNLLRAVSNTLKARQTLELYSCTSEEIDHEDITKDKTSTVEACLPLELTMNESCLASREILITNGSCLASGKASFMTVLCLSSIYEDLKMYQMEFKAMNAKLLMDPKRQFLDQNMLTAIDELLQALNFNSVTVPQKSSLEEPDFYKTKIKLCILLHAFIRAVTIDRMMSYLNASKGELEGKPIPNPLLGLDSTRTGHHHHHH

Predicted aminoacid sequences of the recombinant proteins used in the vaccine: Cysteine Peptidases A and B and a single-chain construct of canine IL-12.

The reciprocal of the dilution of the sample that resulted in half maximal proliferation of cells corresponded to the number of units per added volume of sample. This was used to calculate the number of units per ml. The batch of rIL-12 used in this experiment had an activity of 45,900 units per ml, corresponding to 353 Units/µg protein.

Animals and vaccinations

Fifteen healthy male beagle dogs were used in the study (Harlan, France) these were conventional dogs of approximately 6 months old at the start of the experiment. The animals were housed in pairs in conventional kennels and were fed a standard commercial diet (Canex; Hope Farms, the Netherlands) once daily according to their individual needs, water was provided *ad libitum*.

The animals were allocated to three groups as they came to hand. The first group was vaccinated with 50 µg of each of rCPA and rCPB combined with 10 µg of rIL12 in 2 ml isotonic PBS. Vaccine 2 (group 2) consisted of 50 µg rCPA and rCPB, 1 µg rIL12 and 50 µg Quil A in 2 ml of isotonic PBS. Group 3 served as non- vaccinated control. The vaccines were administered subcutaneously, twice at a four week interval. Three weeks after the booster vaccination, all dogs were challenged intravenously with 5 x 10^7 stationary phase promastigotes of *L. infantum* JPC strain . During the 13-month follow up, samples of blood, bone marrow and lymph nodes were taken at regular intervals.

Dogs were necropsied at the end of the study. Samples of spleen, liver, bone marrow and lymph node were taken aseptically for parasite culturing. Samples of spleen, liver, skin and lymph node were taken for histopathology.

Serum antibody detection

To determine *Leishmania*-specific IgG antibody titres, an ELISA using soluble *Leishmania* antigen (LSA) was performed as described earlier (Poot et al., 2005). Antibodies against rCPB and rCPA were determined by ELISA essentially as described for LSA. Briefly, plates were coated with either rCPA (1 µg/ml) or rCPB (1 µg/ml) diluted in carbonate buffer. After incubation with blocking buffer (PBS/ 1% w/v skimmed milk powder), plates were washed and subsequently incubated with two fold dilutions of the test samples. Excess antibody was washed off the plates and bound antibody was detected using goat anti-dog IgG conjugated to horseradish peroxidase (KPL, maryland, U.S.). Finally, plates were washed and incubated with TMB substrate solution, reactions were stopped by adding 2 N sulphuric acid and optical densities were read at 450 nm. Negative and positive control sera were included on each plate and used to calculate the end-point dilution (Multicalc© algorithms).

Detection of cytokine transcription

Quantitative RT-PCR was used to detect transcripts of interferon gamma (IFNγ), interleukin 4 (IL-4) and, as a control, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G_3PDH) in peripheral blood mononuclear cells (PBMCs). This was performed as described earlier (Poot et al, 2005). Briefly, RNA was isolated from heparinized blood using the RNeasy mini kit (Qiagen, USA). RNA was transcribed to cDNA using a cDNA synthesis kit (Platinum® Quantitative RT-PCR Thermoscript™ One-step system, Life Technologies, USA). Separate Real-Time PCR reactions were subsequently performed for IFNγ, IL-4 and G_3PDH, molecular beacons were used to detect the PCR amplicons. The PCR reaction was performed in an iCycler

(Bio-Rad, USA) that measured fluorescence of the samples and of a standard range of template cDNA Real-Time. The iCycler software calculates the template starting quantity for each sample by interpolation of the standard curve. The relative amount of IFNγ or IL-4 transcript was subsequently calculated for each sample (template starting quantity IFNγ or IL-4 / template starting quantity G_3PDH).

Delayed type hypersensitivity test

Dogs were tested for delayed type hypersensitivity against two antigen preparations: *Leishmania* stationary phase promastigotes in 0.5% phenol/PBS, 3 x 10^6 parasites per dose (0.1 ml) and CP's, 0.05 µg of each of rCPA and rCPB per dose (0.1 ml). Control preparations were 0.5% phenol/PBS and control *E. coli* lysate (no recombiant protein expression, "empty") processed in the same way as the recombinant proteins (Ni-affinity purification). All four were administered intradermally, reactions were checked at 48 and 72 hours after injection.

Clinical examination and blood chemistry

Clinical examination included the assessment of the size of prescapular, axilar and popliteal lymph nodes (0: normal, 1: slight enlargement 1 or 2 nodes, 2: several nodes severely enlarged), general body condition (0: normal, 1: decreased, 2: emaciated) and skin/coat condition (0: normal, 1: slight scaling and/or alopecia, 2: severe alopecia and/or lesions). Obvious clinical abnormalities were recorded if present. Serum samples for clinical chemistry were taken at two monthly intervals, determination of serum protein content and electrophoresis was performed according to standard procedures at the Dept. of Companion Animal Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

Parasite detection and histopathology

Starting 2 months after the challenge infection, biopsies of bone marrow and lymph nodes were taken several times for parasitological diagnosis. Bone marrow biopsies were taken from the pelvis, lymph node biopsies were taken from the popliteal node. The materials obtained were cultured at 27˚C in HO-MEM medium supplemented with 20% (v/v) hiFCS and 50 µg/ml gentamycin (Berens et al, 1976) as well as in the biphasic Evans' modified Tobie's medium supplemented with 50 µg/ml gentamycin. Cultures were incubated until positive or for a maximum period of six weeks.

Histopathological findings were analysed as described before (Poot et al, 2005). Briefly, samples of spleen, liver, skin and lymph node were evaluated and subsequently given a histological score ranging from 'no abnormalities' (0) via 'findings indicative of a *Leishmania* infection' (1) to 'findings characteristic of a *Leishmania* infection' (2). The percentage of the maximum score was calculated for each dog (total of scores for all tissues investigated / (number of tissues x maximum score) x 100).

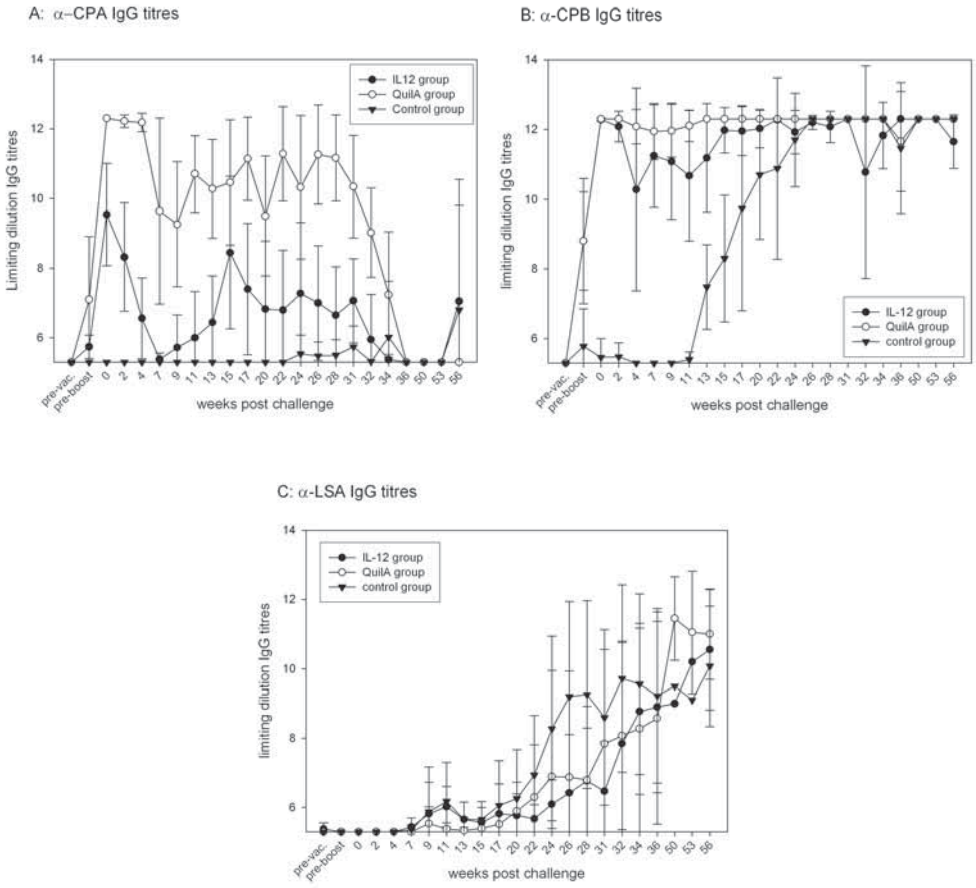

Figure 1: Antibody titres against recombinant and crude Leishmania antigens.
Panels A and B show, respectively, the average CPA and CPB-specific IgG titres that were established using a recombinant protein ELISA. Bars represent the standard deviation. Positive titres against both antigens developed after the first vaccination for both vaccinated groups (IL-12 group and QuilA group) and increased to high levels after the booster injection. In the control group titres against CPB were found from 11 weeks post challenge and increased to high levels wereas antibodies against CPA were only detected from 22 weeks post challenge at very low levels. Panel C shows IgG titres against crude Leishmania antigens. Positive antibody titres were found from 7 weeks post challenge in all groups and continued to increase until the end of the experiment.

Results

Serology

After vaccination, IgG titres against rCPA and rCPB were detectable in both vaccinated groups (figure 1). In the "QuilA group", vaccinated with rCP's plus rIL-12 plus QuilA, titres against rCPB reached the detection limit at the third sampling (4 weeks post-vaccination) and remained at high levels until the end of the study. Anti-rCPB titres in the "IL12 group", vaccinated with CP's plus rIL-12, were on average high although slightly lower than in the QuilA group. In the control group, titres against rCPB appeared at around 3 months post challenge and steadily increased thereafter reaching high levels at 6 months post challenge. CPB antibodies in the control group were already present at the time of DTH testing and must therefore have been induced by the parasites. After vaccination, antibody titres against rCPA were higher in the QuilA group compared to the IL-12 group. Whereas titres in the QuilA group remained medium-high up to 8 months post-vaccination, titres in the IL12 group were only low-positive in this period. In both groups, antibody titres decreased to non-detectable at 1 year post vaccination. In the control group very little antibody against rCPA was detected. The small increase in average anti-rCPA titres between 5 and 7 months post challenge and at the end of the study is almost exclusively due to one animal. Antibodies against Soluble *Leishmania* antigen (SLA) appeared in all three groups at 2 months post challenge. Differences between the groups were small and, on average, titres gradually increased and reached high levels at one year post challenge for all groups.

Cytokine transcription

The first results for IFNγ transcription were obtained at 11 weeks post challenge. Transcription in controls and vaccinates was generally similar (figure 2). IFNγ transcription was found in all animals and the level gradually increased in all groups, reaching the highest levels at week 28 post infection. Average transcription of IL-4 was very variable. Transcripts of IL-4 were not detected in all dogs, but IL-4 positive samples were found in all three groups.

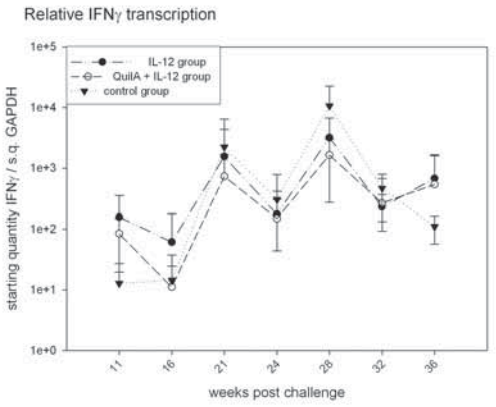

Figure 2: Transcription of IFNγ in peripheral blood mononuclear cells.
This figure shows the average relative IFNγ transcription levels as measured by quantitative RT-PCR in the PBMC's of rCP + rIL-12 vaccinated (IL-12 group), rCP + rIL-12 + QuilA vaccinated (QuilA + IL-12 group) and non-vaccinated control dogs (control group). The quantity of the IFNγ transcript was normalized using the amount of G_3PDH transcript in the same sample. Bars represent the standard deviation.

The first transcripts were detected in week 28 post challenge, 1 dog in the Quil A group, 1 in the control group. In week 32 one dog in the Quil A group was positive wereas in week 36 one dog in the IL-12 group and two dogs in both the Quil A and the control group were positive for IL-4 transcription. IL-4 transcription was found to be comparable in all three groups.

Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) against *Leishmania* promastigotes and recombinant CP's was tested after challenge to avoid any influence on vaccine-induced immunity. The test was performed at 13 weeks post-challenge, just prior to the onset of clinical signs in experimentally infected dogs and just after the start of parasite-induced serological responses. No reactivity was detected against either antigen in any of the dogs.

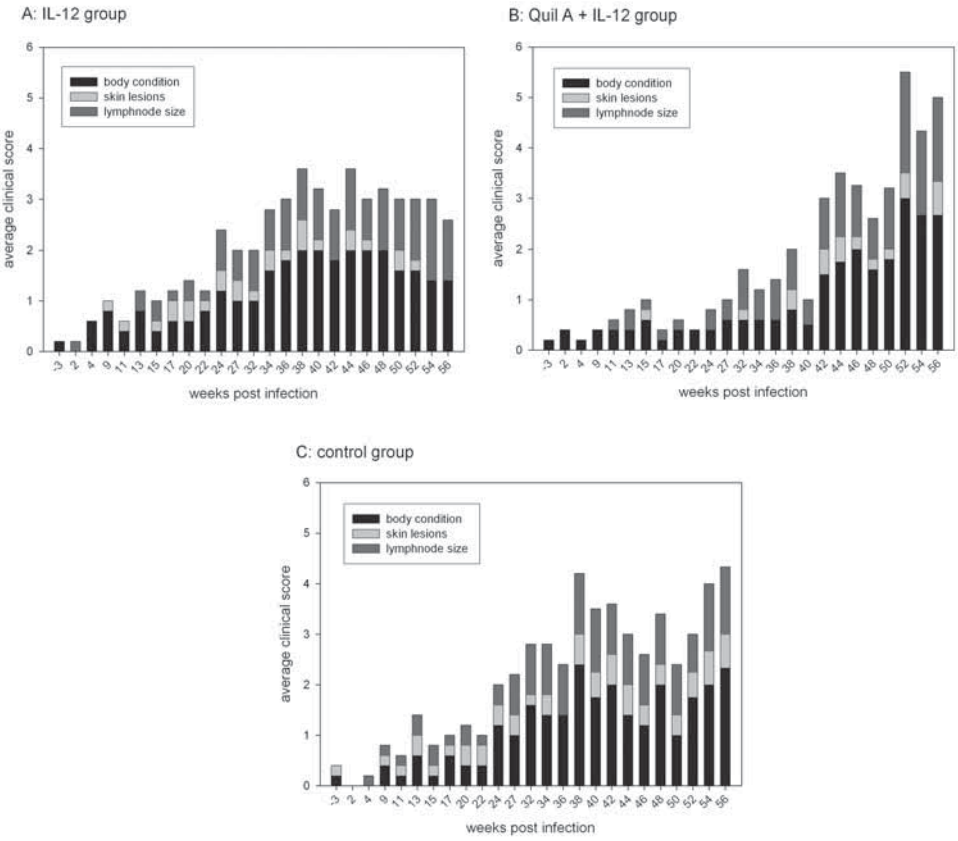

Figure 3: Average clinical score per group.

Average clinical scores of rCP + rIL-12 vaccinated (panel A), rCP + rIL-12 + Quil A vaccinated (panel B) and non-vaccinated control dogs (panel C) are shown. The relative contribution of body condition, skin lesions and lymphnode size to the total average clinical score at each timepoint are indicated by shading of the bars (Clinical score = body condition (black) + skin (light grey) + lymph node (dark grey)). These three observations are scored between 0 (normal) and 2 (severe abnormalities), the maximum clinical score therefore is 6.

Clinical examination

Before the time of challenge, clinical scores were normal. All dogs developed clinical signs of leishmaniasis during the course of the study although the severity and duration of these clinical abnormalities varied. Averages for body condition, lymph node size and skin lesions are shown in figure 3.

Blood chemistry

Between 7 and 30 weeks post-challenge, the albumin-globulin ratio of all but one dog (control group) decreased, with the decrease ranging from -0.2 to -3.5 units. By 30 weeks post-challenge, the ratio reached abnormally low levels (below 2) in 2 dogs in the IL12 group, 1 in the QuilA group and 4 in the control group.

Parasite detection and histopathology

Parasites were detected by culturing of bone marrow and lymph node samples. In the Quil A and IL12 groups, the number of parasite-positive animals was similar to that in the control group throughout the duration of the study. All dogs were found parasite positive at some point during the study.

Average histopathological scores for the different groups were calculated from individual data. The results were 81% for the IL12 group, 79% for the QuilA group and 69% for the control group.

Discussion

Experimental infection of dogs with *L. infantum* almost invariably results in parasite dissemination, development of an immune response dominated by antibody production and ultimately disease symptoms that can result in death of the animal (Martinez-Moreno et al., 1995; Rhalem et al., 1999). Although this disease profile is also observed in naturally infected dogs, several authors have reported the existence of resistant dogs in the field. Characteristically these dogs do not harbour a (detectable) parasite burden and have developed positive leishmania skin tests and/or lymphocyte proliferation (Cabral et al., 1998; Solano-Gallego et al., 2000). These findings appear to open possibilities with respect to the development of a protective vaccine for dogs.

Promising results from vaccine studies against *L. infantum* in dogs have been published recently: autoclaved promastigotes plus live Bacille Calmette Guerin (BCG) (Mohebali et al., 2004), Fucose-Mannose Ligand plus saponin (Borja-Cabrera et al., 2002; da Silva et al., 2000), culture supernatant plus muramyl dipeptide (MDP) (Lemesre et al., 2005) and recombinant chimeric protein Q plus live BCG (Molano et al., 2003) were all successfully used to protect dogs from parasite challenge. There are, however, some disadvantages associated with potential commercial use of these candidates; the use of live BCG in dogs can bear a safety issue (Mohebali et al, 2004) and could render dogs a potential source of infection to humans. The use of native, (purified) parasite-derived proteins potentially poses problems for large scale production of the vaccine.

A vaccine consisting of recombinant protein or DNA with an appropriate safe adjuvant would be preferred over the above mentioned candidates. However, results that have been achieved with vaccines containing recombinant proteins such as H1 and/or HASPB1 adjuvanted with Montanide (Moreno et al., 2005), TSA-LmSTI1-LeIF trifusion protein in combination with either monophosphoryl lipid A (MPL®) or AdjuPrime™ (Gradoni et al., 2005),or LACK DNA/Vaccinia (Ramiro et al., 2003) are less encouraging compared to those mentioned above. Although results in other experimental models (mice, primates) were positive for the recombinant vaccines (Campos-Neto et al., 2001; Gonzalo et al., 2001; Gonzalo et al., 2002; Skeiky et al., 2002; Stager et al., 2000), the induced level of protection in dogs (50%, 0% or 60%, respectively) was not sufficiently high and parasites were readily detectable in vaccinates after challenge.

Cysteine peptidases have been successfully used as vaccines in mouse models of infection (Pollock et al, 2003; Rafati et al, 2001). A study in dogs has also been reported wherein 10 animals were injected with a vaccine containing CPA and CPB-DNA: a priming and a booster injection with CP DNA plus CpG's were followed by a final booster containing recombinant CPA and CPB proteins, CpG and Montanide™. This vaccination schedule resulted in protection of 10 out of 10 dogs (Rafati et al., 2005). In the study we report here, vaccination of dogs with recombinant CPA and CPB in combination with recombinant canine IL-12 induced a detectable immune response, but convincing evidence for protection was not found. This contrasts with the results in the mouse model of infection using *L. mexicana* CP's and rIL-12 (Pollock et al, 2003) and also with the results in dogs using CP DNA in combination with proteins(Rafati et al, 2005).

The observed lack of efficacy of CP's in this study could be due, completely or in part, to a number of factors associated with the vaccine antigen, the adjuvant or host-parasite interactions. The *L .infantum* recombinant CPB used in this study contains a C-terminal extension. Over time, various functions for the C-terminal domain have been suggested and subsequently rejected, but there is some evidence for a role in immune evasion (Gonzalez et al., 1996; Martinez et al., 1993; Nakhaee et al., 2004). The presence of the C-terminal domain in our recombinant protein may have induced a disease-enhancing response or it may have reduced protective responses by divergence of the immune system. However, both full-length and truncated forms of *L. mexicana* CPB have been shown to confer protection in mice, indicating that in this model the C-terminal extension has little or no influence on protective responses (Aebischer et al, 2000; Pollock et al, 2003). There are examples of *Leishmania* proteins that confer protection in their native form but fail to do so in recombinant form (Handman et al., 1990; Sjolander et al., 1998). For CP's it was shown in the mouse model that recombinant enzymatically active enzyme could confer protection. Therefore, although it is possible that the conformation of the proteins or their recombinant nature is the cause of the lack of protection in this study, it appears unlikely.

The choice of adjuvant is another important factor influencing efficacy of any vaccine, and leishmaniosis vaccines in particular. In mice the use of rIL-12 has been very successful in combination with several *Leishmania* antigens and specifically with CP's (Aebischer et al, 2000; Afonso et al., 1994; Gonzalo et al, 2001; Pollock et al, 2003), and the important role of IL-12 in T-helper cell differentiation and subsequent protection against *Leishmania* infection has been the topic of many studies (Heinzel, 1994; Reiner and Locksley, 1995).

In contrast, little is known about the use of rIL-12 in dogs. The dose of rIL-12 used in this study was therefore mainly based upon extrapolation from the dosage for mice. The combination of a low dose of rIL-12 plus Quil-A was chosen as adjuvant because it was found that the addition of a saponin (QS-21) can potentiate the effects of rIL-12 (Hancock et al, 2000). Moreover, saponins are proven successful adjuvants for leishmania vaccines (Borja-Cabrera et al, 2002). Although it has been shown that timing of application can be essential when using IL-12 as an adjuvant (Noormohammadi et al., 2001), antigen and adjuvant were co-administered in this study; this is the most practical way of application. The desired effect - induction of a balanced immune response - apparently was not achieved; in both the IL-12 and Quil A/IL-12 groups, a significant level of specific antibodies was detected after vaccination, which further increased after challenge. This effect appeared to be slightly stronger in the Quil A/IL-12 group compared to the Il-12 group as the α-CPA IgG titres in the former were somewhat higher. The increased antibody production may have been caused by the addition of Quil A since this adjuvant is known to induce both cell-mediated responses and antibodies. Differences in α-CPB IgG titres were much smaller; perhaps the immunogenicity of this molecule made the effect of the adjuvant less pronounced. Although there was no direct measurement for cellular responses after vaccination, we found no indication for the presence of such responses after challenge (evidenced by the negative DTH response). The increase of IFNγ transcripts in PBLC's that was detected during the course of the study occurred simultaneously with the strong rise in antibody titres. Rises in IFNγ transcripts have previously been reported in infected susceptible dogs (Santos-Gomes et al., 2002) and it is not shown yet that IFNγ levels are a good indicator for a protective –balanced - immune response. Therefore, although rIL-12 is expected to induce a Th1- driven immune response, this effect was likely not achieved in our experiment.

The challenge used in this study involved intravenous injection of a high dose of parasites; this is nothing like the natural infection and potentially this strong challenge could have overpowered a protective response present. However, recent publications show that in dogs challenged with 10^8 promastigotes intravenously some partial protection can be obtained (Lemesre et al, 2005; Molano et al., 2003). This makes it highly unlikely that the challenge used in our study was too harsh.

Although our candidate vaccine was similar to preparations successfully used to protect mice from challenge with *L. mexicana*, protection against challenge with *L. infantum* was not achieved. From several studies mentioned above, it is shown that CP's can be applied as protective vaccine candidates, not only in rodents but also in dogs. It appears that the addition of immuno-regulatory molecules plays an essential role in the outcome of the effects induced.

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CHAPTER 7

Vaccination of hamsters against Leishmania infantum with recombinant Cysteine peptidases, culture supernatant or autoclaved promastigotes in combination with various adjuvants.

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Summary

A preventive vaccine for canine leishmaniasis would be a valuable tool in veterinary medicine and, since dogs serve as the main reservoir for this parasite, could likely also be and aid in the reduction of the incidence of zoonotic visceral leishmaniasis. With respect to the practical aspects of such a commercial vaccine a subunit approach would be most suited. In this study we tested several known antigens in combination with different adjuvants in the hamster model in order to evaluate their potential for a canine leishmaniasis vaccine. Cysteine Peptidases are known virulence factors of *Leishmania* and have been used successfully in experimental vaccines; here we evaluated the use of *L. infantum* rCPB and rCPA. Two types of native parasite antigen were tested; promastigote culture supernatant (*Li*ESAp) and autoclaved promastigotes (A*Li*). Different adjuvants were used in order to stimulate either cell mediated immunity (live BCG), humoral responses (oil-in-water emulsion, MDP) or both (saponin). Results show that the use of live Bacillus Calmette-Guérin, regardless of the antigen, causes a significant reduction in parasite burden. At the same time however, the mycobacteria also appear to be the cause of severe histopathological abnormalities making this a less suitable candidate for a commercial vaccine. Vaccination with MDP alone appeared to be partially protective but the addition of (any) antigen abolished this effect. When combined with saponin or oil-in-water emulsion, vaccination with rCP's slightly increased susceptibility to infection, possibly due to the immune modulating potential of these molecules. Combining CP's with strong Th1 inducing adjuvants like recombinant Interleukin 12 or immunostimulatory oligodeoxynucleotides (CpG's) may reverse deleterious effects and result in protection.

Introduction

Canine leishmaniasis, caused by *Leishmania infantum*, is a severe and potentially fatal disease that occurs in the Mediterranean and in large parts of South America (*L. chagasi*). This parasite, which is transmitted by the bites of infected sandflies, not only causes veterinary problems but can be the cause of zoonotic visceral leishmaniasis in children and immune compromised adults as well. A preventive vaccine for dogs would be a valuable tool for the prevention of canine leishmaniasis and, since dogs serve as the main reservoir for this parasite, this would likely also reduce the incidence of zoonotic visceral leishmaniasis (Dye, 1996; Tesh, 1995).

Many different candidate *Leishmania* vaccine preparations have been tested in past and recent years, with approaches ranging from virulent live parasites (leishmanization) to DNA vaccines. The potential of various candidate antigens for a canine leishmaniasis vaccine has been reviewed by Gradoni (Gradoni, 2001). Apart from the question of efficacy of these different approaches, it is evident that a subunit vaccine -and preferably a recombinant protein- is the most appropriate candidate for a world-wide commercial application of a canine leishmaniasis vaccine. Live vaccines may pose problems associated with safety, public health, shelf life and storage method whereas vaccines composed of (partially) purified parasite proteins may be problematic with regard to yield and cost of production. DNA vaccination often requires heterologous prime-boost regimes with combinations of DNA, recombinant viruses and or protein in order to reach optimal effects; implicating development and production of two different products. Vaccines based on recombinant proteins have drawbacks as well, the main disadvantage probably being the need to purify the protein -at least when it is produced in bacteria- but these appear minor compared to the above mentioned challenges.

Cysteine peptidases (CP's) are virulence factors of *Leishmania* parasites. The Cathepsin L-like enzymes CPA and CPB are thought to be involved in various processes that are of key importance to the parasite, many of which are associated with immune modulation (Mottram et al., 2004). Because of these vital functions, the *Leishmania* CP's are not only potential drug targets but also of interest for vaccine development. A double knockout (Δ*cpa/cpb*) mutant was found to be strongly attenuated, not causing any lesion development in mice. Moreover, vaccination with this Δ*cpa/cpb* double knockout provided partial protection against subsequent challenge with wild type *L. mexicana* (Alexander et al., 1998). Vaccination with recombinant CPB protein (rCPB) of *L. mexicana* was found to induce a Th2-type of response in mice, which led to exacerbation of the disease. However, in combination with recombinant interleukin 12 (rIL-12), vaccination of mice with rCPB induced a Th1 response and resulted in partial protection against a challenge infection with wild type *L. mexicana* (Aebischer et al., 2000; Pollock et al., 2003). Protection of mice against a challenge infection with *L. major* was found after vaccination with native CP purified from amastigotes formulated in Freunds' complete adjuvant (Rafati et al., 2000). In the same model, vaccination with a combination of plasmids containing *CPA* and *CPB* genes was found to be protective, whereas vaccination with either of the plasmids separately was not (Rafati et al., 2001).

The use of (partially purified) native antigen of *Leishmania* in vaccines against canine leishmaniasis has been tested by several groups. A field study with a vaccine consisting of partially purified lysate (LiF2) and muramyl dipeptide (MDP) rendered the dogs more susceptible to infection (Dunan et al., 1989). The use of culture supernatant, *Leishmania* excretion secretion antigen or *Li*ESAp, was more successful; vaccination of dogs with *Li*ESAp and MDP induced protection against challenge in 11 out of 12 animals (Lemesre et al., 2005). Another candidate vaccine was successful both in the laboratory and in the field; this consisted of autoclaved *Leishmania major* (and *L. infantum*) promastigotes in combination with live bacillus Calmette-Guérin (BCG) and induced protection in 70% (field) to 90% (lab) of dogs (Mohebali et al., 1998; Mohebali et al., 2004). In order to evaluate the use *Li*ESAp and autoclaved promastigotes in-house, similar antigens were prepared and tested in hamsters in combination with different adjuvants.

The choice of adjuvant can be essential for the efficacy of any vaccine. In leishmaniasis the general consensus is that vaccines need to elicit Th1-type responses and adjuvants are likely important tools to help achieve this. Although much used adjuvants like Freunds' incomplete adjuvant and Aluminum Hydroxide are known to induce mainly antibody responses, several other adjuvants are known to elicit Th1 responses (rIL12, live BCG, *C. parvum*) whereas others result in the production of antibodies as well as cellular immune responses (Saponins, Freunds' complete adjuvant). The type of immunity needed to protect dogs from infection with *L. infantum* is not known. Th1 responses are likely to be important as this has been shown in many models of infection and in vaccination trials in model animals. However, it cannot be ruled out that successful preventive vaccination may work through the induction of an immune response that is quite different, possibly depending on antibody production. Therefore, in order to evaluate the feasibility of a *L. infantum* subunit vaccine we tested the candidate antigens in combination with several adjuvants, together representing the whole spectrum of immune stimulation, in a hamster challenge model.

Materials and methods

Experimental design

Three separate experiments were performed, each testing one or several vaccine preparations for efficacy against *L. infantum* in hamsters. In experiment A one group of five hamsters was vaccinated with *L. infantum* recombinant Cysteine Peptidase B (rCPB) and Quil A, twice with a four week interval. A second group of five hamsters served as non-vaccinated controls and all animals were challenged three weeks after the second vaccination. The size of the parasite burden was estimated three months later at necropsy.

Experiment B involved three groups of five animals each, the first was vaccinated with rCPA + rCPB + μDiluvac forte® (Intervet, the Netherlands), the second with rCPA + rCPB + Q-vac (Nor-Vet, Denmark) and the third group served as non-vaccinated control. Again, animals were vaccinated twice with a four-week interval and challenged three weeks after the second vaccination. The size of the parasite burden was estimated at three months post infection.

The third and largest experiment (experiment C) comprised eleven groups of nine hamsters each. Eight groups were vaccinated with different combinations of three antigens (promastigote culture supernatant (similar to *Li*ESAp), rCPB or autoclaved *L. infantum* promastigotes (ALi)) and two adjuvants; live BCG or muramyl dipeptide (MDP)), two control groups were injected with the adjuvant alone and one group served as non-vaccinated control. Hamsters were vaccinated twice with a four-week interval. All groups were challenged at three weeks after the booster vaccination and the parasite burden was determined at one, three and six months after challenge. Table 1 summarizes the treatments for the different groups in all three experiments.

Animals

Six-week-old female *spf* golden hamsters (Harlan, the Netherlands) were used in experiment A whereas male hamsters of the same age and from the same supplier were used in experiments B and C. Animals were divided over the groups as they came to hand. Hamsters were not individually marked but different groups were kept in separate marked cages. Standard commercial feed ("RMH-B 2181", Arie Blok BV, the Netherlands) and water were provided *ad libitum*. Hamsters were allowed to acclimatize for one week before the start of each experiment.

Antigens and adjuvants

For the production of promastigote culture supernatant (*Li*ESAp) *L. infantum* JPC strain (MCAN/ES/98/LLM-724) promastigotes were expanded at 27°C in HO-MEM medium supplemented with 20% fetal bovine serum. Cultures were maintained until they reached the end-log phase, promastigotes were then washed and resuspended in serum-free HOMEM/199 medium (HOMEM + 2% medium 199H 10x (199H with Hanks salts; ICN Biomedicals Inc., U.S.A.) + 0.0005% Bovine Hemin (Sigma, USA).

After an additional four to five days of incubation, the culture supernatant was harvested by centrifugation (2,000 g, 10', 4ºC) and subsequently sterilized by filtration (Millex-GV 0.22µm, Millipore USA). The supernatant was concentrated by filtration (Centricon 10-YM, Millipore USA) and the protein content determined using a BCA protein assay kit (Pierce, Rockford, U.S.A.).

Table 1: groups and treatments

exp.	group	antigen	antigen dose	adjuvant	adjuvant dose	challenge dose, route	Necropsy (month p.i.)
A	1	rCPB	17 µg	Quil A	25 µg	10^8, i.p.	3
	2	-	-	-	-	10^8, i.p.	3
B	3	rCPA+rCPB	25 µg+25 µg	Diluvac	n.a.	10^8, i.p.	3
	4	rCPA+rCPB	25 µg+25 µg	Q-vac	25 µg	10^8, i.p.	3
	5	-	-	-	-	10^8, i.p.	3
C	6	ESP	100 µg	BCG	10^6 *cfu*	10^8, s.c.	1, 3, 6
	7	ESP	10 µg	BCG	10^6 *cfu*	10^8, s.c.	1, 3, 6
	8	rCPB-GFP	10 µg	BCG	10^6 *cfu*	10^8, s.c.	1, 3, 6
	9	ALi	10 µg	BCG	10^6 *cfu*	10^8, s.c.	1, 3, 6
	10	-	-	BCG	10^6 *cfu*	10^8, s.c.	1, 3, 6
	11	ESP	100 µg	MDP	100 µg	10^8, s.c.	1, 3, 6
	12	ESP	10 µg	MDP	100 µg	10^8, s.c.	1, 3, 6
	13	rCPB-GFP	10 µg	MDP	100 µg	10^8, s.c.	1, 3, 6
	14	ALi	10 µg	MDP	100 µg	10^8, s.c.	1, 3, 6
	15	-	-	MDP	100 µg	10^8, s.c.	1, 3, 6
	16	-	-	-	-	10^8, s.c.	1, 3, 6

Sixteen groups of hamsters divided over three experiments (exp. A, B and C) were vaccinated by subcutaneous injection; twice with a four week interval. Different combinations of antigen and adjuvant were used as shown in the table. Antigens were recombinant Cysteine Peptidase B (rCPB), recombinant Cysteine Peptidase A (rCPA) both produced in E. coli; recombinant Cysteine Peptidase B – Green Fluorescent Protein fusion (rCPB-GFP) produced in insect cells; Leishmania infantum promastigote culture supernatant (ESP) and autoclaved L. infantum promastigotes (ALi). Adjuvants were Quil-A, Q-vac, µDiluvac forte (Diluvac), live bacille Calmette-Guérin (BCG) and muramyl dipeptide (MDP). For BCG the dose was calculated in colony forming units (cfu). All vaccines were formulated in phosphate buffered saline, the dose volume was 1 ml for experiment A and B and 0.2 ml for experiment C. At three weeks after the second vaccination hamsters were challenged via intraperitoneal (i.p.) or subcutaneous (s.c.) routes. Necropsy was performed at 1, 3, or 6 months post infection (p.i.).

Autoclaved promastigotes (A*Li*) were prepared from end-log phase cultures of *L. infantum* JPC strain. Parasites were harvested by centrifugation (2,000g, 10') washed in sterile isotonic PBS (0.04M; pH 7.4), diluted to the appropriate concentration and subsequently autoclaved.

In experiment A and B recombinant cysteine peptidases were produced in *E.coli* as described previously (Poot et al., 2006). Briefly, the his-tagged proteins were purified from E.coli cell lysate using Ni-agarose beads (BD Talon™, Clontech USA). The resulting purified protein solution was analysed by SDS-PAGE and Western blotting, protein content was determinedas described above. For rCPA as well as rCPB a small portion of the recombinant protein appeared to be degraded, probably by autolysis.

For experiment C, recombinant cysteine peptidase B-GFP fusion protein was produced in a Baculovirus system. The coding sequence of *L. infantum* CPB was cloned from the E.coli expression vector into the baculovirus vector pFBhisGFP (Kaba et al., 2002). Virus production, transfection into *Sf9* insect cells and protein expression were performed using the Bac-to-Bac® Baculovirus expression system according to the manufacturers protocol (Invitrogen, U.S.A.). Recombinant protein was detected inside the insect cells only; therefore cells were harvested by centrifugation and subsequently disrupted by sonication. Specific (rCPB-GFP) protein content was estimated by SDS-PAGE. The cell lysate was inactivated by incubation with triton and subsequent γ-irradiation (25 kGray).

A stock suspension of 1mg/ml muramyl dipeptide (gMDP; Gerbu, Germay) in isotonic PBS was prepared and used to formulate the different vaccines. Live BCG: Freeze-dried bacillus Calmette-Guérin was obtained from Organon (US; OncoTice ®). One vial containing approximately $5*10^8$ *cfu* of BCG was reconstituted in isotonic PBS at a concentration of 10^7 *cfu* per ml and 100 µl per dose was added to the antigen preparation just prior to injection.

Parasites

For the challenge infections, *L. infantum* JPC strain (MCAN/ES/98/LLM-724) promastigotes were expanded at 27°C in HO-MEM medium supplemented with 20% fetal bovine serum. Cultures were maintained until they reached the end-log phase, promastigotes were then washed (2,000g, 10', 4°C) and resuspended in PBS at a concentration of $2{\cdot}10^8$/ml. Hamsters were challenged with 0.5 ml of parasite suspension via the intra peritoneal route in experiment A and B and via subcutaneous injection in the neck in experiment C.

Delayed type hypersensitivity testing

Stationary phase promastigotes were prepared to be used as antigen for Delayed Type Hypersensitivity testing. Parasites were washed twice with isotonic PBS and resuspended in 0.5% phenol/PBS. In experiment B 100 µl containing $1{\cdot}10^6$ parasites was injected intradermally on the back. In experiment C 3 hamsters from each group were tested for DTH responses at 1 month p.i., this was performed by injection of 50 µl containing $1{\cdot}10^7$ parasites in the dermis of the ear. In both cases 0.5% phenol/PBS was used as a control, intradermally on the back (exp. B) or in the opposite ear (exp. C).

Parasite quantification

At necropsy parts of liver and spleen were removed aseptically and used for parasite quantification by culture microtitration as described previously (chapter 5).

Lymphocyte Proliferation Assay

Lymphocytes were prepared from the spleens removed at necropsy. The test was performed as described before (chapter 5). Proliferative responses were expressed as stimulation indices (SI), which represent the ratio of mean proliferation after stimulation with mitogen (concanavilin A) or antigen (*Leishmania* soluble antigen) to the mean proliferation of medium controls.

Antibody detection

In experiment A, Western blots were used to detect specific antibodies against *Leishmania* (promastigote) antigen and rCPB.

In experiment B and C an ELISA was performed essentially as described before (chapter 5). Briefly, 96-well plates were coated with 5 µg/ml *Leishmania* soluble promastigote antigen in carbonate buffer. Plates were blocked with 1% Skimmed milk powder in PBS and subsequently incubated with test sera at a starting dilution of 1:40, diluted serially in the plate. After washing bound antibody was detected with goat-anti-hamster IgG H+L HRPO conjugate diluted 1:1000 in blocking buffer. Finally plates were developed and the absorbance was read.

Similarly an ELISA was performed using recombinant CPB purified from *E.coli* by Ni-affinity chromatography. Plates were coated with rCPB at a concentration of 1µg/ml and further steps were performed as described for the LSA ELISA.

Necropsy and histopathology

At the end of each experiment, hamsters were anaesthetised using either zoletil 100 ® (tiletamin and zolazepam; 0.3 ml s.c.; experiment A) or ether (experiment B and C). Subsequently animals were bled and killed humanely; part of the liver and spleen was collected aseptically for parasite culturing. Samples of spleen, liver, lymph node and skin were taken for histopathology. Histological findings were divided in three categories; no abnormalities, findings indicative or findings characteristic for a Leishmania infection. The results were summarized in a histological score as described before (chapter 5). In experiment C both ears were collected and processed for histological evaluation of DTH responses.

Results

Parasite quantification

The method used to determine the parasite burden was by limiting dilution culturing in all experiments. In experiment A, however, the input of spleen tissue was measured in grams, whereas in later experiments (B and C) this was measured as cell number. It is therefore difficult to compare values across experiments with regard to spleen parasite burden. In experiment A, no statistically significant differences in parasite burden of spleen or liver were found between vaccinates and controls. The average spleen and liver parasite burdens in this experiment are shown in figure 1.

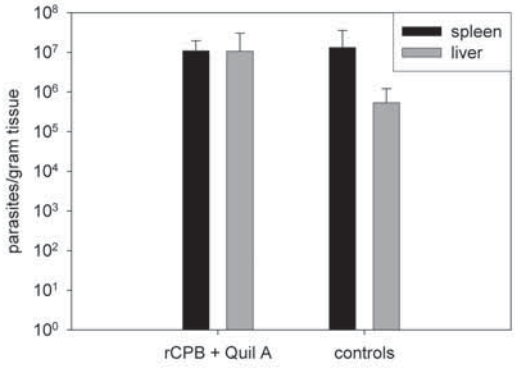

Figure 1:
Parasite burdens in experiment A
Parasite burdens were determined by limiting dilution culturing at 3 months post challenge and depicted in this graph as parasites per gram of tissue (spleen or liver). Results are shown for group 1 that was vaccinated with rCPB plus Quil A (rCPB + Quil A) and the control group 2 (both experiment A). Error bars indicate the standard deviation.

For the spleen parasite burdens found in experiment B a similar conclusion applies; no significant differences were found. However, significant differences were found in the liver; the average parasite burden in both vaccinated groups was higher compared to the controls. Although differences were not statistically significant, the average spleen parasite burden appeared also higher in the vaccinated groups (figure 2).

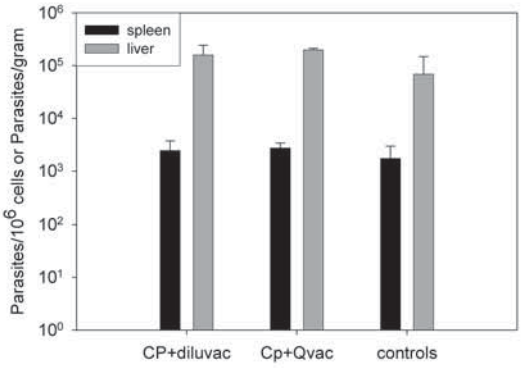

Figure 2:
Parasite burdens in experiment B
Parasite burdens were determined by limiting dilution culturing at 3 months post challenge and depicted in this graph as parasites per10^6 cells (spleen) or per gram of tissue (liver). Results are shown for group 3 that was vaccinated with rCPB and rCPA in µDiluvac forte (CP+Diluvac), group 4 that was vaccinated with rCPB and rCPA plus Q-vac (CP+Qvac) and the control group 5 (all experiment B). Error bars indicate the standard deviation.

Parasite burdens in experiment C were determined at three different time points after the challenge infection (figure 3). As expected, few parasites were detected either in the spleen or in the liver at one month post infection (p.i.).

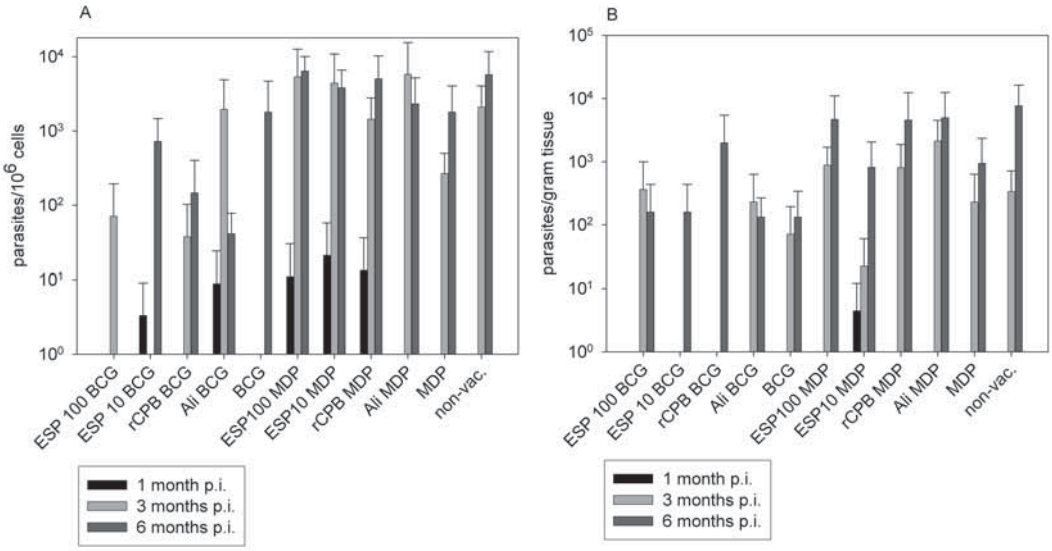

Figure 3: Parasite burdens in experiment C

Parasite burdens were determined by limiting dilution culturing at 1, 3 and 6 months post challenge (p.i.) and depicted in this graph as parasites per 10^6 cells for the spleen in panel A) or per gram of tissue for the liver in panel B. Results are shown for groups 6 to 16 (all experiment C) that were vaccinated with 100 or 10 µg of promastigote culture supernatant (ESP 100 and ESP 10 respectively) or recombinant Cysteine Peptidase B (rCPB), or autoclaved promastigotes (ALi) in combination with either live bacille Calmette-Guérin (BCG) or muramyl dipeptide (MDP). Adjuvant controls (BCG; MDP) were included as well as non vaccinated controls (non-vac.). Error bars indicate the standard deviation.

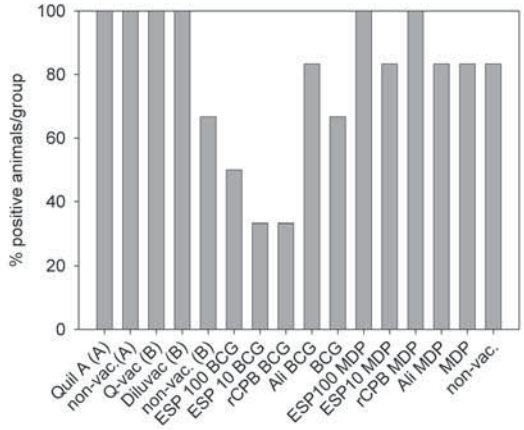

Figure 4:
Parasite positive animals per group
The number of animals that was parasite positive in any of the tissue samples investigated (spleen, liver, bone marrow) is shown here as a percentage of the total number of animals in a group. All groups in experiments A, B and C are included in this graph, for details see table 1.

At three months p.i. the average spleen parasite burdens in most groups had increased to levels comparable to those found in experiment B. Exceptions are groups 7 (ESP 10 + BCG) and 10 (BCG) where no parasites were detected in the spleen at 3 months p.i.. At six months p.i. high parasite burdens were found in the spleen of controls and in all "MDP groups" (groups 11-16). Spleen parasite burdens were also high in group10 (BCG) but were medium high in groups 7, 8 and 9 (ESP 10 + BCG; rCPB-GFP 10 + BCG; ALi 10 + BCG) whereas no parasites were detected in group 6 (ESP 100 + BCG) at six months p.i..

In the liver, parasite burdens at three and six months p.i. were a factor 100 lower compared to the numbers found in experiment B. This may well be due to the different challenge route (subcutaneous) that was used in experiment C.
Between the groups in experiment C parasite burdens were comparable except for groups 7 and 8 (ESP 10 + BCG; rCPB-GFP 10 + BCG) that were parasite negative at three months p.i..
Due to the small number of animals per group per time point and due to large individual variation, differences between the groups were not statistically significant at any of the three time points or for the whole experiment.

Whereas all but one of the animals in experiments A and B were parasite positive, quite a few remained negative in experiment C. This is summarized in figure 4, showing the percentage of animals per group that was parasite positive at three months (exp. A and B) or at three and six months p.i. (exp. C). In groups 7 and 8 (ESP 10 + BCG; ALi 10 + BCG) only 33% was parasite positive (2 out of 6 animals), whereas in group 16 (controls) 83% was found positive (5 out of 6 animals).

Differences between control groups and vaccinates with regard to average parasite burden are summarized in table 2 for all three experiments.

Table 2 : Reduction in parasite burden.

treatment	rCPB QuilA	rCPA/B Diluvac	rCPA/B Qvac	ESP 100 BCG	ESP 10 BCG	rCPB BCG	ALi BCG	BCG	ESP 100 MDP	ESP 10 MDP	rCPB MDP	ALi MDP	MDP
Group	1	3	4	6	7	8	9	10	11	12	13	14	15
Spleen	17%	-42%	-58%	99%	91%	98%	75%	77%	-50%	-5%	17%	-3%	74%
Liver	-1876%	-132%	-188%	93%	98%	75%	95%	97%	30%	89%	33%	11%	85%

Percentage reduction of the average parasite concentration in spleen (parasites per10^6 cells) and liver (parasites per gram) of vaccinated groups compared to their respective non-vaccinated control groups. Group 1 was compared to group 2, groups 3 and 4 to group 5 and groups 6 to 15 were compared to group 16 (see table 1). Negative values indicate an increase in the parasite concentration compared to controls.

Histopathology

Results of the histopathological evaluation of samples of spleen, liver, skin and lymph nodes are shown for all groups in figure 5. Average scores at three months p.i. in experiments A and B were high and not significantly different between vaccinates and controls, albeit average scores in the vaccinates were again higher.

For experiment C, data obtained at three and six months p.i. are depicted. Histopathology scores in the "BCG-groups" were very high at all time points. This high score did not stroke with the relatively low parasite burden determined in culture and since pathology caused by mycobacteria is very similar to what is detected in the course of a *Leishmania* infection we decided to perform an immuno histochemistry test for these groups (6-10).

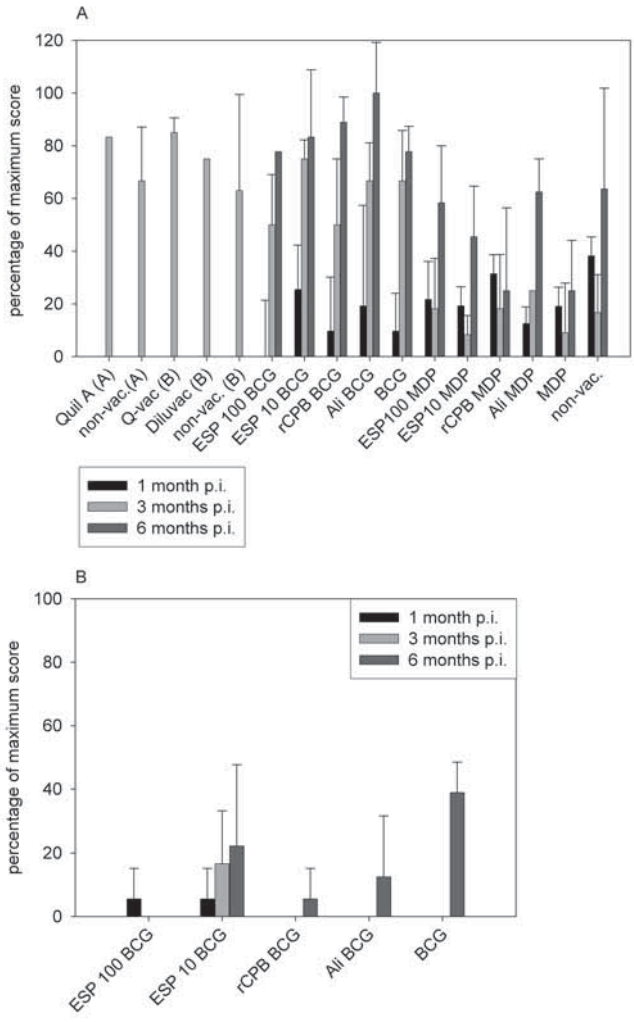

Figure 5: Histopathology scores

Panel A shows the average histopathology scores for all groups plotted as percentage of the maximum score. In experiment A and B (indicated between brackets) samples were taken at three months after challenge (3 months p.i.) only. In experiment C samples were taken at one month (1 month p.i.), 3 months (3 months p.i.) and six months (6 months p.i.) post challenge. For details regarding the vaccinations see table 1. Error bars indicate the standard deviation.

Panel B shows the average immuno histochemistry score for groups vaccinated with bcg as adjuvant, plotted as a percentage of the maximum score.

Results of the immuno histochemistry were scored 0, 1 or 2 for 'no indication of infection', 'suspected *Leishmania* stages' and '*Leishmania* stages present' respectively (data shown in figure 5). At three months p.i. scores are negative in groups 6, 8, 9 and 10 (ESP 100 + BCG; rCPB-GFP + BCG; ALi + BCG and BCG), whereas scores are low in the other groups. At six months p.i. scores are somewhat higher in most groups but in groups 6, 8 and 9 (ESP 100 + BCG; rCPB-GFP + BCG and ALi + BCG) scores were still very low.

Lymphocyte proliferation

Lymphocyte proliferation assays were performed in experiments B and C, results are shown in figure 6. Cell proliferation after stimulation with the mitogen (conA) was detected in samples from the majority of animals both in experiment B and C, thus showing viability of the cells used. Incubation with antigen (LSA, rCPA, or rCPB) resulted in stimulation indices that were not significantly different from 1, indicating no proliferation compared to non-stimulated control cells.

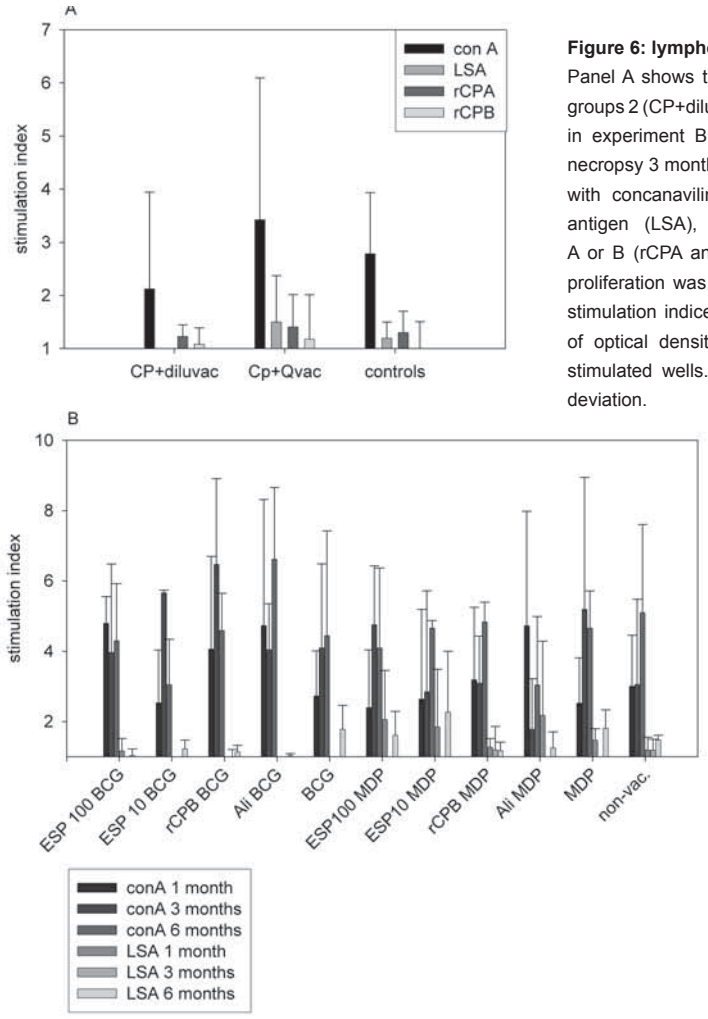

Figure 6: lymphocyte proliferation

Panel A shows the average stimulation indices for groups 2 (CP+diluvac), 3 (CP+Qvac) and 4 (controls) in experiment B. Spleen cells were harvested at necropsy 3 months post challenge and co-cultured with concanavilin A (con A), Leishmania soluble antigen (LSA), recombinant cysteine peptidase A or B (rCPA and rCPB) or with no addition. Cell proliferation was measured by BrdU incorporation; stimulation indices were calculated as the quotient of optical density values of stimulated and non-stimulated wells. Error bars indicate the standard deviation.

Panel B shows the average stimulation indices for groups 6 - 16 in experiment C (table 1). Spleen cells were harvested at 1, 3 and 6 months post challenge and co-cultured with con A or LSA. For each group the first three bars indicate proliferation after stimulation with mitogen (con A) and the last three bars proliferation after stimulation with antigen (LSA).

Delayed type hypersensitivity

In experiment B, animals were injected intradermally (on the back) with a suspension of inactivated promastigotes. Some reactivity in the form of skin redness was found in all animals at 24 and 48 hours after injection. However, no nodules were detected in any of the animals and no difference was found between the groups. Therefore, no indication was found for the presence of a specific cellular immune response.

In experiment C the parasite suspension was injected in the dermis of the ear. The ears were checked for the appearance of swelling and/or redness at 48 hours after injection. Some swelling of 1-2 mm. was detected in the test-ears of all hamsters whereas the control-injected ears remained unchanged. The exact size of the swelling was not recorded but histology of the ears was subsequently evaluated; all test-ears showed moderate to severe mononuclear infiltration except for group 6 (ESP 100 + BCG) which showed granulomatous inflammation with epitheloid cells. The control ears showed minimal to mild mononuclear infiltration. After a DTH-challenge perivascular cuffing is one of the major effects expected to be found microscopically. The observed diffuse mononuclear infiltration is however more consistent with a general inflammatory reaction to parasite antigen application than with a delayed type hypersensitivity reaction. No difference was found between the groups (vaccinated or control).

Leishmania-specific antibody titres

Antibodies against *Leishmania* promastigote antigen (LSA) were detected by Western blot in experiment A. Serum taken at the time of challenge was negative in the controls and in all but one of the vaccinated hamsters which showed a single band at 66 kD.
All sera taken at three months p.i. reacted strongly to the LSA except for one hamster in the control group. The parasite burden in this serologically negative animal was 1,000-fold lower compared to the average burden of the controls. Antibodies against rCPB were only detected in sera taken at three months p.i.: two of the vaccinates and two control hamsters were seropositive.

Both vaccinates and controls in experiment B developed positive antibody titres against crude promastigote antigen after the challenge infection to a similar extend (figure 7). Background levels in the rCPA ELISA were relatively high and average titres did not reach the cut off level in any of the groups throughout the experiment. Background levels in the rCPB ELISA were similarly high; however average titres in both vaccinated groups were just above the cut off level after vaccination and at three months p.i.. Although the average titre in the control group appeared to have increased somewhat after challenge, it did not exceed the cut off level (figure 7). The high background in both rCPA and rCPB ELISA is probably caused by the presence of contaminating *E.coli* proteins in the purified recombinant protein preparations. At one month after the challenge infection average antibody titres against LSA were positive in most groups in experiment C (figure 8).

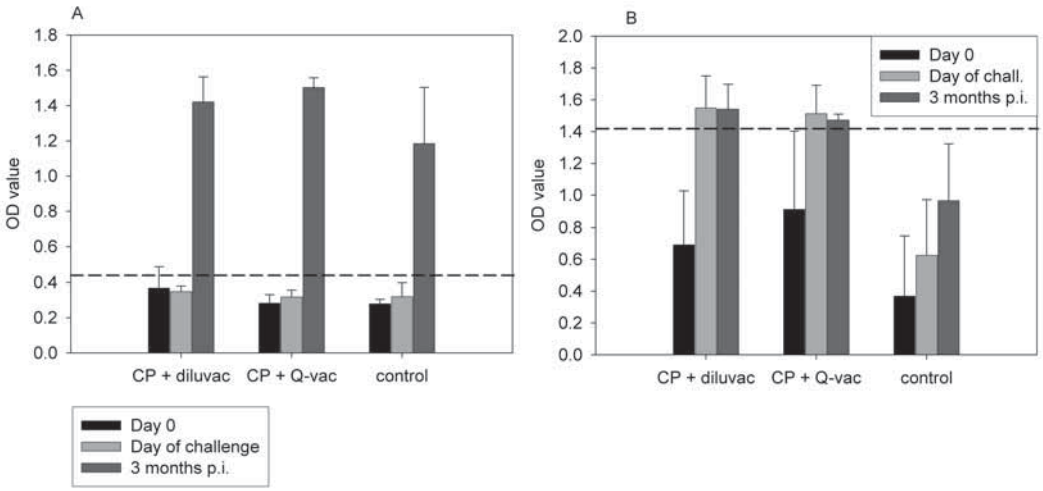

Figure 7: antibody titres to Leishmania antigen and rCPB in experiment B
In panel A results of the Leishmania soluble antigen (LSA) ELISA are depicted as average optical densities (average OD value) for groups 3 (CP + diluvac), 4 (CP + Q-vac) and 5 (control) of experiment B (table 1). Serum samples were taken at the start of the experiment (Day 0), after vaccination (Day of challenge) and at necropsy 3 months post challenge (3 months p.i.). The dotted line indicates the cut off value calculated as the average of all negative samples plus twice the standard deviation. Error bars indicate the standard deviation.
Panel B shows the results of the rCPB ELISA for experiment B. Again the dotted line indicates the cut off value and error bars indicate the standard deviation.

The two groups vaccinated with a high dose of ESP (ESP 100 + BCG and EPS 100 + MDP) had significantly higher titres compared to the rest of the groups, the combination of ESP 100 with MDP gave significantly higher titres compared to the BCG adjuvant. Groups that received a vaccine containing MDP had higher average titres compared to their BCG-counterparts. Only titres in group 14 (ALi + MDP) were unexpectedly low and even lower than the average titre in group 9 (ALi + BCG).

At three months p.i. most groups had high antibody titres. Differences between the groups were not statistically significant; however titres in some of the BCG-vaccinated groups (7, 8 and 9) appear to be somewhat lower. At 6 months the titres had again increased, differences were not statistically significant but the BCG-groups had somewhat lower titres compared to the MDP-groups.

Titres against rCPB were very low overall and not significantly different between the groups. It is striking that even in the rCPB-GFP vaccinated groups (8 and 13) titres did not develop. At six months post challenge average titres in some of the groups exceeded the threshold value, mostly due to one animal (figure 8).

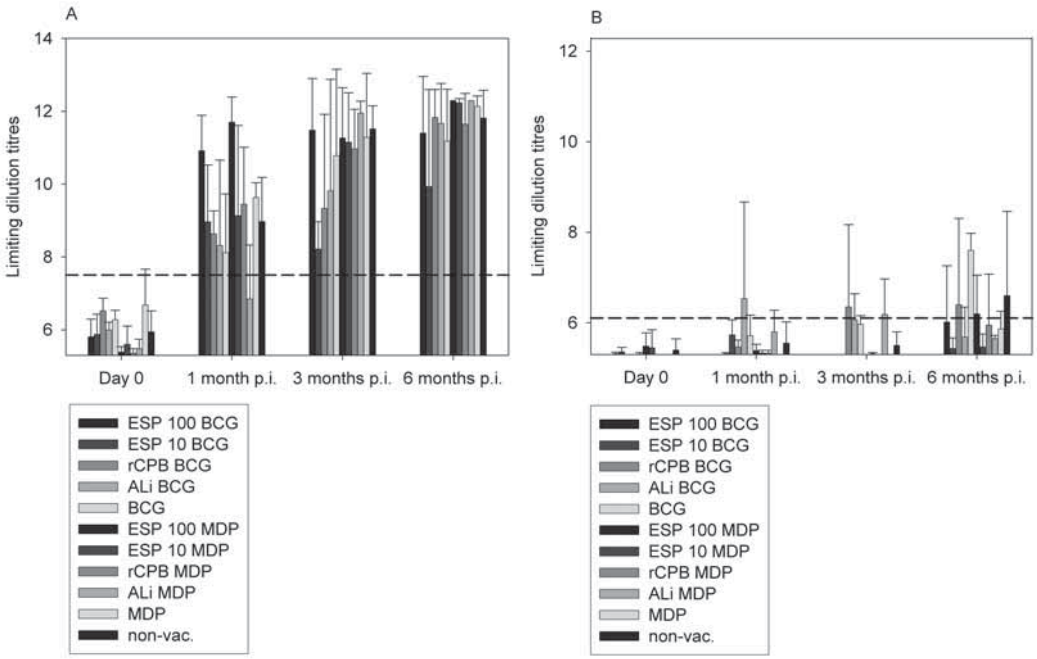

Figure 8: antibody titres to Leishmania antigen and rCPB in experiment C

Panel A shows the average limiting dilution titres calculated for the Leishmania soluble antigen (LSA) ELISA performed on serum samples from experiment C (groups 6 to 16). Groups are indicated by the type of vaccine received; the sequence shown in the legend from top to bottom is the same order as was used in the graph from left to right. Serum samples were taken at the start of the experiment (Day 0) and at one, three and six months post challenge (1 month p.i., 3 months p.i. and 6 months p.i. respectively). The dotted line indicates the cut off value calculated as the average of all negative samples plus twice the standard deviation. Error bars indicate the standard deviation.

In panel B the average limiting dilution titres from the rCPB ELISA are plotted for all groups in experiment C (groups 6 to 16, table 1). Again the dotted line indicates the cut off value and error bars indicate the standard deviation.

Discussion

In all three experiments (A, B and C) the use of recombinant Cysteine Peptidases as vaccine antigens against *L. infantum* was investigated. In order to widen the spectrum of immune reactions induced by the different preparations, different adjuvants were used to formulate the candidate vaccines. The saponins (Quil A or Q-vac) are known to induce both humoral and cell mediated responses (Newman et al., 1992; Wu et al., 1992), the oil in water emulsion (µDiluvac) and MDP in saline are expected to induce mainly antibodies whereas live BCG should induce macrophage activation and help the induction of cell mediated immunity (Pappas et al., 1983; Youngner and Salvin, 1973).

In experiments A and B vaccination –if anything- appeared to enhance susceptibility to infection rather than induce protection. This may indicate that a specific immune reaction was induced, but this had deleterious effects. As was shown in the mouse model, vaccination with *L. mexicana* rCPB alone enhanced lesion growth associated with the induction of a Th2 response. The addition of rIL12 as an adjuvant to the *L. mexicana* rCPB resulted in the induction of Th1 responses and partial protection (Pollock et al., 2003). In experiments A and B the Cysteine peptidases (CP's) may have had some enhancing effect on the *L. infantum* infection possibly due to the absence of an adjuvant with a strong Th1-stimulating activity.
Although it has been reported that the addition of rCPA encoding DNA significantly improved the efficacy of an rCPB DNA vaccine in a mouse model for cutaneous leishmaniasis (*L. major*) (Rafati et al., 2001) in this study we did not see an effect –either negative or positive- of the addition of rCPA to the vaccine.

The combination of rCPB-GFP and MDP did not appear to induce protection. Although the parasite burden was slightly decreased in this group, the number of parasite positive animals was higher compared to the controls. Again, this may be due to potential immune modulating effects of the antigen and failure of the adjuvant (MDP) to convert this immune response to a protective Th1 type of response. The administration of MDP in aqueous solutions has been reported to have predominantly antibody-inducing properties and lack the ability to induce cell mediated cytotoxicity that is induced by MDP formulated in water in oil emulsion (Azuma et al., 1976). MDP formulated in water (without the addition of antigen) was reported to reduce the *L. donovani* parasite burden by approximately 25% (not statistically significant)(Pal et al., 1991). In the study mentioned above MDP was administered to hamsters at day -7 and +7 before and after infection, although we administered the adjuvant once at day -21 before infection, the reduction in parasite burden may have been caused by a similar non-specific immunestimulation.

Although differences between vaccinates and controls were not statistically significant, the average parasite burden in the rCPB-GFP + BCG vaccinated group was reduced by 98% in the spleen and by 75% in the liver compared to the controls. Vaccination with a combination of leishmania native antigen (*Li*ESAp and A*Li*) and BCG or the use of live BCG alone reduced the parasite burden to a similar extent. The effect of vaccination with A*Li* plus live BCG we observed is similar to what has been reported for *L. donovani* in hamsters. Vaccination of hamsters with live BCG in combination with autoclaved promastigotes of *L. major* (ALM) or *L. donovani* (ALD) resulted in partial protection against *L. donovani* challenge as shown by 90% reduction in parasite burden a decrease in the number of parasite positive animals of 30-50% and an increased survival time(Srivastava et al., 2003). Similar to what we have found, vaccination with BCG alone has been reported to decrease the parasite burden after *L. donovani* challenge (Jarecki-Black et al., 1984; Srivastava et al., 2003). There may be a slight difference between the BCG-preparations because vaccination with *Li*ESAp or rCPB-GFP plus BCG resulted in a reduced number of parasite positive animals whereas A*Li* plus BCG or BCG alone did not. It appears from the reduction in parasite burden that a major part of the effect was due to the presence of BCG.

Results of histopathology were generally comparable to the results of parasite quantification, except for the groups vaccinated with live BCG. In these groups spleen size was greatly enhanced and histopathological changes were also more severe compared to the non-vaccinated and MDP-vaccinated groups (results not shown). This effect is most likely due to the infection with BCG which has previously been described to induce similar lesions in hamsters (Jarecki-Black et al., 1984; Zwilling and Davis, 1976).

Antibody titres against crude *Leishmania* antigen reflect the size of the parasite burden in non-vaccinated animals but this may be influenced by vaccination (chapter 3). In groups 3 and 4 this appears not to be the case as the titres against LSA are very similar to the control group 5. This may be explained by the surprisingly low antibody titres against CP's in these groups that are probably not high enough to interfere with the LSA ELISA. An explanation for the low antibody response despite the use of antibody-enhancing adjuvants may be that a relatively low amount of antigen was used (25 µg per antigen per dose) in the vaccine preparations; this may have restricted antibody induction. In the rCPB-GFP vaccinated groups (8 and 13) rCPB specific titres were hardly detected. Although this could also be an effect of the amount of antigen or of the adjuvant used (live BCG and MDP) it may also be due to the fact that the vaccine antigen was produced in baculovirus whereas the ELISA was performed with *E.coli* produced material. In the *E.coli*-CPB vaccinated hamsters an unknown part of the antibody titre will have been directed to host cell material present both in the vaccine and on the ELISA plate, this 'background' reactivity was eliminated by vaccinating with baculovirus derived material (rCPB-GFP). Another factor may be the difference in recombinant rCPB protein; the *E.coli*-derived protein was a HIS-fusion whereas the baculovirus derived protein was a GFP-fusion and, moreover, rCPB-GFP was probably glycosylated in contrast to the *E. coli* material. In groups 6 and 11, vaccinated with ESP 100, LSA antibody titres are relatively high, indicating that there are probably several cross-reactive epitopes between the vaccine antigen and the crude soluble antigen. A tenfold lower dose of antigen (groups 7 and 12) significantly decreased the antibody titre. The use of MDP appears to induce slightly higher antibody titres than live BCG which could be expected form the respective adjuvant properties.
Lymphocyte proliferation tests and Delayed type hypersensitivity testing were performed in order to detect stimulation of cellular immune responses. Unfortunately such responses were not detected while at the same time a reduction in parasite burden and therefore –partial-protection was found in several groups. Enhanced lymphoproliferative responses of hamsters to leishmania antigen have been reported after vaccination with autoclaved promastigotes plus BCG (Srivastava et al., 2003). The discrepancy between these results could possibly be explained by the type and dose of antigen that was used for stimulation; while we used 2 µg/ml of promastigote lysate, these authors used 10 µg/ml of autoclaved promastigotes. Surface antigens, not present in the lysate, may have played an important role in the induction of T-cell proliferation.

In conclusion, partial protection of hamsters against challenge with *L. infantum* was achieved by vaccinating with preparations containing live BCG. Much of the observed effect appeared to be attributable to the use of BCG in these vaccines. Hamsters developed major histopathological abnormalities upon infection with BCG and although the general health of the hamsters appeared not to be seriously affected, live BCG is possibly not safe as a vaccine adjuvant. Although vaccination with MDP alone appeared to be partially protective, the addition of (any) antigen abolished this effect. This is surprising since the combination of MDP and ESP has been reported to be protective in dogs (Lemesre et al., 2005). Vaccination with rCP's appeared to increase susceptibility to infection, possibly indicating their immune modulating potential and at the same time stressing the importance of these antigens. Combining CP's with strong Th1 inducing adjuvants such as recombinant Interleukin-12 or CpG oligodeoxynucleotides may reverse deleterious effects and result in protection.

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CHAPTER 8

Summarizing discussion

Introduction

Canine leishmaniasis is caused by *L. infantum* (syn. *L. chagasi*) and transmitted by the bite of infected sandflies. The infective metacyclic promastigotes are taken up by the host's macrophages and transform to amastigotes which survive and multiply within the macrophage. Dogs suffer from persistent infections causing a chronic and ultimately fatal disease. Even though treatment usually results in clinical improvement, the parasite is not completely cleared from its canine host and relapses are the rule rather than the exception. Moreover, infected dogs form the reservoir for this parasite species that can also cause zoonotic visceral leishmaniasis in children and immune compromised adults.

The research described in this thesis was performed in order to establish a suitable challenge model in dogs and subsequently test candidate vaccines for canine leishmaniasis. Although much is known about *Leishmania* and its possible interactions with the host immune system this knowledge is mainly derived from disease models using inbred mouse strains. At the onset of this project information on the experimental infection of dogs was scarce and sometimes contradictive. The first aim was therefore to establish an experimental model in dogs that could be used for vaccination-challenge experiments; this is described in chapter 2. Since the number of dogs that can be used is limited, a second model was established in hamsters that enables the screening of larger numbers of candidate vaccines for their efficacy (chapter 3). The first vaccine candidates to be tested were live attenuated parasites, generated by knockout of the virulence factors cysteine peptidase A and B. The residual virulence and protective potential of different knockout strains was tested in the hamster model as described in chapters 4 and 5. As a second line of investigation the efficacy of a number of subunit vaccines was tested; recombinant cysteine peptidases A and B were tested in dogs (chapter 6) as well as hamsters (chapter 7) while two preparations based on native parasite antigens, one consisting of autoclaved promastigotes the other of culture supernatant, were tested in hamsters only (chapter 7).

Challenge models

The first steps in our studies were to establish challenge models in dogs and hamsters in our laboratory, to optimize these models and to confirm the virulence of the JPC challenge strain.

Dog challenge model

Dogs are naturally infected by the bite of infected sandflies. A bite may contain between ten and several thousand infectious metacyclic promastigotes (Warburg et al., 1986) and dogs in endemic areas can receive up to seven infected bites per night during the transmission season. Use of the natural infection route in laboratory models is complicated; a sandfly colony would need to be maintained and standardization of a challenge by bites is virtually impossible. Intravenous injection of a relatively high dose of parasites, either promastigotes or amastigotes, is most often used to challenge dogs. For vaccination-challenge experiments it is probably advantageous to use promastigotes as this is infective stage in natural infections. It has been concluded by a

number of authors that promastigotes are less infective compared to amastigotes (Abranches et al., 1991a; Campino et al., 2000; Santos-Gomes et al., 2003). Analysis of a larger number of studies revealed that infectivity of amastigotes and promastigotes must be similar; when differences in infective dose, follow up period and method of parasite detection are omitted, 30 out of 33 dogs were found parasite positive after promastigote challenge (91%) while 37 out of 40 were positive after amastigote challenge (93%) (Campino et al., 2000; Martinez-Moreno et al., 1995; Molano et al., 2003; Paranhos-Silva et al., 2003; Riera et al., 1999);(Binhazim et al., 1993; Campino et al., 2000; Leandro et al., 2001; Oliveira et al., 1993; Rhalem et al., 1999; Santos-Gomes et al., 2002). Although in most studies mentioned above almost all dogs became parasite positive, the reported percentage of dogs with clinical signs was lower. For a good experimental challenge model, the appearance of parasites as well as clinical signs in all dogs is a great advantage. This enhances the difference between control groups and treated (protected) groups and therefore reduces the number of dogs needed.

We found a 100% infection rate after intravenous challenge with a medium high dose ($5x10^7$) of *L. infantum* JPC strain promastigotes (chapter 2). Differences between our result and infection rates reported in some of the above mentioned papers is probably due to the method of parasite detection and to the length of the follow up period. In order to further improve the challenge model we tried to find (early) parameters of disease by monitoring clinical signs, blood chemistry, serology, lymphocyte proliferation and cytokine production. In our study clinical signs were found in the majority of animals; this confirms results from a number of studies that have employed intravenous challenge but contradicts results found in two studies where clinical signs were not found (Campino et al., 2000; Oliveira et al., 1993). The reason for this apparent contradiction is probably that subtle changes in the clinical appearance were not taken into account by these authors. We found however that repeated abnormal findings are a sign of disease, even when the changes are minor, and should therefore be recorded regularly and accurately in order to be useful for establishing (in retrospect) the onset of disease.

While the earliest onset of clinical disease was around 30 weeks post infection, antibody titres developed from as early as 2 weeks p.i. and were positive in all dogs at 15 weeks p.i.. We found that the combination of clinical, serological and parasitological parameters was sufficient to allow evaluation of disease progression in the canine experimental leishmaniasis model. It is however essential to use the most sensitive methods for parasite detection and perform detailed clinical examination regularly.

Many authors have reported the existence of asymptomatic dogs and it has been suggested that an asymptomatic form of the infection mainly develops after experimental infection with promastigotes while infection with amastigotes causes clinical disease (Abranches et al., 1991b; Campino et al., 2000; Gradoni et al., 1980; Oliveira et al., 1993). From our studies however (chapter 2), it appears that after experimental infection with promastigotes all dogs progress from “subpatent infection” to “asymptomatic patent infection” and finally to “symptomatic patent infection” according to the new definitions proposed by Gradoni (Gradoni et al., 2005b).

Others have also observed the slow progression of disease in all dogs infected, usually starting with the appearance of either parasites or antibodies but invariably resulting in the appearance of clinical signs. This was found both in experimentally infected dogs (Martinez-Moreno et al., 1995) and in a longitudinal study of naturally infected dogs (Gradoni et al., 2005a).

The detection of cellular immune responses may further improve the challenge model in dogs. It is known from the mouse model of infection that the development of a T-helper type 1-associated cell mediated immune response is correlated with disease resistance while the development of Th2-directed immune response results in uncontrolled development of disease. The detection of cellular immune responses in dogs is generally hampered by a lack of immunological markers and tools; only few studies have addressed this. We have used lymphocyte proliferation, delayed type hypersensitivity and cytokine expression to analyze cellular immune responses but were unable to detect any relation between test results and disease progression (chapter 1 and 6). This corroborates data from a number of other studies and supports the new insight that the Th1-Th2 paradigm, explaining development of resistance or disease by the presence of a polarized immune response in the mouse model, may not apply to canine leishmaniasis (Gradoni et al., 2005a; Martinez-Moreno et al., 1995; Quinnell et al., 2001; Santos-Gomes et al., 2002). Since we have not been able to protect dogs by vaccination, it cannot be concluded whether the monitoring of cellular immune responses can be a tool to identify protected dogs; this needs further investigation.

The experimental challenge we have used may carry the risk of under- or overestimation of vaccine efficacy due to the 'unnatural' dose, route, life cycle stage and chemical composition of the inoculum. This has also been suggested by Gradoni to explain observed discrepancies between vaccine efficacy found after experimental and natural challenge (Gradoni et al., 2005b). Although dogs can be infected by dermal injection of parasites, the development of disease is even more prolonged compared to the i.v. challenge method (Paranhos-Silva et al., 2003; Santos-Gomes et al., 2000). This appears to be an effect similar to what we found in experimentally infected hamsters; the 'peripheral' injection of parasites appeared to lower the initial number of successfully established amastigotes, subsequently delaying the onset of disease. During natural transmission, sandflies deliver low numbers of metacyclic promastigotes by regurgitation into the skin of a mammalian host but this inoculum also contains promastigote secretory gell and sandfly derived substances like salivary proteins (Rogers et al., 2004; Sacks et al., 2001). The addition of sandfly-derived material may offer an opportunity to improve dermal experimental infection models. The effect of adding sand fly saliva to a challenge dose has been tested in the mouse model with results ranging from marked enhancement of infection (Belkaid et al., 1998; Castro-Sousa et al., 2001; Morris et al., 2001; Titus et al., 1988; Warburg et al., 1994) to no measurable effect (Belkaid et al., 1998; Castro-Sousa et al., 2001; Morris et al., 2001; Titus et al., 1988; Warburg et al., 1994). In dogs challenged by intradermal injection of metacyclic promastigotes, the addition of sand fly saliva did not enhance infection (Paranhos et al., 1993; Paranhos-Silva et al., 2003). A parasite-derived promastigote secretory gel (PSG) generated within the fly was shown to accompany infecting metacyclic promastigotes during fly bite and

enhance infection in a mouse model of infection (Rogers et al., 2004). We tested the use of PSG in the dog challenge model; no obvious enhancement of the infectivity was detected and we decided not to use this as our standard for vaccination-challenge experiments (chapter 2).

Hamster challenge model

In contrast to dogs, outbred mice are not very susceptible to *L. infantum* and the disease tends to self-cure (Trotter et al., 1980), making this a less appropriate model for canine leishmaniasis. Hamsters are however highly susceptible to *L. infantum*. Nonetheless, few studies have been published about the experimental infection of hamsters with *L. infantum* or on the use of this model in vaccine research. We therefore initiated an experiment to evaluate the virulence of the JPC challenge strain in hamsters, establish the optimal challenge dose and route and evaluate parameters for detection of disease progression.

Limiting dilution culturing of spleen and liver was shown to be an excellent tool for estimation of the final parasite burden in hamsters while *Leishmania*-specific antibody titres were shown to be useful for monitoring the development of parasites during the course of the experiment (chapter 3). Although all infection routes were successful, the intraperitoneal route of infection was shown to be more virulent compared to subcutaneous and intradermal routes. Decrease of the challenge dose resulted in an apparent delay in the development of the parasite burden and a similar effect was observed when the route of infection was changed from intraperitoneal to peripheral. These results are similar to what was found in a study with *L. donovani* in hamsters in which infection with 10-fold dilutions of amastigotes resulted in gradually increasing time to patency (Ott et al., 1967).

With the highest challenge dose of 10^8 promastigotes i.p. a 'plateau' was not reached as reduction of the challenge dose had a direct effect on the parasite burden. Thus it should be possible with this model to detect effects on parasite burden due to vaccination or treatment. Infection with lower doses of parasites requires longer incubation times (up to 1 year) and larger groups in order to achieve similar results which is not convenient for screening of vaccine candidates. Vaccination-challenge experiments were therefore performed with a high challenge dose of 10^8 stationary phase promastigotes of the JPC strain. Both the intraperitoneal and the subcutaneous route can be used for experimental challenge. These routes do not appear to induce a different disease spectrum but compared to intra peritoneal injection the subcutaneous route caused a delay in the onset of parasite development. A more subtle challenge can be advantageous in vaccination-challenge experiments as it may help detect lower levels of protection.

Testing of candidate vaccines

Cysteine peptidases (CP's) are thought to be involved in various processes that are of vital importance to *Leishmania* spp., including facilitating colonisation of the mammalian host (McKerrow, 1989). The cathepsin L-like CP's CPA, CPB and CPC of *Leishmania* are

virulence factors that are involved in immune modulating activities such as the induction of IL-4 production, inhibition of macrophage IL-12 production and degradation of MHC class II molecules in the parasitophorous vacuole (Mottram et al., 2004). The *Leishmania* CP's are therefore considered to be potential drug targets as well as vaccine candidates (McKerrow, 1989). CP knockout mutants of *L. mexicana* (Δ*cpa/cpb*) were shown to have significantly reduced virulence in mice and induce a shift of the immune response toward a protective Th1-type (Alexander et al., 1998). The potential of recombinant cysteine peptidases (rCP's) as components of leishmaniasis vaccines has been investigated in mouse models of infection and in dogs. Mice vaccinated with *L. mexicana* rCPB exhibited enhanced lesion growth after challenge; this infection potentiating property could however be reversed by addition of rIL-12 to the antigen, resulting in partial protection against challenge with *L. mexicana* (Pollock et al., 2003). Vaccination with a cocktail of plasmid DNA encoding *L. major* CPA and CPB genes was found to be protective in mice albeit injection of either of the genes separately was not (Rafati et al., 2001). The same authors have found a vaccination regime consisting of a priming injection with a cocktail of plasmids encoding CpG's and CPA and CPB genes of *L. infantum* followed by booster vaccinations with rCPA and rCPB protein, to be protective in dogs (Rafati et al., 2005). We have performed experiments in hamsters to test the efficacy of live attenuated *L. infantum* strains generated by disruption of CP genes. The efficacy of recombinant CP proteins was evaluated in hamsters and dogs.

CP knockouts as live attenuated vaccines

Attenuated knockout strains of *L. infantum* were generated by targeted disruption of cysteine peptidase genes. This strategy was successful for the single copy gene cysteine peptidase A (CPA) (chapter 4) but failed to completely knock out the multicopy CPB gene. This was not expected since the same strategy was previously successfully applied for the generation of *L. mexicana* double Δ*cpa/*Δ*cpb* knockouts (Alexander et al., 1998). Experiments in hamsters showed that both the Δ*cpa* knockouts and the *CPB/*Δ*cpb* heterozygotes had markedly reduced virulence (chapter 5). This was not expected since *L. mexicana* Δ*cpa* knockouts had significant residual virulence (Alexander et al., 1998). The effect of disruption of one of the alleles of the CPB gene could not be compared to a similar knockout strain. It is possible that the loss of one allele caused a virulence phenotype but further investigation would be needed to confirm whether this is the only mechanism behind the observed reduction of virulence.

Since the knockouts and heterozygotes were found to be attenuated, their protective potential was evaluated in hamsters. Although the virulence phenotype could be confirmed in this experiment, protection against wild type challenge could not be established (chapter 5). Mice have been shown to be (partially) protected by vaccination with attenuated lines of *L. chagasi, L. major, L. mexicana* and *L. tarentolae* (Breton et al., 2005; Daneshvar et al., 2003; Rivier et al., 1993; Streit et al., 2001). However, similar successes have not been reported in hamsters or dogs; at this moment it is questionable whether this approach can be 'translated' from the mouse model to these more susceptible species. An essential difference between mice on the one hand and dogs and hamsters on the other hand is the ability of the former species to self-cure visceral

leishmaniasis. When applying live attenuated vaccines it is exactly this ability to clear the parasite by generating a specific immune response that is exploited to induce protection against wild-type challenge. Since recent research has generated data indicating that self-cure does not occur in dogs infected with *L.infantum* a live attenuated vaccine approach now appears to be less appropriate.

Subunit vaccines based on CP's

We tested the efficacy of various combinations of rCP's and adjuvants in hamsters; the parasite burden in the vaccinates was mostly similar to the controls although enhanced susceptibility to infection was observed after vaccination (chapter 7).The vaccine failure may have been caused by immunomodulatory activity of the antigen and if so, it may be possible to reverse this effect by the addition of stronger adjuvants as was shown for *L. mexicana* CP. In dogs a preparation containing *L. infantum* rCPA and rCPB in combination with rIL-12 was tested for efficacy. The recombinant proteins were not able to confer protection in this study. Again, the potential immunomodulatory activity of the CP's may require other –stronger- adjuvants or other approaches like DNA vaccination. DNA vaccination is known to induce Th1-type immune responses and was already shown to be effective in mice and dogs (Rafati et al., 2001; Rafati et al., 2005).

Addition of live Bacille Calmette-Guérin to a number of Leishmania antigens (rCP's, *Li*ESAp or A*Li*), or even vaccination with live BCG alone, resulted in strong reduction of spleen and liver parasite burdens in hamsters (75% to 98%) (chapter 7). Although slight differences appeared to exist between the different vaccine preparations with or without antigen, in general the effect was similar and it is therefore thought to be caused mainly by the presence of live BCG. Vaccination with live BCG has been found to be both prophylactic and therapeutic in BALB/c mice against challenge with *L. donovani* and prophylactic in hamsters against *L. donovani* challenge (Jarecki-Black et al., 1984; Smrkovski et al., 1977). Infection with BCG causes activation of macrophages which is essential for the elimination of *Leishmania* amastigotes (Tokunaga, 1980). BCG has been used as adjuvant in a number of successful experimental vaccines against leishmaniasis (Aebischer et al., 2000; Misra et al., 2001; Santos et al., 2002; Streit et al., 2000) including several in dogs (Mayrink et al., 1996; Mohebali et al., 1998; Mohebali et al., 2004; Molano et al., 2003). The application of live BCG in a commercial canine leishmaniasis vaccine would however be hampered by safety and public health issues (Safety of BCG in dogs, J. Poot, unpublished results). The efficacy of such a vaccine was therefore not tested in dogs.

Concluding remarks

It was shown in this thesis that despite previous positive results in a mouse model of infection, hamsters could not be protected with live attenuated strains of *L. infantum* generated by disruption of CP genes. In dogs vaccination with recombinant CP's plus rIL-12 did not confer protection against challenge with *L. infantum* although a similar vaccine was shown to be partially protective in mice. Results from our challenge studies as well as from other studies reported in literature suggest that the majority of dogs do not develop resistance to *L. infantum*. In contrast to the self-curing infections in most mouse models of infection, dogs and hamsters develop a slow chronic infection that can be kept under control for a variable time period. Uncontrolled multiplication of the parasite and subsequent clinical signs generally develop after the onset of specific immunity (antibodies). The start of this cascade appears to be influenced by factors like malnutrition, concurrent infections or chemical immune suppression (chapter 2) all of which may exert their effect through the innate immune system. If effective specific immunity does not occur in infected dogs, this means most mouse models are less appropriate for screening of vaccine candidates. Hamsters appear to be more appropriate hosts and the development of immunological tools would greatly enhance the value of this model. Another consequence is that vaccines designed to mimic natural development of immunity –for instance live attenuated vaccines- cannot be expected to be efficacious in dogs. With current limited knowledge of canine leishmaniasis and canine immunity it is very difficult, if not impossible, to predict from results in mouse models which vaccine candidates would work in dogs. It therefore remains essential to perform experiments in dogs, both for testing new vaccine candidates and for analysis of immune responses. Results of such experiments are valuable, whether or not protection will be achieved, in order to advance knowledge about canine leishmaniasis and to give direction to future protection strategies.

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Dutch summary
Nederlandse samenvatting

Samenvatting

Leishmania infantum is een protozaire parasiet van honden die een ernstige chronische ziekte (leishmaniasis) kan veroozaken. Bij kinderen en immuungecompromiteerde volwassenen kan zoönotische viscerale leishmaniasis het gevolg zijn van een infectie. Dit ééncellige organisme wordt overgebracht door zandvliegen (*Phlebotomus spp*. in de oude wereld of *Lutzomyia spp*.in de nieuwe wereld). Het verspreidingsgebied van de parasiet strekt zich uit van het Mediterrane gebied tot in het Midden-Oosten, de voormalige USSR en China. In de noordelijke helft van Zuid-Amerika komt een parasiet voor (*L. chagasi*) die niet te onderscheiden is van *L. infantum* en daarom doorgaans beschouwd wordt als dezelfde soort.

Bij de beet van een geïnfecteerde zandvlieg worden infectieuze metacyclische promastigoten vanuit de maag geregurgiteerd in de bijtwond. De promastigoten worden opgenomen door macrophagen en transformeren naar amastigoten. Amastigoten vermeerderen zich in het phagolysosoom wat uiteindelijk leidt tot de dood van de cel en vrijkomen van de parasieten die vervolgens weer nieuwe macrophagen infecteren. Wanneer een zandvlieg een bloedmaal neemt van een *Leishmania* geïnfecteerde hond kunnen macrophagen met amastigoten worden opgenomen. Amastigoten komen vrij in de maag van de mug, transformeren tot promastigoten en na vermenigvuldiging transformeren deze weer tot infectieuze metacyclische promastigoten.
Leishmaniasis in honden wordt veelal gekenmerkt wordt door conditie verlies en huidafwijkingen. Veel verschijnselen zijn terug te voeren op de extreem hoge (polyclonale) antilichaamtiters en circulerende immuuncomplexen; zonder behandeling is de ziekte doorgaans fataal. Een aantal verschillende medicijnen is beschikbaar maar enkele hiervan geven vrij ernstige bijwerkingen zoals nierschade. Therapie geeft vaak wel een vermindering van de verschijnselen, maar genezing wordt vrijwel nooit bereikt. Recidive is dan ook de regel.

De enige preventieve maatregelen die momenteel beschikbaar zijn richten zich op de bestrijding van de vector. Het gebruik van een halsband geïmpregneerd met delthamethrin (scalibor®) geeft maandenlange bescherming tegen zandvliegen. Veldproeven hebben aangetoond dat de halsband niet alleen bescherming bied aan de hond maar ook aan mensen in de omgeving. Het grootste nadeel van de halsband is dat honden deze kwijt kunnen raken. Een preventief vaccin zou een grote stap voorwaarts zijn bij de bestrijding van leishmaniasis bij de hond. De toepassing van een honden vaccin zou bovendien ook de incidentie van zoönotische viscerale leishmaniasis bij de mens kunnen verlagen.

Het is al langer bekend dat proteases essentieel zijn voor de virulentie van een groot aantal parasieten. In *Leishmania* zijn met name de cysteïne proteases bekend als virulentie factoren. Deze enzymen hebben ondermeer een belangrijke functie bij het onschadelijk maken van de immuunrespons van de gastheer. Een *L. mexicana* stam waarbij twee cysteïne protease genen waren uitgeschakeld bleek zodanig geattenueerd te zijn dat geen laesies meer werden veroorzaakt in muizen. Vaccinatie met deze knock-out kloon had bovendien tot gevolg dat de dieren beschermd waren tegen infectie met het wild-type van de parasiet. Van recombinante cysteïne

proteases van *L. mexicana* is eveneens een effect op het verloop van een infectie gevonden: hier werd de vorming van laesies juist verergerd door vaccinatie. De toevoeging van een cytokine (interleukine-12) met een sterk stimulerend effect op de celgemediëerde immuniteit zorgde er echter voor dat het recombinant cysteïne protease gedeeltelijke bescherming bood tegen de infectie.

Om de werkzaamheid van kandidaat-vaccins te kunnen testen is een laboratorium model van de ziekte in honden nodig. In hoofdstuk 2 van dit proefschrift wordt de optimalisatie van het laboratorium model voor honden beschreven. Het is gebleken dat intraveneuze injectie van promastigoten een infectie veroorzaakt in alle honden en bovendien in het merendeel van de dieren klinische verschijnselen opwekt. Het is ook duidelijk geworden dat voor een optimaal resultaat de gezondheids status van de honden nauwkeurig gevolgd moet worden tot ongeveer een jaar na de infectie.

Omdat het niet mogelijk, en ook niet wenselijk, is om grote aantallen honden te gebruiken voor het zoeken naar een geschikt vaccin is nog een tweede diermodel toegepast. Muizen worden veel gebruikt als model in onderzoek naar *Leishmania* vaccins. De meeste inteeltlijnen bij muizen zijn echter van nature resistent tegen de ziekte en zullen de parasiet direct, of na bepaalde tijd zelf 'opruimen'. Hamsters daarentegen zijn, net als honden, heel gevoelig voor de infectie en daarom geschikt om te dienen als model. In hoofdstuk 3 wordt beschreven hoe het hamster model voor *L. infantum* is geoptimaliseerd. Het belangrijkste verschil tussen infecties met verschillende doses parasieten is de lengte van de prepatent periode en het aantal positieve dieren in de periode tot 6 maanden na infectie. Optimaal is daarom het gebruik van een relatief hoge dosis promastigoten aangezien een lagere dosis een langere incubatie tijd tot gevolg heeft en daarom langere proeven met eventueel ook meer dieren tot gevolg zou hebben.

Twee verschillende benaderingen voor een *L. infantum* vaccin zijn getest in hamsters en honden. Ten eerste een levend geattenueerd vaccin dat werd verkregen door het vernietigen cysteïne protease genen in *L. infantum*. In *L. infantum* bestaan drie verschillende cysteïne protease genen: CPA, CPB en CPC. Met name CPB en in mindere mate CPA worden belangrijk geacht voor de virulentie, daarom is geprobeerd deze twee genen uit te schakelen. In hoofdstuk 4 is beschreven hoe twee onafhankelijke Δ*cpa* stammen werden verkregen. Het vernietigen van het CPB gen was minder succesvol; een aantal verschillende heterozygote stammen werd gegenereerd maar het bleek niet mogelijk het tweede allel van dit gen uit te schakelen. Het testen van de virulentie en het beschermend vermogen van een aantal kloons, (Δ*cpa* en Δ*cpb/CPB)* vond plaats in hamsters en is beschreven in hoofdstuk 4 en 5. Zowel de CPA knockouts als de CPB heterozygoten waren duidelijk minder virulent dan het wildtype. Terugplaatsen van een CPA gen had echter geen herstel van de virulentie tot gevolg, ondanks de bevestiging door middel van RT-PCR dat het gen wel werd afgelezen. Het is daarom onduidelijk waardoor het verlies aan virulentie is veroorzaakt. Uit een vaccinatie proef in hamsters met een aantal van de kloons bleek dat de knock-outs geen beschermend effect hadden (hoofdstuk 5).

Een tweede benadering voor een vaccin vormt de toepassing van recombinante cysteïne proteases van *L. infantum* in combinatie met een adjuvans. Gezien het succes van de combinatie van cysteïne protease met Interleukine-12 in muizen tegen *L. mexicana* werd een soortgelijk vaccin getest in honden. Dit experiment is beschreven in hoofdstuk 6. Het vaccin, bestaande uit recombinant cysteïne protease A en B en recombinant honden-IL-12, had geen beschermend effect.

In hamsters werd een aantal verschillende vaccins getest. Naast het recombinant cysteïne protease B of de combinatie van cysteïne protease A en B werden ook nog twee natieve antigenen getest; geautoclaveerde promastigoten en promastigoten kweeksupernatant. Van de combinatie van geautoclaveerde promastigoten met levend Bacillus Calmette-Guérin was eerder een succesvol experiment in honden beschreven. Voor de combinatie van kweeksupernatant met muramyl dipeptide geldt dit eveneens. Verschillende combinaties van bovengenoemde antigenen (cysteïne proteases, geautoclaveerde promastigoten, kweeksupernatant) met een aantal adjuvans werden daarom getest. De gebruikte adjuvantia; saponine, muramyl dipeptide of levend Bacillus Calmette-Guérin hebben allemaal een stimulerend effect op de cellulaire immuunrespons. Het resultaat van deze experimenten is beschreven in hoofdstuk 7. Toepassing van vaccins met levend BCG had een duidelijk verlagend effect op de parasietenlast van de hamsters. Helaas was in de tussendtijd duidelijk geworden dat het gebruik van levend BCG in honden een probleem is vanwege veiligheid en volksgezondheid. Deze vaccins zijn dan ook niet verder getest.

Een algemene conclusie die uit dit onderzoek kan worden getrokken is dat het ziekte verloop in honden (en hamsters) sterk afwijkt van dat in muizen modellen. Bij muizen kan in verreweg de meeste inteeltstammen specifieke beschermende immuniteit tegen *Leishmania* worden opgewekt, hetzij door een infectie, hetzij door vaccinatie. Uit onze resultaten en die van anderen komt naar voren dat de meeste honden een infectie met *Leishmania* niet kunnen overwinnen. Honden en hamsters ontwikkelen een chronische infectie die gedurende een zekere periode onder controle kan worden gehouden. Het lijkt erop dat niet-specifieke immuniteit hierbij een belangrijke rol speelt. Opvallend hierbij is dat de ontwikkeling van specifieke immuniteit (antilichamen) eigenlijk altijd vooraf gaat aan het ontstaan van klinische symptomen.
De muis is dus minder geschikt als model voor *Leishmania* bij de hond. Hamsters zijn, gezien het vergelijkbare ziekte verloop, een beter model. Bovendien is het moeilijk om resultaten van vaccinaties in de muis te 'vertalen' naar de hond. Vaccinatie experimenten in honden zijn dan ook essentieel voor evaluatie van de effectiviteit van een vaccin kandidaat. Resultaten van zulke experimenten zijn zeer waardevol, ongeacht het resultaat, omdat ze de kennis over de immuniteit en het ziekteproces vergroten en daarmee richting kunnen geven aan toekomstig onderzoek naar een vaccin voor honden tegen *Leishmania*.

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Curriculum Vitae

Jacqueline Poot werd geboren op 19 oktober 1971 te Leiden. In 1990 behaalde zij haar VWO diploma aan het Pieter Groen College te Katwijk. In datzelfde jaar werd begonnen met de studie Diergeneeskunde aan de Universiteit Utrecht die in 1998 werd afgesloten met het dierenartsexamen specialisatie landbouwhuisdieren. Binnen deze studie periode werd ook een jaar lang onderzoek gedaan in het kader van het excellent tracé, dit werd uitgevoerd op de afdeling Parasitologie en Tropische Diergeneeskunde van de faculteit Diergeneeskunde en in 1995 afgesloten met het behalen van de titel Master of Veterinary Science. Aansluitend aan het behalen van het dierenarts diploma werkte zij als dierenarts in D.A.P. Ede en D.A.P. Ouder-Amstel. Vanaf augustus 1999 is zij werkzaam als projectleider bij de afdeling Parasitology R&D van Intervet, hier werd het werk zoals beschreven in dit proefschrift uitgevoerd.