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VACCINE AGAINST CANINE VISCERAL LEISHMANIASIS

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2. TABLE OF CONTENTS

2. TABLE OF CONTENTS	2
3. Executive summary	3
4. Research objectives	4
5. Methods and Results	4
Israel	4
i. Antigens for Diagnosis and Vaccination	4
ii. Analysis of Antigens	6
iii. Alternative Adjuvants	10
Morocco	11
i. Field Work	11
ii. Laboratory Experiments	13
iii. Vaccine Field Trial	19
6. Impact, Relevance and Technology Transfer	23
7. Project Activities/Outputs	24
8. Project Productivity	25
9. Future Work	26
10. Literature Cited	26

3. EXECUTIVE SUMMARY

Canids are the major reservoir host for *Leishmania donovani infantum* and *L. d. chagasi* in many regions. These parasites cause fatal visceral leishmaniasis both in dogs and humans. The objective of this research proposal was to develop and test a vaccine for canine visceral leishmaniasis (CVL) thereby breaking the cycle of transmission from dogs to humans in the peridomestic environment. Development of a dog vaccine will be easier and involve fewer restrictions than a similar vaccine for humans. While many leishmanial antigens have been shown to protect mice against a challenge with *Leishmania*, none have been tested in dogs. The purpose of this project was to test the efficacy of three potential vaccine candidates for CVL using an experimental dog model that was established in Rabat. In addition, the immunological status of the dogs was monitored during natural infection, following recovery from infection after drug treatment and following immunization and latter challenge with an infective *L. d. infantum* isolate. Experiments in dogs immunized with gp63 or crude *L. infantum* antigen, using BCG as adjuvant, were carried out. While no protection was observed in dogs immunized with the crude antigen, those receiving gp63 were protected and showed no signs of disease for at least nine months after challenge with the parasite. Based on these results a field test of the defined vaccine was carried out in Khemisset, Morocco, a region of high parasite transmission and canine disease. A total of 30 dogs were immunized. Half of the animals received gp63 plus adjuvant and half adjuvant alone. The clinical and immunological status of the dogs was monitored for about 18 months. Only 1/15 dogs immunized with gp63 showed clinical signs of visceral leishmaniasis while 3/15 dogs immunized with adjuvant alone had signs of disease suggesting that vaccination with gp63 may be effective in protecting dogs against visceral leishmaniasis. Additional antigens, both pure and recombinant were prepared in Israel and sent to Morocco for use in these studies. Several of the recombinant proteins were also evaluated for use as diagnostic antigens. An ELISA using rHSP70 was shown to be highly specific and sensitive for both human and canine visceral leishmaniasis. Various technical skills including the purification of proteins, immunological assays and polymerase chain reaction were transferred to Morocco. The ability immunize and evaluate vaccines against canine diseases using locally produced parasite antigens was one large benefit of this project. Problems with transfer of funds and communication between Morocco and Israel over the last 16 months of this project severely slowed the research progress.

4. RESEARCH OBJECTIVES

The overall aim of this project is to reduce the incidence of human visceral leishmaniasis (VL) caused by *Leishmania donovani spp.* through development of a canine vaccine. No good drug exists to treat canine disease. It was expected that the production of an inexpensive and effective vaccine for this parasite in dogs would break the transmission cycle to humans. The original specific objectives of this proposal were:

1. To test pure (dp72, gp63), recombinant and crude vaccine candidates in a laboratory dog model in Morocco.
2. To monitor the immunological responses in vaccinated and naive dogs following a laboratory challenge with local *L. donovani infantum* isolates.
3. To optimize protocols and identify new adjuvants for use in a dog vaccine.
4. To monitor the epidemiology of canine visceral leishmaniasis (CVL) and identify a site in Morocco where a field test of a vaccine for CVL can be carried out.
5. To field test the vaccine in an endemic site where the prevalence of canine disease is high.

It is now accepted theory that the most effective approach to prevent human visceral leishmaniasis, caused by *L. infantum* or *L. chagasi,* will be through vaccination of dogs against these parasites. Few studies have been undertaken to vaccinate dogs. In recent years significant work on the immunology of canine visceral leishmaniasis has been initiated improving our understanding of this disease in dogs. This study provides important immunological and clinical parameters on vaccination against this disease using one of the major parasite antigens, gp63. It complements and extends other work in now underway on the immunology of canine visceral leishmaniasis.

5. METHODS AND RESULTS

ISRAEL

I. ANTIGENS FOR DIAGNOSIS AND VACCINATION

Recombinant Proteins: Clones containing the partial sequence of three separate genes recognized by sera from dogs with CVL were isolated from a *L. d. chagasi* expression library and subcloned into the glutathione S-transferase (GST) pGEX1 high expression vector. A summary of the clones and the genes encode by them are given in Table 1.

Table 1. Analysis of *L. d. chagasi* clones*

Clone #	Gene id	Insert size (kb)	mRNA size (kb)	Amas.	Promas.
pDog1	Kinesin	3.57	9.2	+	+
pDog2	HSP70	0.87	3.8	+	+
pDog3	HSP83	1.7	4.0	+	+

*Identification of the genes encoded for by each clones was based on homology to DNA or predicted amino acid sequence found in the various gene banks. Amas. = amastigote, Promas. = promastigote. Size of major mRNA transcript is shown.

A comparison of the nucleotide sequence of clone pDog1 (3572 bp) to DNA sequences deposited in the gene bank showed that the 5'-end of the clone had high identity with kinesins from organisms, such as *Chlamydomonas reinhardtii* (52.3% over 667 nucleotides, nt). The predicted amino acid (aa) sequence of this clone showed that the 5'-region of the clone had 23.8% identity over 252 amino acides (aa) to a kinesin from *Syncephalastrum racem.* A similar level of homology was seen with kinesins from other organisms, including *Leishmania donovani* (Genbank accessioin No. L07879; 21.9% over 237 aa). Interestingly, the 3'-region of pDog1 showed 22.6% identity over 243 aa to RNaseE from *E. coli.*

The fusion polypeptide encoded for by rHsp70 codes for about 41% of *L. d. chagasi* HSP70, including the complete COOH-terminus (HSP70c). Preliminary experiments using λ–Zap II showed that this clone is expressed at higher levels than the other pDog clones. The expression of HSP70c was optimized on a small scale by following its induction with IPTG for different periods of time and temperature. Large quantities (40-80 mg) of rHSP70c and the GST control polypeptide were expressed and the fusion polypeptide purified from the bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (Pharmacia). The pure protein eluted from the column with reduced Glutathione and was analyzed by SDS-PAGE. Polyclonal rabbit antibodies to HSP70 recognized this material. Half of each recombinant protein was dialyzed extensively against sterile RPMI-1640 medium, sterile filtered and stored at 4°C. Protein concentration was examined by the Bradford Protein assay. Cleavage of the GST leader using the protelolytic enzyme Xa was accomplished, though western blotting and ELISA using the GST leader peptide alone showed that it is not recognized by human or dog sera tested. Therefore, it is not necessary to remove the GST peptide from the fusion polypeptide in order to produce a specific diagnostic assay.

Problems were encountered in expressing the full-length polypeptides for rHsp83 and rKinesin. These were solved over the last year of the project by subcloning these genes into a different expression vector (the pDEST15, Invitrogen). Both proteins were successfully expressed as GST fusion polypeptides and purified on Glutathione Sepharose 4B. Preliminary experiments using the pure recombinant proteins showed

that both rHSP83 and rKinesin were recognized by CVL sera (data not shown).

Purified native proteins: Ascites fluid containing the monoclonal antibodies (mAb) D2 (anti-gp70) and D13 (anti-dp72) were produced in BALB/c mice. The mAb were purified on Protein A Sepharose 4B affinity resins and the purity checked by SDS-PAGE. Activity of the antibodies was tested by ELISA on crude *L. donovani* membranes. The mAbs were coupled to activated Sepharose 4B to produce affinity resins for the purification of gp70 and dp72.

The hybridoma cells producing D13 proved to be extremely unstable and it was difficult to produce enough ascites fluid containing the required mAb. Coupling of D13 to Sepharose 4B at low protein to resin ratios was tested. The capacity of these affinity resins was limited and large amounts of the parasite antigen could not be purified.

New mAb were produced to dp72 by immunizing BALB/c mice with the pure protein and fusing the immune spleen cells with a mouse NS-1 myeloma cell line. Culture supernatants from the hybridoma cells were screened both by ELISA and western blotting on dp72 previously purified by affinity chromatography on D13. Two new mAbs against dp72 were produced, 3A3-C6 and 2C6-C6. Large quantities of ascites fluid were produced for 3A3-C6 and the mAb purified on Protein A Sepharose as described above. After checking mAb purity, affinity resins were prepared on a small scale using three mAb:resin coupling ratios, 4-8 mg/ml; 8-10 mg/ml and 10-15 mg/ml. The capacity of each affinity resin was checked using a fixed amount of *L. infantum* membranes (4 mg protein) for purification of dp72. The resin prepared with 10-15 mg mAb/ml resin had the highest capacity and a large batch (10 ml) of affinity resin was prepared using this coupling ratio. Using the new affinity resin we successfully isolated 400 μg dp72 from *L. infantum* membranes in one purification. This material has been lyophilized and stored in aliquots (50 μg).

II. ANALYSIS OF ANTIGENS

Recombinant and pure:
Three proteins (gp70, dp72 and rHSP70c) were purified for use in immunological assays. Prior to lyophilization and shipping to Morocco they were tested for use in ELISA, western blotting and lymphocyte proliferation using local infected dog sera and peripheral blood lymphocytes. Problems were encountered in the stability of dp72 and it appeared to degrade upon lyophilization.

The stability of the purified rHSP70c was tested prior to shipment. Aliquots of the recombinant antigen were prepared and half were stored frozen at -20°C and half-lyophilized. After one month on the bench at room temperature the lyophilized antigen was resuspended and a

crosshatched titration of the two antigens with infected and normal dog sera carried out. No differences between the frozen and lyophilized antigen in optimal sera or antigen concentrations for use in ELISA or western blotting were noted (data not shown). The rHSP70c was also tested for use in lymphocyte proliferation. Mice were immunized either with crude *L. infantum* promastigote antigen (IPT1), rHSP70c or just the GST polypeptide from the pGEX vector. After two weeks the spleen cells were removed and used for lymphocyte proliferation with each antigen. The results for mice immunized with IPT1 are shown in Table 2. As can be seen the rHSP70c is not toxic and

Table 2. Proliferation of BALB/c mouse spleen cells with rHSP70c.

	PROLIFERATION (cpm)		
ANTIGEN*	IPT1	rHSP70c	pGEX
None	83	272	272
0.05 (1)	543	30120	1053
0.5 (5)	1315	25168	2935
5 (10	2030	21549	8939

*Concentration of antigens used for proliferation are for IPT1 - $1\ 0^5$, $5 \times 1\ 0^5$ and 10×10^5; for rHSP70 and pGEX - 0.05, 0.5 and 5 µg.

induced strong proliferation in the IPT1 immunized mice. Proliferation induced by the GST portion of the polypeptide and crude parasite antigen was much lower.

The rHSP70c polypeptide was tested by ELISA and western blotting (flexible PVC 96-well microtiter plates, Dyanatek) for use as an antigen to diagnosis human VL. Antigen concentrations from 25 to 500 ng/well were examined by crosshatched titration with VL patient and healthy control serum, and a 500 ng/well concentration was chosen to coat the plates. After optimizing the conditions for both western blotting and ELISA using rHSP70, a total of 112 human and 94 dog sera were examined by these techniques.

The ELISA on rHSP70c using human sera was highly specific (Fig. 1 and data not shown) and sensitive. Of the VL sera tested 87% (13/15) gave strong positive reactions with the antigen, > 1 O.D. A few weak false positive reactions were observed using sera from patients with cutaneous leishmaniasis (5/42) or African trypanosomiasis (2/6). No false positive reactions were observed with sera from patients with schistosomiasis (n=3), Chagas' disease (n=8), leprosy (n=4), lupus disease (n=6), toxoplasmosis (n=8), malaria (n=8) or the healthy controls (n=18).

HSP70 could also be used for diagnosis of dogs with VL (CVL) by ELISA or western blotting (Fig. 2 and data not shown). The results obtained by ELISA using rHSP70c and crude antigen is shown below. The two assays show a good correlation. Additional studies indicate that rHSP70c may be useful for early diagnosis of CVL.

Pooled CVL sera (1:100) recognized rKinesin and rHSP70c by western blotting. CVL sera did not react with the negative control GST peptide. No reaction or very weak reaction with the fusion polypeptides was seen with a pool of three normal dog sera (results not shown).

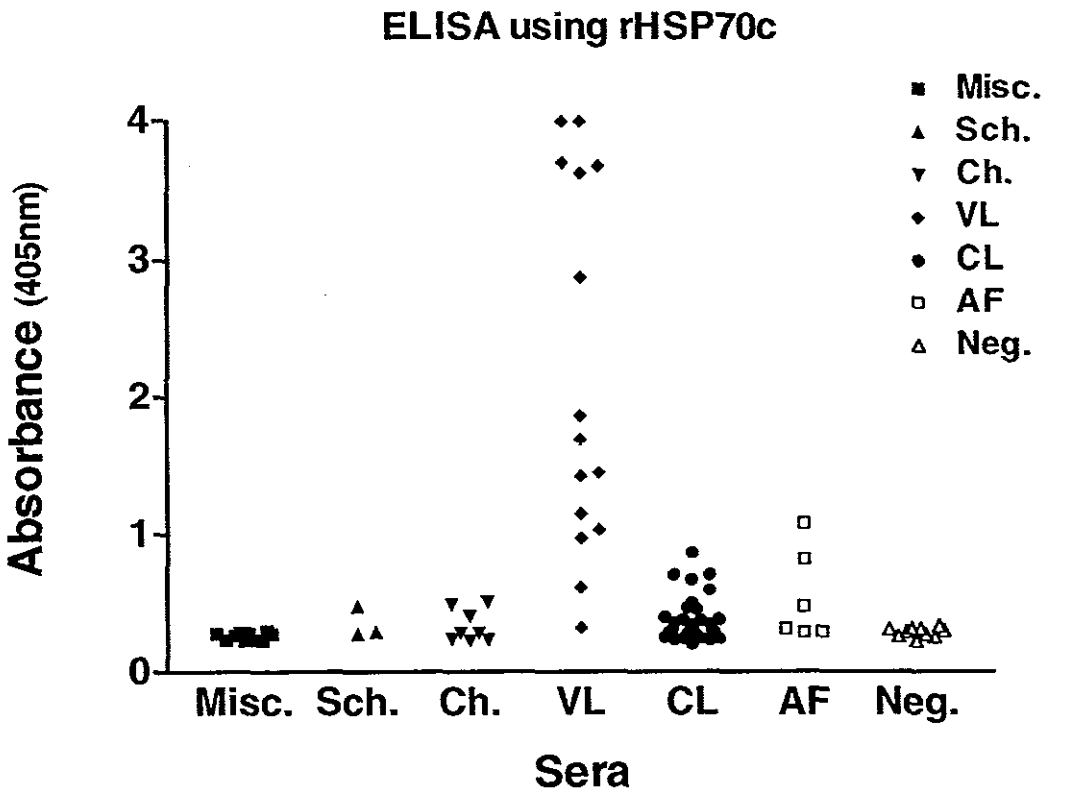


Figure 1. Diagnosis of human visceral leishmaniasis on rHSP70c. ELISA plates were coated with rHSP70c (500 ng/well) and incubated overnight with different human reference sera. Antibody binding was detected using Protein A conjugated to Horseradish Peroidase and ABTS as substrate. Sch – Schistosomiasis; Ch – Chagas' disease; VL – visceral leishmaniasis; CL – cutaneous leishmaniasis; AF – African Trypanosomes; Neg – Healthy negative control sera; Misc – Systemic Lupus Erythrotomous and Leprosy.

<u>Axenic amastigote antigen:</u> A *L. infantum* isolate from a dog in Israel with VL was obtained. It was adapted to grow at acidic pH and 37°C, conditions which induce the transformation of promastigotes to axenic amastigotes. The transformation was examined by western and northern blotting using antibodies and oligonucleotide probes, which react with stage specific antigens and genes. Antibodies to the A2 antigen, an amastigote specific protein, and to lipophosphoglycan, a promastigote specific protein were used (Turco, et al., 2001). Northern blots using total RNA isolated from the promastigotes and axenic amastigotes were hybridized with probes to *genes B and C, rKinesin* and *Lmpkac1* (Siman-Tov et al., 1996; McKean et al., 1997). These experiments (data not

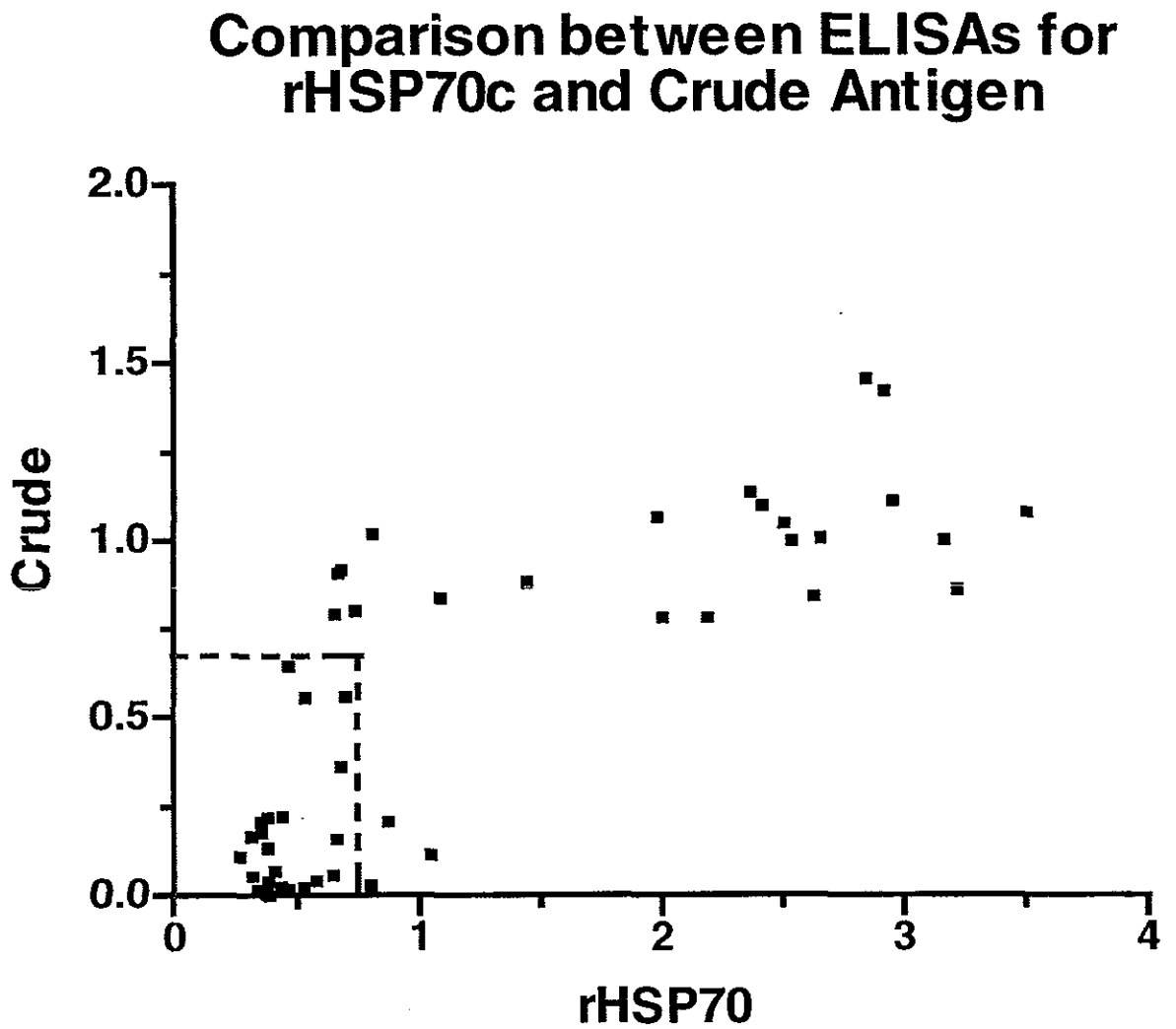


Figure 2. Comparison of ELISA results for canine visceral leishmaniasis using crude parasite antigen and rHSP70c. ELISA plates were coated with rHSP70c (500 ng/well) or crude *L. donovani* antigen (Jaffe et al., 1988) and incubated overnight with different sera from dogs from an endemic area for CVL in Israel. Antibody binding was detected using Protein A conjugated to Horseradish Peroidase and ABTS as substrate.

shown) demonstrated that the axenic amastigotes were indeed amastigote-like in their molecular and antigenic properties. Antigen was prepared from promastigotes and amastigotes of this isolate an a preliminary comparison of their antigenic properties by western blotting, ELISA and lymphocyte proliferation was carried out prior to shipment to Morocco. The proliferation results comparing the amastigote and promastigote antigens using mice immunized with crude *L. infantum* promastigote antigen (IPT1) is shown in Table 3. The best stimulation index (SI) was observed using the particulate amastigote antigen.

Table 3. Comparison of lymphocyte proliferation induced by amastigote and promastigote antigens in *L. infantum* immunized BALB/c mice.

	PROLIFERATION (SI)			
ANTIGEN (μg)	**PS***	**PP**	**AS**	**AP**
0.5	1.6	4.1	3.6	10.9
5	2.3	n.d.	2.3	13.0

*Antigens used: PS - soluble promastigote, PP - particulate promastigote, AS - soluble amastigote and AP - particulate amastigote.

III. ALTERNATIVE ADJUVANTS

Use of AS101 as adjuvant in mice: BALB/c mice were immunized freeze/thawed *Leishmania* antigen using AS101 as adjuvant. Two groups of mice were immunized, half with *Leishmania major* antigen and half with *L. donovani* antigen. Several different protocols of immunization were examined, see table 4.

Table 4. Schedule of immunization of BALB/c mice with AS101 and leishmanial antigen.

Group[a]	**Treatment**[b] **(days)**				
	-28	**-14**	**-3**	**0**	**10**
PBS	p	p	p	+	p
2X AS alone	AS	AS	p	+	p
2X AS + L	AS/L	AS/L	p	+	p
1X AS + L	AS/L	L	p	+	p
L alone	L	L	p	+	p
1X AS	AS	p	p	+	p
AS post-infection	p	p	p	+	AS
AS -3d	p	p	AS	+	p

Footnotes: a. Each group contains 5 BALB/c mice b. As, received 20 μg of AS101; L, received 2 x 10^7 killed promastigotes as antigen. The parasites were freeze/thawed 3 times and sonicated before use; p, mice were injected with phosphate buffered saline i.p.; +, mice were challenged with either 10^7 *L. donovani* or *L. major* stationary phase promastigotes.

Mice challenged with *L. donovani* promastigotes were sacrifice after 21 days. In the mice challenged with *L. major* the infected and non-infected rear foot thickness was measured weekly and the ratio of the infected/non-infected thickness calculated. Immunization with crude leishmanial antigen alone did not result in protection against a challenge by *L. major* promastigotes. Instead lesion development was more rapid in these mice (Leish alone) than the PBS control animals. Interestingly, when AS101 was included as an adjuvant together with the parasite antigen

(A101 + Leish), disease progression was identical to the mice given antigen alone. No difference in disease development was seen whether one or two injections of adjuvant plus antigen were given. Likewise, the effect of AS101 alone on disease progression was investigated since this compound has been successful used to treating certain types of cancers. However, when AS101 was given, either immediately before or after parasite infection, no effect on disease progression was noted (results not shown). .

Analysis of results from the *L. donovani* challenge experiment showed that AS101 was not effective either as an adjuvant for vaccination or directly in treatment post-infection. No difference liver parasite burden was seen for mice immunized with AS101 plus crude parasite antigen and control mice receiving either AS101 or antigen alone (results not shown). Vaccine studies using this material as an adjuvant were discontinued.

MOROCCO

I. FIELD WORK.

Survey: Visits were made to the Khemisset region in order to establish contacts with the local authorities and explain the aims and objectives of the project. Agreements to provide facilities and assistance were established with local health and agricultural departments. Work continued all during this project on the epidemiology of CVL in this region and contact was made with farmers in the chosen localities were the vaccine trial was planned. Farmers were recruited as animal keepers.

Initially, work was carried out at six locations in the Khemisset foci. Four hundred and twenty one stray and pet dogs were examined for clinical signs of leishmaniasis. Blood was collected from 375 dogs and tested by ELISA at a 1/100 dilution. Seroprevalence varied from 3 to 26% in the six locations. The highest seropositivity was found in Sid Elghndour (26%). Based on these initial studies the number of potential vaccine trial sites was narrowed down to three locations. An additional 175 sera in these 3 foci were collected and analyzed for anti-leishmanial antibodies. Active transmission was confirmed by the detection of new seropositive cases in animals previously diagnosed as clinically healthy and serologically negative. Seroprevalence in Sid Elghndour increased from 26 to 29.7% in the first 6 months of 1998 and showed that there is continuous and active transmission of the disease in this focus. Sampling was increased in order to understand better the evolution of the disease in this focus and during the second half of 1998 a total of 367 dogs were examined by indirect immunoflourescence and ELISA. Of these dogs 91 were seropositive and 276 seronegative. A *L. infantum* strain was isolated from a dog suffering from a severe VL in Sid Elghndour locality for use in vaccine studies.

The disease progression was examined in the seropositive dogs in order to develop a scoring system for the different disease stages. This system was used to classify clinical disease during the vaccine experiments. ELISA technique and western blotting were used to follow antibody production and characterize the parasite antigens recognized by the dog sera at different stages of the disease. Clinical status was scored using the following parameters: 1) general status (emaciation and onychogryposis); 2) skin lesions (alopecia, dermatitis, dandruff production, ulcers); 3) ocular lesions (conjunctivitis, blue keratitis, blindness); 4) digestive signs (diarrhea) and 5) visceral signs (lymphadenopathy, spleenohepatomegaly). These results are summarized in Table 5.

Table 5. Pattern of disease in clinically and serologically positive dogs.

Clinical signs	% dogs	Clinical signs	% dogs
General Status		**Skin Lesions**	
Thinness	86.6	Alopecia	20
Illness	60	Dermatitis	46.6
Emaciation	73.3	Dandruff production	33.3
Onychogryposis	100	Ulcers	33.3
Digestive signs	20	**Ocular lesions**	33.3
Visceral signs		Conjunctivitis	33.3
Lymphadenopathy	93.3	Blue keratitis	0.6
Splenohepatomegaly	0.6%	Blindness	0.0
Nervous signs			
Paralysis of limbs	0.6%		

After death two dogs were necropsied and the histological changes investigated. Lesions were recorded and portions of the lymph nodes, liver, spleen, kidney and lungs were fixed in 10% neutral formalin. Tissue blocks were embedded in paraffin and sectioned at 5 µ thickness. The slides were stained with hematoxylin and eosin. The results are as follows:

Lymph nodes:

- Severe infiltration by mononuclear cells.
- Hyperplasia of macrophage cells with a high number of amastigotes in the cytoplasm.

Spleen:

- Severe infiltration by mononuclear cells.
- Hyperplasia of macrophages with a high number of amastigotes in the cytoplasm.

Kidney:

- Interstitial nephritis.
- Chronic glomerulonephritis.

Liver:

- Severe infiltration by mononuclear cells.

Lungs:

- Infiltration of alveoli by polynuclear cells.
- Edema and thickness of alveolar septa.

Western blot analysis: Sera from naturally infected sick dogs were found to react with antigens of different molecular weights ranging from 10 to 116 kDa. At least 23 antigens were recognized. This diversity is probably the due to the overproduction of antibodies during acute canine leishmaniasis. All of the sick dogs had antibodies against gp63 and gp70.

Sera from asymptomatic cases reacted with fewer antigens, 5-9 antigens depending on the dogs examined. The protein gp63 was not recognized by any of these animals.

Analysis of cell mediated responses: The cell proliferation of naturally infected sick dogs was almost completely inhibited. This lack of cell mediated activity was verified with crude promastigote antigen, gp63 and Concanavalin A (data not shown and Table 7). These results showed evidence of immune suppression and explain the invasive nature of this disease. Interestingly, all six naturally infected asymptomatic dogs examined showed strong proliferative responses, especially to gp63.

In conclusion, our investigations in this focus suggest that sick dogs exhibit multiple clinical signs that vary from dog to dog. However, the following signs: onychogryposis, thinness/emaciation and adenopathy were frequently observed. Sick dogs produce high levels of anti-parasite antibodies, but did not exhibit a cellular response to parasite antigen, including gp63. These parameters were used to score experimental infections in the canine leishmaniasis model and in future field studies.

II. LABORATORY EXPERIMENTS

Immune responses before and after drug treatment:

Dogs (n=10) were infected with amastigotes (i.v. 105 parasites/kg body weight) and clinical symptoms monitored. Within 20 weeks all the infected dogs were polysymptomatic. Five dogs were treated with Lomidine (4 mg/kg body weight), five were used as controls. In addition, seven other dogs were also examined and included: two asymptomatic dogs, two healthy *Leishmania* free dogs and three naturally infected dogs

Table 6.

Change in anti-*leishmanial* antibodies following treatment with pentamidine

Dogs[a]	Before treatment	After treatment (one month)	After treatment (six months)
Ext 1	0.98	0.90	0.31
Ext 2	0.87	0.86	0.27
Ext 3	1.01	1.00	0.55
Ext 4	1.11	1.10	0.22
Ext 5	0.91	0.91	0.30
Exu 6	0.91	0.90	0.92
Exu 7	1.01	1.08	died
Exu 8	0.89	0.88	0.95
Exu 9	0.92	0.90	0.98
Exu 10	1.11	1.12	died
Nat 11	1.32	1.30	0.55
Nat 12	1.51	1.00	0.55
Nat 13	1.22	0.91	0.42
Asy 1	0.12	0.11	0.10
Asy 2	0.09	0.09	0.08
Control 1	0.05	0.05	0.05
Control 2	0.04	0.04	0.05

[a] Serum antibodies were measured at 1 : 1000 dilution on crude *L. infantum* antigen (Khemisset strain) and the absorbances 405 nm measured. Group 1: Ext 1 to Ext 5 — experimentally infected treated dogs. Group 2: Exu 6 to Exu 10 — untreated sick dogs (experimentally infected). Group 3: Nat 11 to Nat 13 — naturally infected treated dogs. Group 4: Asy 1 and Asy 2 — asymptomatic dogs. Group 5: Control 1 and Control 2 — dogs free of leishmaniasis

brought from Khemisset region. The naturally infected dogs were also treated with Lomidine. The dogs were tested for humoral and cell mediated responses before and after treatment. All of the treated dogs showed marked clinical improvement and were clinically healthy and parasitologically negative by the end of the study (Rhalem et al., 1999).

All clinically sick dogs had high anti-parasite antibodies prior to treatment. The titer of antibodies decreased gradually to low levels by six months after treatment, while the level remained high in the six control dogs (Fig. 6). The antigen specific lymphocyte proliferation was also measured before and after drug treatment. In the experimentally infected dogs it was also examined in the prepatent phase of the infection. Experimentally infected dogs showed good proliferation during the prepatent phase of the infection as compared to the control *Leishmania* free animals (Table. 7). Peripheral blood mononuclear cells (PMBC) proliferated well to both crude leishmanial antigen and gp63. However, when these dogs entered the patent stage of disease a dramatic suppression of parasite specific proliferative responses was observed. Six months following drug treatment the dogs were no longer immunosuppressed and their PMBCs proliferated strongly to leishmanial antigens (Table 7).

Table 7

Proliferative response of PBMC (OD at 405 nm)

Dogs[a]	Prepatency phase[b]			Patency phase			After treatment		
	crude Ag 1 µg	gp63 0.5 µg	Con A 2.5 µg	crude Ag 1 µg	gp63 0.5 µg	Con A 2.5 µg	crude Ag 1 µg	gp63 0.5 µg	Con A 2.5 µg
Ext 1	0.24	0.38	0.65	0.09	0.13	0.42	0.48	0.68	0.63
Ext 2	0.19	0.27	0.55	0.07	0.12	0.31	0.34	0.53	0.58
Ext 3	0.20	0.31	0.68	0.10	0.11	0.48	0.44	0.61	0.70
Ext 4	0.22	0.28	0.55	0.08	0.09	0.40	0.45	0.57	0.61
Ext 5	0.21	0.38	0.55	0.09	0.09	0.55	0.48	0.58	0.60
Exu 6	0.22	0.28	0.60	0.10	0.11	0.59	0.10	0.13	0.58
Exu 7	0.30	0.38	0.58	0.08	0.09	0.58	died		
Exu 8	0.28	0.30	0.55	0.08	0.08	0.55	0.07	0.08	0.62
Exu 9	0.29	0.30	0.58	0.08	0.08	0.58	0.08	0.08	0.53
Exu 10	0.23	0.29	0.63	0.08	0.09	0.60	died		
Nat 11	ND	ND	ND	0.12	0.13	0.56	0.38	0.41	0.61
Nat 12	ND	ND	ND	0.06	0.09	0.48	0.41	0.49	0.48
Nat 13	ND	ND	ND	0.10	0.09	0.56	0.39	0.41	0.56
Asy 1	ND	ND	ND	0.63	0.82	0.68	0.68	0.82	0.65
Asy 2	ND	ND	ND	0.58	0.67	0.81	0.58	0.71	0.80
Control 1	0.05	0.07	0.51	0.05	0.06	0.61	0.05	0.05	0.51
Control 2	0.06	0.09	0.58	0.05	0.05	0.61	0.06	0.05	0.58

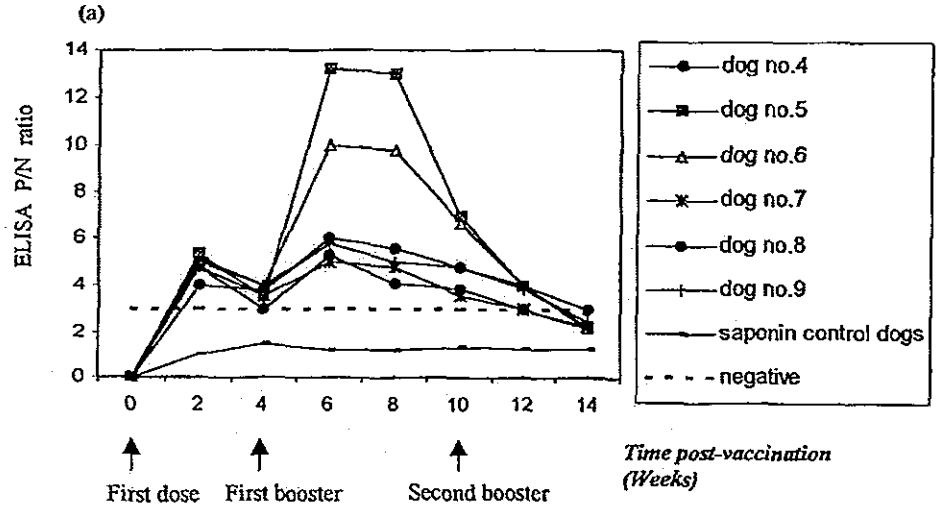
[a] Group 1: Ext 1 to Ext 5 — experimentally infected treated dogs. Group 2: Exu 6 to Exu 10 — untreated sick dogs (experimentally infected). Group 3: Nat 11 to Nat 13: — naturally infected treated dogs. Group 4: Asy 1 and Asy 2 — asymptomatic dogs. Group 5: Control 1 and Control 2 — dogs free of leishmaniasis.
[b] ND, not done.

Vaccination with crude antigen:

The immune responses of dogs vaccinated with autoclaved *Leishmania major* promastigotes (ALM) were investigated using two different adjuvants, saponin and BCG. ALM was kindly provided by the WHO TDR programme. ALM was initially tested in these studies because several human vaccine trials against cutaneous and visceral leishmaniasis with this antigen were underway at this time in Iran and the Sudan, respectively. Twelve dogs were divided into two groups of six. One group received three injections of ALM + BCG and the second group received three parallel injections of ALM + saponin. An additional two groups of control dogs, three each group, only received BCG or saponin. Each dog was sampled and sera were collected for immunological studies at approximately 15-day intervals, up to 10 months. The results are shown below in Figure 3. Interestingly dogs immunized with ALM + saponin showed high anti-parasite antibody titers (Fig. 3a). These titers peaked approximately 2 – 4 months after the first boost (second injection) and gradually returned to normal by 14 months, even though the dogs were give a second booster injection after 10 months. If instead the dogs were immunized with the ALM + BCG mixture poor antibody responses were seen barely reaching three times background (Fig. 3b). Western blotting using sera collected from the two groups gave a similar pattern of

reaction (26 -108 kDa) with the parasite antigen, even though the titers were different (Lasri et al., 1999). Interestingly, the lymphocyte proliferative responses in the two groups of dogs were exactly the opposite the antibody responses. The dogs receiving the ALM + BCG mixture showed good proliferative responses, while those immunized with ALM + saponin were no different than those seen using cells from naïve or control animals (Lasri et al., 1999).

Protection of dogs immunized with crude *L. infantum* promastigotes (CPA) using BCG as an adjuvant was investigated using a experimental model for canine visceral leishmaniasis (CVL). Each dog received two subcutaneous injections (20 μg CPA + BCG or BCG alone) at 4 week intervals. One more booster than in the studies described above. Immunized animals were challenged with 10^5 amastigotes / kg body weight. Results obtained approximately one year after infection show that this immunization protocol was not effective. Out of the six dogs immunized with CPA + BCG 83% showed clinical signs of disease. Parasites were isolated from the spleen of all the vaccinated dogs. Antibody titers and patterns of reactivity by Western bloting using immune sera following infection were similar to those seen in the control animals that only received BCG and in naturally infected dogs identified in the field. The cellular response that was seen following the final boost with CPA + BCG was lost 25 weeks following challenge with parasites. The following symptoms and lesions were observed in a majority



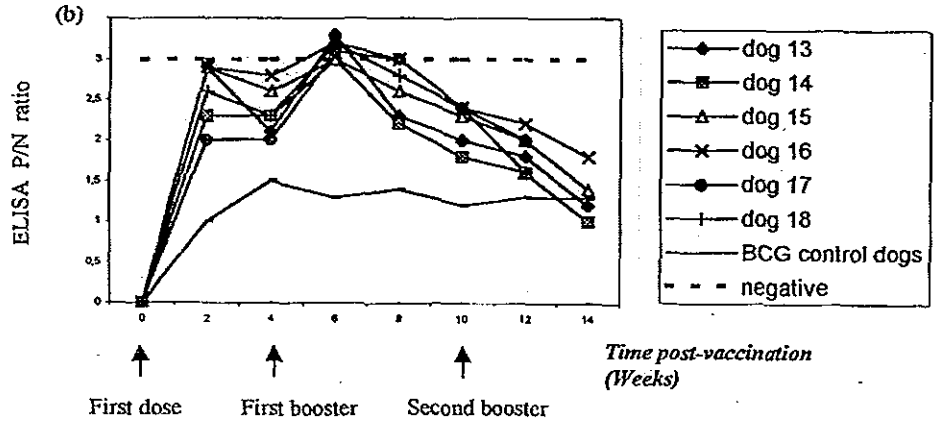


Figure 3. Evolution of anti-leishmanial antibodies in vaccinated dogs. At each time point indicated, a sample of peripheral blood was taken to measure anti-parasite antibodies by ELISA. Postive/negative (P/N) serum ratios >3 were considered positive. a) Dogs vaccinated with ALM plus saponin; b) dogs vaccinated with ALM plus BCG.

of the infected dogs: thinness, emaciation, onychogryphosis, conjunctivitis, dandruff and hepatospleenomegaly. Dogs immunized with only adjuvant, BCG, show varied signs of clinical leishmaniasis and loss of cellular immune responses to leishmanial antigens. Therefore vaccination with CPA + BCG does not appear to provide protection against CVL.

Vaccination with gp63: Immune responses of dogs vaccinated with pure gp63 were compared to several other groups of animals (Table 8). BCG was used as adjuvant. Interestingly, the dogs immunized with gp63 and BCG showed no antibodies to the antigen, but instead very strong cellular responses. The immune responses of these dogs are similar to those observed in following pentamidine treatment (Lomidine) and clinical recovery of dogs with CVL.

These dogs were challenged with 10^5 amastigotes / kg body weight (i.v.) and their clinical and immunological status monitored. Nine months after challenge no clinical signs typical of CVL were noted in the immunized group as compared with the control group that only received BCG. All of the dogs in the control group showed signs of CVL and two of these dogs died
within 28 weeks after parasite challenge. These results are summarized in Tables 9 and 10.

Table 8. Immune responses of dogs to gp63.

	ELISA 1/100	ELISA 1/1000	Western blot	Cellular response
Sick dogs*	+2	+2	+4	-
Asympt.	+2	+2	+	+
Drug Treat	+2	-	+	+4
gp63 + BCG	-	-	-	+4
gp63 + Saponin	+2	-	+2	+
Gp63 alone	+2	-	+	-

*Sick dogs, dogs from Sid Elghandour with clinical infections; Asympt., asmyptomatic dogs; Drug treat, clinically cured dogs following drug treatment.

Table 9. Change in anti-leishmanial antibodies in dogs immunized with gp63.

DOG No.	BEFORE-INF.*	POST-INFECTION	
		3 months	9 months
Gp63 + BCG			
1	0.159	0.459	0.319
2	0.213	0.856	0.978
3	0.089	0.389	0.514
4	0.158	0.512	0.323
5	0.203	0.374	0.450
6	0.178	0.411	0.727
BCG alone			
7	0.072	1.402	Died
8	0.054	0.785	0.988
9	0.022	0.622	1.089
10	0.083	1.125	1.155
11	0.073	1.300	Died
12	0.051	0.845	0.985
Dogs with other clinical status			
Sick^	1.227	1.515	1.429
Asymptomatic	0.120	0.118	0.108
Treated	0.092	0.093	0.087

*Serum antibodies were measured by enzyme-linked immunosorbent assay (ELISA) at a 1/1000 dilution on gp63 and the absorbance at 405nm determined. *Before-inf., before infection.

These results show that immunization of the dogs with gp63 + BCG provides good protection and based on this a field trial using this procedure was initiated in Sid Elghandour.

Immune response to recombinant rHSP70c: This protein produced in the Israeli lab was sent to Morocco. The immune responses of infected and immunized dogs to rHSP70c were examined. All of the dogs immunized with either CPA + BCG, ALM + BCG or ALM + saponin as adjuvant reacted weakly with this protein. Sick dogs suffering from CVL also showed a lymphoproliferative response to rHSP70c. However, the proliferative response to this protein was much higher using PMBC obtained from asymptomatic dogs or dogs recovering from CVL following drug therapy.

Table 10. Proliferative response of peripheral blood lymphocytes in dogs vaccinated with gp63.

	PREPATENCY PHASE 3 mon. post-infection			PATENCY PHASE 9 mon. post-infection		
DOG	Crude*	Gp63	ConA	Crude	Gp63	ConA
gp63 + BCG						
1	0.445	0.838	0.651	0.565	0.621	0.680
2	0.298	0.575	0.555	0.449	0.739	0.638
3	0.340	0.633	0.689	0.601	0.657	0.831
4	0.286	0.555	0.554	0.438	0.584	0.618
5	0.453	0.780	0.550	0.480	0.580	0.600
6	0.533	0.628	0.600	0.722	0.712	0.815
BCG Alone						
7	0.150	0.380	0.587	Died		
8	0.281	0.247	0.556	0.222	0.180	0.540
9	0.312	0.198	0.587	0.200	0.189	0.330
10	0.338	0.290	0.634	0.182	0.238	0.444
11	0.359	0.433	0.517	Died		
12	0.402	0.417	0.462	0.146	0.177	0.342
Dogs with other clinical status						
Sick^	0.124	0.188	0.442	0.122	0.134	0.419
Asymp	0.570	0.877	0.685	0.590	0.783	0.704
Drug	0.639	0.798	0.568	0.664	0.773	0.769

Lymphocyte proliferation was measured using a commercial ELISA kit obtained from Boeringher-Mannheim for measurement ofbromodeoxyuridine incorporation into proliferating cells. *Crude Ag - crude antigen, 1 µg; gp63, 0.5 µg and ConA -concanavalin A, 2.5 µg.

III. VACCINE FIELD TRIAL

Thirty dogs were chosen for inclusion in a field trial of the gp63 vaccine and their owner's permission was obtained. The dogs were divided into two groups, a) Group 1 containing 15 dogs vaccinated with gp63 + BCG, and b) Group 2 containing 15 dogs vaccinated with BCG alone. The first immunization was carried out in December 1998. The dogs received booster injections in January 1999 and then again during March 1999. Dogs were placed at chosen farms in Sid Elghandour, Khemisset region as described previously in the project. The course of infection was followed at least for 18 months. Clinical and histological change in infected dogs was monitored. This study also allowed us to follow the clinical and pathological evolution of canine leishmaniasis using naïve dogs introduced into an endemic site. The parasitological and immunological status of the dogs was also monitored. The presence of the *Leishmania* was confirmed by blood smears and/or culture of biological material

collected from spleen or lymph nodes of the vaccinated dogs. All of the results are summarized in Table 11 and 12. Data were analyzed by the Student's t-test.

All of the dogs vaccinated with gp63 + BCG stilled showed good lymphocyte proliferation and a weak humoral response to this protein three months after the last boost (Tables 13 and 14). Minor changes in these parameters could be noted at 6 months post-vaccination and marked differences were apparent at 15 months post-vaccination. As found in the experimental dog model (Table 10) the cell mediated response to this immunization procedure is much more pronounced that the humoral response. After 15 months 4/15 of the vaccinated dogs showed a distinct rise in the antibody titers to gp63. Of these dogs 3/4 showed a decrease or loss of lymphocyte proliferation to gp63 and parasites were cultured from two of these dogs.

Among the 15 vaccinated dogs only one animal showed symptoms related to leishmaniasis (6%). The main histological changes recorded in this dog consisted mainly of a widespread infiltration by mononuclear cells and hyperplasia of lymph nodes, spleen and liver and the cellular immune response was abolished against gp63. The finding of disease in only 1/15 of the vaccinated dogs is a significant result when compared to control dogs (inoculated only with BCG) where 3/15 dogs showed clinical symptoms (20%).

Serological data also revealed significant differences between the two groups. Even though both groups showed a similar percentages of seroconversion when measured by ELISA on crude antigen, vaccinated (4/15, 26%) as compared to the control (5/15, 33.3%) group, there was an obvious difference between these dogs regarding serum reactions with both gp63 and rK39. All five of the dogs with CVL in the control group (BCG alone) had antibodies to both pure antigens. These dogs were also parasite positive (4/5) and showed very weak proliferative responses to gp63. On the other hand, only 2/5 dogs in the gp63 vaccinated group had antibodies to rK39 and only 1/4 had antibodies to gp63. This suggests that the presence of serum antibodies and strength of reaction against rK39 or gp63 is indicative of disease development and perhaps the loss of immunity. The increase of antibody production to crude antigens is the consequence of introduction of antigenic material following natural infection.

During the last 3 years Morocco and the Khemisset region is in the midst of a serious drought. This climatic situation may have strongly influenced sand fly vector distribution and perhaps explains the decrease in the number of symptomatic cases during the course of our field trial.

Based on serological data on crude antigen there doesn't appear to be a significant difference between vaccinated dogs and control animals. However, if clinical symptoms or antibodies to rK39 and gp63 are used to measure protection, this data suggests vaccination with gp63 + BCG imparts partial protection under field conditions. Further optimization of

the conditions used for immunization and perhaps the addition of a booster injection at yearly intervals may increase the level of protection observed.

Further monitoring of the dogs vaccinated, as part of this field study, was hampered by funding problems over the last 16 months. During this period funds earmarked for Rabat were not transferred or received by the Moroccan investigators.

Table 11. Summary of results in dogs showing natural *Leishmania* infection

Dogs	Sympt*	Parasites	Serology on*			Prolif.
			crude Pro's	gp63	rK39	gp63
Vaccine						
Kh18	-	+	+	-	++	+
Tem04	-	-	+	-	-	+
Ghn2	+++	++	+++	+++	+++	-
Skh4	-	-	+	-	-	++
Control						
Skh 17	++	++	+++	++	++	-
Tem31	-	-	++	+	+	+
Tem24	+++	++	++++	++++	+++	-
Rab05	-	+	++++	+	++	+
Skh11	+++	+++	+++	++++	++	-

*Sympt – symptoms; parasites – cultures positive for parasite growth, Serology – ELISA on crude Pro's (promastigote antigen), gp63 or rK39 and prolif. – lymphocyte proliferation to pure gp63.

Table 12. Clinical pattern of the disease

Dogs.	Weight loss	Symptoms*
Vaccinated Group		
Ghn2	52	Em, On, Di, Dp, Ai, Sp, Ud, He, Ts
Control Group		
Skh17	52	Em, On, Ad, Dp, Ai, Sp, Ud, He, Ts
Tem24	63	Em, On, Co, Dp, Sp, Ud, He, Ts
Skh11	37	Em, On, Co, Ad, Sp, He, Ts

*Symptoms: Em, emaciation; On, onychogryphosis; Dp, dandruff production; Co, conjunctivitis; Ud, ulcerous dermatitis; Ts, thinness, Ai, alopecia; Sp, splenomegaly; He, hepatomegal

Table 13. Humoral responses of dogs vaccinated with Gp63/BCG

Dogs	Time post- immunization (months)		
	3	6	15
Kh03	0.299	0.214	0.180
Kh11	0.258	0.310	0.171
Kh18!o	0.362	0.633	**0.732**
Tif05	0.377	0.451	0.222
Tif01	0.278	0.301	0.292
Tem04o	0.411	0.588	**0.918**
Tem7	0.348	0.412	0.144
Ra109	0.277	0.512	0.266
Sal12	0.319	ND	ND
Sal11	0.282	0.355	0.214
Ghn23	0.350	0.461	0.150
Ghn2*!o	0.286	0.558	**1.425**
Skh4o	0.344	0.464	**1.114**
Skh16	0.297	0.291	0.168
Skh07	0.266	0.230	0.146

ELISA on pure gp63; *symptomatic;

°seropositive or !culture parasite positive.

Table 14. Cellular immune response in dogs vaccinated with gp63/BCG+

Dogs	Time post- immunization (months)		
	3	6	15
Kh03	0.744	0.711	0.318
Kh11	0.551	0.601	0.404
Kh18[!o]	0.507	0.464	**0.182**
Tif05	0.341	0.558	0.219
Tif01	0.525	0.602	0.277
Tem04[o]	0.575	0.270	**0.198**
Tem7	0.460	0.642	0.141
Ra109	0.344	0.412	0.271
Sal12	0.596	ND	ND
Sal11	0.513	0.436	0.209
Ghn23	0.302	0.385	0.177
Ghn2[*!o]	0.444	0.287	**0.071**
Skh4[o]	0.390	0.315	**0.322**
Skh16	0.221	0.326	0.169
Skh07	0.465	0.505	0.143

+Incubation of *PBMLs* with pure gp63;
[*]symptomatic; [o]seropositive or [!]culture parasite positive.

6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

Scientific Impact of Collaboration - Israeli scientists have been involved in the purification and characterization of new parasite antigens for use in immunological and vaccine studies. Many of the purification and diagnostic techniques developed in Israel were transferred to Morocco where they have been implemented and used in this project. Following initial characterization of the toxicity and properties of the antigens they were sent to Morocco for inclusion in the studies on the dog CVL model. This model doesn't exist in Israel. The Moroccan group has carried out all of the preliminary vaccine and epidemiological studies, as well as a field trial using the gp63/BCG vaccine for CVL.

Project Impact - Results from the preliminary laboratory vaccine study in the experimental dog model for CVL look very promising. This vaccine based on gp63 + BCG as adjuvant was implemented in a field study carried out in an endemic site for CVL in Morocco. This study suggests that gp63 may be useful in a vaccine against CVL. A vaccine for this disease can be used in all countries where CVL is a problem.

Strengthening of Developing Country Institute - Several Ph.D. candidates received training in the Moroccan laboratory as part of this project and they include: El Bed Ana-Igor, Lasri Saadia and Natami Amaleare. One student, El Bed Ana-Igor, visited the Israeli lab in1999 for additional training in polymerase chain reaction for leishmanial species characterization and parasite diagnosis. Several times the Israel group has ordered reagents from scientific supply companies that don't have representatives in Morocco and sent them to Morocco by express airfreight to facilitate and speed up receipt of material needed for the project.

7. PROJECT ACTIVITIES/OUTPUTS

Visits

A Ph.D. student from Morocco, Anas Igor Elbied, visited the Israeli laboratory in 1999 for training in polymerase chain reaction for use in diagnosis and characterization of *Leishmania.*

Workshops

i. Leishmaniasis and tools for diagnosis. Workshop for Post graduate student. March, 2001.

ii. The first workshop for regional veterinarians and technicians on the Epidemiology and Diagnosis of Canine Leishmaniasis. June, 2001.

Original Publications

i. Rhalem A, Sahibi H, Lasri S, Jaffe CL. Analysis of immune responses in dogs with canine visceral leishmaniasis before, and after, drug treatment. Vet Immunol Immunopathol. 1999 Oct 1;71(1):69-76.

ii. Lasri S, Sahibi H, Sadak A, Jaffe CL, Rhalem A.
Immune responses in vaccinated dogs with autoclaved Leishmania major promastigotes. Vet Res. 1999 Sep-Oct;30(5):441-9.

iii. Rhalem A, Sahibi H, Guessous-Idrissi N, Lasri S, Natami A, Riyad M, Berrag B. Immune response against Leishmania antigens in dogs naturally and experimentally infected with Leishmania infantum. Vet Parasitol. 1999 Mar 1;81(3):173-84.

iv. Natami A, Sahibi H, Lasri S, Boudouma M, Guessouss-Idrissi N, Rhalem A. Serological, clinical and histopathological changes in naturally infected dogs with *Leishmania infantum* in the Khemisset Province, Morocco. Vet Res. 2000 May-June;31(3):355-63.

v. Abdelkebir RHALEM et Hamid SAHIBI. Les leishmanioses : Etat de

connaissances actuelles et perspectives de développement de vaccins. Animalis, Vol(1) : 53-57., 2001.

vi. Hamid SAHIBI, Amale NATAMI, Saadia LASRI, Ahmed Moufaid, Charles JAFFE & Abdelkebir RHALEM. Clinical and Sero-epidemiological Study of Canine Leishmaniasis in Northern Morocco. Actes. Vol (21) 4: 65-71., 2001.

vii. Saadia LASRI, Hamid SAHIBI, Charles Jaffe and Abdelkebir RHALEM. Western Blot analysis of *Leishmania infantum* antigens using sera from Pentamidine treated dogs. Submitted to Veterinary Immunology and Immunopathology. Oct 2001.

viii. Hamid SAHIBI, Saadia LASRI, Ahmed Moufaid, Charles Jaffe and Abdelkebir RHALEM. Immune response against Gp63 antigen in infected dogs with *Leishmania infantum.* Inpreparation.

Theses

i. **El Bed Ana-Igor**. Etude du pouvoir immunogen d'un vaccin inactive anti-leishmanien chez le chien. Dec. 1998. These de doctorat Veterinaire, pp. 1-94. Institut Agronomique et Veterinaire Hassan II-Rabat, Maroc.

ii. **Natami Amale.** Leishmaniose Canine: Contribution à l'étude sero-epidémiologique dans le Nord du Maroc. Faculté des sciences, Université Hassan II, Casablanca. PhD, 9 May 2000.

iii. **Lasri Saadia**. Leishmaniose Canine: Profil de la réponse immunitaire dans le cas de l'infection et de l'administration d'immunogènes. Faculté des Sciences, Université Mohamed V, Rabat, PhD. 9 May 2000.

8. PROJECT PRODUCTIVITY

On the whole this project progressed essentially as described in the time schedule of the contract. This occurred, even considering that problems existed to a greater or lesser extent during the project in transferring funds to Morocco. Except for the following goal, testing the live recombinant BCG vectors (BCG/pMV261::gp63 and BCG/pMV261) in the CVL model, the objectives described in the original proposal were completed. We were unable to acquire the recombinant BCG vectors even though we received a letter promising us access to the vectors prior to the project. Plans for these experiments were discontinued at an early stage and new experiments were added.

The following objectives described in the time schedule of work were completed. Testing of AS101 for use as an adjuvant. The properties of

this compound were examined in mouse models using crude antigens. No protection was observed and further studies discontinued. The antigens, dp72, gp63, rHSP70, crude promastigotes and amastigotes, were purified or produced by recombinant technology and used into the project either for vaccine or immunological studies with CVL. A field site to test the gp63 vaccine was identified and vaccination of dogs in this site was carried out. The immunological parameters characteristic of protective immunity were analyzed. This research utilized an experimental dog model for visceral leishmaniasis (CVL) and several vaccine candidates were tested during the project. Additional vaccine candidates and antigens were prepared by the Israeli group and supplied to the Moroccans during the course of these studies. In parallel the Moroccan group acquired the technical knowledge for the production of native antigens or recombinant polypeptides.

9. FUTURE WORK

Due to the recurrent Palestinian – Israeli problems there has been a slow down in overt co-operation between Israel and Morocco. However, the two groups are still in e-mail contact and will try to obtain alternative funding to continue this project once the political situation allows this. We still have several co-authored publications to prepare and submit.

10. LITERATURE CITED

Jaffe CL, Keren E, Nahary O, Rachamim N and Schnur L (1988) Trans. Royal Soc. Trop. Med. Hyg. 82: 852-853.

Lasri S, Sahibi H, Sadak A, Jaffe CL and Rhalem A (1999) Vet. Res. 30: 441-449.

McKean PG, Delahay R, Pimenta PF and Smith DF (1997) Mol. Biochem. Parasitol. 85: 221-231

Turco SJ, Spath GF and Beverley SM (2001) Trends Parasitol. 17: 223-226.

Rhalem A, Sahibi H, Lasri S and Jaffe CL (1999) Vet. Immunol. Immunopahthol. 71: 69-76.

Siman-Tov MM, Aly R, Shapira M and Jaffe CL (1996) Mol. Biochem. Parasitol. 77: 201-215.