

## **ANNUAL REPORT**

Covering Period 01/01/98 to 12/31/98

Submitted to the U S Agency for International Development, Bureau  
for Global Programs, Field Support and Research, Center for  
Economic Growth

### **VACCINE AGAINST CANINE VISCERAL LEISHMANIASIS**

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Project No C16-026

**Grant Number TA-MOU-96-C16-026**

Grant Project Officer William Crane

Project Duration January 1, 1997 to December 31, 1999

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## Executive Summary

Dogs are the major reservoir host in many regions for *Leishmania donovani infantum* and *L d chagasi* that cause fatal visceral leishmaniasis. The objective of this research proposal is to develop vaccine for canine leishmaniasis and break the cycle of transmission to humans in the peridomestic environment. Development of a dog vaccine will be easier and involve fewer restrictions than a similar vaccine for humans. The efficacy of three potential vaccine candidates: dp72, gp63 and crude parasite extracts, in protecting against canine visceral leishmaniasis will be examined using different adjuvants. The immunological status of the dogs will be monitored following immunization and challenge with infective *L d infantum* isolates. Antigens preventing or delaying the onset of disease will be the focus of further studies to optimize protocols. A field test of the vaccine will be carried out in Khemisset, Morocco, a region of high parasite transmission and canine disease. Over the past year protection experiments in dogs immunized with gp63 and crude *L infantum* antigen, using BCG as adjuvant, were carried out. No protection was observed in dogs immunized with the crude antigen, but dogs receiving gp63 show no signs of disease nine months after challenge with the parasite. A field test of the gp63 vaccine in dogs has been initiated. Additional antigens were prepared in Israel and sent to Morocco for use in these studies.

## SECTION I

### A Research Objectives

- The objectives of this project are to
- 1 Identify a site in Morocco where a field test of a vaccine for canine visceral leishmaniasis (CVL) can be carried out
  - 2 Test candidate antigens purified from *L infantum* or produced by recombinant in a experimental model of CVL and identify potential vaccine antigens
  - 3 Study the immunological parameters of the vaccine antigens in the CVL model
  - 4 Carry out a field test of potential vaccines for CVL in the field site identified in objective no 1

### B Research Accomplishments

#### Israel

**Recombinant Proteins** Clones containing part of the genes for the three proteins *Hsp70*, *Hsp83* and *pDog1* 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
1	pDog1	3 57	9 2	+	+
2	HSP70	0 87	3 8	+	+
3	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.

Comparison of the nucleotide sequence of pDog1 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 pDog1 showed that the 5'-region of the clone had 23.8% identity over 252 aa to a kinesin from *Syncephalastrum racem*. A similar level of homology was seen with kinesins from other organisms, including *Leishmania donovani* (21.9% over 237 aa). Interestingly, the 3'-region of this clone shows 22.6% identity over 243 aa to RNaseE from *E coli*.

Plasmid DNA was prepared from a number of colonies for all three inserts, and restriction digestion or DNA sequencing carried out in order to identify and confirm that the subclones were in the correct orientation for protein expression. Subclones in the correct orientation were obtained for *Hsp70*, *Hsp83* and *pDog1*, optimal

conditions to expressing all three fusion polypeptides were determined by carrying out small scale experiments

The fusion polypeptide encoded for by Hsp70 codes for about 41% of *L. d. chagasi* HSP70, including the complete COOH-terminus (HSP70c). Preliminary experiments using  $\lambda$ -Zap II showed that this clone is expressed at higher levels than the other pDog clones. The expression of HSP70 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 (figure 1A). Polyclonal rabbit antibodies to HSP70 (figure 1B) 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 proteolytic enzyme Xa is in progress.

Problems were encountered in expressing the full-length polypeptides for *Hsp83* and *pDog1*. The orientation of pDog1 in pGEX was determined by restriction analysis with BanII and confirmed by nucleotide sequencing of the vector containing the insert. In a preliminary experiment a fusion polypeptide, 85 kDa, expressed by clone pDog1-1 was observed. Expression peaked at 3-4 hr and appeared to decrease after longer periods, overnight, due to protein degradation. No fusion polypeptide was observed when pDog1-8, opposite orientation, was used. Optimization of pDog1 expression system was obtained at 30°C and 3 hr, and the fusion polypeptide purified by affinity chromatography. The size of the polypeptide obtained, 85 kDa, was smaller than the predicted size of the GST-kinasin fusion product, estimated  $M_r = 170$  kd. We do not yet know the reason for this size difference. This 85 kDa polypeptide is recognized by antiserum to GST and serum from dogs with visceral leishmaniasis (VL), however two proteins of lower  $M_r$  react even more strongly with these antiserum, suggesting proteolytic degradation of the fusion polypeptide. Alternative purification protocols and host bacteria are being examined in an attempt to solve the degradation problems.

Sequencing and restriction enzyme analysis of the pGEX/hsp83 subclone showed that the insert is the correct orientation, however the size of the fusion polypeptide was essentially the same as for pGEX alone. This clone encodes approximately 74% of HSP83 protein. Since the estimated  $M_r$  of the fusion polypeptide, 90 kDa, is much larger than the product observed, it appears that the fusion

polypeptide is not stable in pGEX We are presently subcloning the *Hsp83* gene into a different expression vector

**Purified native and recombinant proteins** Three proteins (gp70, dp72 and HSP70c) have been 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

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 rHSP70 was also tested for use in lymphocyte proliferation Mice were immunized with either crude *L infantum* promastigote antigen (IPT1), rHSP70 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

Table 2 Proliferation of BALB/c mouse spleen cells with rHSP70

ANTIGEN*	PROLIFERATION (cpm)		
	IPT1	rHSP70	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 -  $10^5$ ,  $5 \times 10^5$  and  $10 \times 10^5$  for rHSP70 and pGEX - 0.05, 0.5 and 5  $\mu$ g

can be seen the rHSP70 is not toxic and induced very strong proliferation in the IPT1 immunized mice Proliferation induced by the the GST portion of the polypeptide and crude parasite antigen was much lower

The rHSP70 polypeptide was also tested by ELISA and western blotting (flexible PVC 96-well microtiter plates, Dyanatek) for use as an antigen to diagnosis VL Antigen concentrations from 25 to 60 ng/well were examined by crosshatched titration with VL patient and healthy control serum, and a 50 ng/well concentration chosen to coat the plates The results shown in table 2 are compared with several other serological assays for VL carried out on the same sera

The ELISA on HSP70c was 100% specific (Table 3) No false positive reactions were observed using sera from patients with cutaneous leishmaniasis, toxoplasmosis, malaria or the healthy controls However, the sensitivity of the assay was low compared to the other assays with almost half, 42%, of the VL sera giving false negative reactions Only 7/12 VL patients, 58% of the sera, reacted with the fusion polypeptide Interestingly, even though 5/7 VL sera reacted strongly with HSP70c (absorbances >0.176), no correlation was observed between the titers observed in other serological assays on crude parasite antigen and the strength of reaction with HSP70c HSP70 could also be used for diagnosis of dogs with VL (CVL) by western blotting (data not shown)

**Table 3 Comparison of visceral leishmaniasis diagnosis using the recombinant antigen HSP70c and other serological tests**

Patient sera	Biopsy for VL	IFA*	tot-ELISA	DAT	HSP70c
VL01	+	256	2048	102400	-
VL02	+	256	2048	51200	0.003
VL03	+	1024	4096	102400	-
VL04	+	256	nd	25600	0.018
VL05	nd	256	nd	102400	0.006
VL06	-	-	-	25600	0.410
VL07	+	4096	16000	102400	0.056
VL08	+	4096	nd	102400	0.611
VL09	+	512	4096	25600	1.035
VL10	+	256	nd	51200	0.006
VL11	nd	128	nd	102400	0.586
VL12	-	64	nd	3200	0.176
CL (n=8)		-	-	-	-
Tox (n=8)		nd	nd	nd	-
Malaria (n=8)		nd	nd	nd	-
Healthy controls (n=8)		-	-	-	-

\*IFA - immunofluorescence assay, tot-ELISA - enzyme-linked immunosorbent assay on crude leishmanial lysate DAT - direct agglutination assay, HSP70c - ELISA on recombinant HSP70c, VL - visceral leishmaniasis CL - cutaneous leishmaniasis Tox - toxoplasmosis nd - not determined

Results for western blotting using pDog1, HSP70, HSP83 and GST were carried out A pool of CVL sera (1/100) recognized pDog1 and HSP70 by western blotting, but not the HSP83 fusion polypeptide

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).

Problems were encountered in the stability of dp72 that appeared to degrade upon lyophilization. We are presently trying to solve this problem.

**Axenic amastigote antigen** A *L. infantum* isolate from a local Israeli dog with VL was obtained. It has been 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, to lipophosphoglycan, a promastigote specific protein were used. Northern blots using total RNA isolated from the promastigotes and axenic amastigotes were hybridized with probes to genes B and C, pDog1 and LPK2. These experiments (data not shown) demonstrated that the axenic amastigotes were indeed amastigote-like in their molecular and antigenic properties. Antigen is now being prepared from promastigotes and amastigotes of this isolate for preliminary comparison of their antigenic properties by western blotting, ELISA and lymphocyte proliferation 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 4. The best stimulation index (SI) was observed using the particulate amastigote antigen.

Table 4 Comparison of lymphocyte proliferation induced by amastigote and promastigote antigens in *L. infantum* immunized BALB/c mice

ANTIGEN (µg)	PROLIFERATION (SI)			
	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

## Morocco

### A FIELD WORK

**1 Survey** Work has continued on the epidemiology of CVL in the region of Sid Elghndouf. Additional visits were made in order to establish contacts with farmers in the localities chosen for the vaccine trial and engage them as animal keepers.

Based on initial studies carried out over the first year the number of sites where the vaccine trial will be carried out has been narrowed down to three locations. An additional 175 sera were collected in these foci 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 has increased from 26 to 29.7% in the first 6 months of 1998, showing that there is continuous and active transmission of the disease in this focus. A total of 52 dogs were seropositive, of which 7 were sick and three died of CVL by the end of Oct 1998. A *L. infantum* strain was isolated from a dog suffering from a severe VL in Sid Elghndour locality for use in vaccine studies.

Sampling was increased in order to understand better the evolution of the disease in this focus. From June to December 1998 a total of 367 dogs were examined by indirect immunofluorescence and ELISA. From these dogs 91 were seropositive and 276 seronegative.

**ii Field Trial** Thirty dogs were chosen for inclusion in a field trial of vaccination and their owners' 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 98 and the first boost in January 99, six weeks later. The final boost is scheduled for March 99. The immunological and clinical status of the dogs is under observation.

## **B LABORATORY EXPERIMENTS**

**i Vaccination with crude antigen** Protection of dogs immunized with crude promastigote antigen (CPA) using BCG as an adjuvant was investigated using the experimental model for canine visceral leishmaniasis (CVL). As previously described, two groups of six dogs were immunized with either CPA+BCG or BCG alone (control group). Each dog received two subcutaneous injections (20 µg CPA+BCG or BCG alone) at 4-week intervals. Immunized animals were challenged with  $10^5$  amastigotes/kg body weight. Results obtained approximately one year later show that this protocol is not effective. To date, 5/6 dogs immunized with CPA+BCG show clinical signs of disease. Parasites were isolated from the spleen of all the vaccinated dogs. Antibody titers and patterns of reactivity in Western blots 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 of the infected dogs: thinness, emaciation, onychogryphosis,

conjunctivitis, dandruff and hepatosplenomegaly Dogs immunized with only adjuvant, BCG, show varied signs of clinical leishmaniasis and loss of cellular immune responses to leishmanial antigens Therefore vaccination with BCG + CPA does not appear to provide protection against CVL

**ii Vaccination with gp63** Immune responses of dogs vaccinated with pure gp63 were compared to several other groups of animals (Table 5) 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 following pentamidine treatment and recovery of sick dogs with CVL

Table 5 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 asymptomatic dogs Drug treat recovered dogs following drug treatment

These dogs were challenged with  $10^5$  amastigotes by the iv route 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 wks after parasite challenge The results of this work is summarized in Tables 6 and 7

Table 6 Change in anti-leishmanial antibodies in dogs immunized with gp63

DOG No.	BEFORE-INF.*	POST-INFECTION	
		3 months	9 months
<b>Gp63 + BCG</b>			
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

<b>BCG alone</b>			
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
<b>Dogs with other clinical status</b>			
<b>Sick<sup>^</sup></b>	1 227	1 515	1 429
<b>Asymptomatic</b>	0 120	0 118	0 108
<b>Treated</b>	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 <sup>^</sup>See the legend to Table 5

Table 7 Proliferative response of peripheral blood lymphocytes in dogs vaccinated with gp63

<b>DOG</b>	<b>PREPATENCY PHASE</b> 3 mon. post-infection			<b>PATENCY PHASE</b> 9 mon post-infection		
	Crude*	Gp63	ConA	Crude	Gp63	ConA
<b>gp63 + BCG</b>						
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
<b>BCG Alone</b>						
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
<b>Dogs with other clinical status</b>						
<b>Sick<sup>^</sup></b>	0 124	0 188	0 442	0 122	0 134	0 419
<b>Asymp</b>	0 570	0 877	0 685	0 590	0 783	0 704
<b>Drug</b>	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 of bromodeoxyuridine incorporation into proliferating cells <sup>†</sup>Crude Ag crude antigen 1 µg gp63 0.5 µg and ConA -concanavalin A 2.5 µg <sup>^</sup>See table 5

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

**III Immune response to recombinant HSP70c** 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 crude *L infantum* promastigotes + BCG (see section B1) or autoclaved *L major* promastigotes using either BCG or 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.

**C Scientific Impact of Collaboration** Israeli scientists have been involved in the purification and characterization of new parasite antigens for incorporation in the immunological and vaccine studies. Following initial characterization of the toxicity and properties of the antigens they are sent to Morocco for inclusion in the studies on the dog CVL model. The Moroccan group has carried out preliminary vaccine and epidemiological studies to lay the ground work for a field trial of vaccines for CVL.

**D Project Impact** At this point results from preliminary vaccine studies in the experimental dog model for CVL are being applied in a field test of the first promising vaccine candidate, gp63. If the field test also looks successful the vaccine can be used in all countries where CVL is a problem.

**E Strengthening of Developing Country Institute** Several Ph.D. candidates, El Bed Ana-Igor, Lasri Saadia, and Natami Amale, in the Moroccan laboratory are receiving training as part of this project. One student, El Bed Ana-Igor, is scheduled to visit the Israeli lab in April 1999 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.

**F Future Work** On the whole this project is progress as described in the time schedule of the contract. Most of the objectives described for the first and second year have been completed with the following exception. We were unable to acquire the live recombinant BCG vectors (BCG/pMV261 gp63 and BCG/pMV261).

for testing in the CVL model, even though prior to the project we received a letter promising us access to the vectors. Plans for these experiments have been discontinued.

The following objectives described in the time schedule of work for the first two years have been 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, have been 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 initiated.

In the final year of the project work will continue on the analysis of the immunological parameters characteristic of protective immunity. This research will be carried out with the experimental dog model for visceral leishmaniasis (CVL) using vaccine candidates identified in the first two years of the project. Additional vaccine candidates and antigens will be prepared by the Israeli group and supplied to the Moroccans for these studies. In parallel the Moroccan group will acquire the technical knowledge for the production of native antigens or production of recombinant polypeptides. Purification of the other antigens for testing is on schedule.

## Section II

**A Managerial Issues** None

**B Budget** No major changes

**C Special Concerns** No changes

### **D Collaboration, Travel, Training and Publications**

Due to family problems, the Moroccan principal investigator was unable to visit Israel this past year and their visit was postponed. A Ph.D. student from Morocco, Anas Igor Elbied, is scheduled to visit the Israeli laboratory in March or April 1999 for training in polymerase chain reaction for use in diagnosis and characterization of *Leishmania*. Dr. Jaffe is tentatively scheduled to visit Rabat in June 1999.

### Original Publications

**Rhalem A, Sahibi H, Lasri S and Jaffe CL** Analysis of immune responses in dogs with canine visceral leishmaniasis before

and after drug treatment Submitted to Veterinary Immunology and Immunopathology

**Lasri S, Sahibi H, Sadek A, Jaffe CL and Rhalem A**  
Immune responses in vaccinated dogs with autoclaved *Leishmania major* promastigotes Submitted Veterinary Research

**Rhalem A, Sahibi H, Geussous N, Lasri S, Natami A, Riyad M and Berrag B** (1999) Immune response against *Leishmania* antigens in dogs naturally and experimentally infected with *Leishmania infantum* Veterinary Parasitology 15 1-12

Thesis

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

Lasri Saadia Etude de la reponse immunitaire chez le chien contre differentes preparation antigeniques PhD thesis, Dec 1999, Rabat, Morocco

Natami Amale Les Leishmanioses Canines Etude de la reponse immunitaire dans des conditions naturelles PhD thesis, Dec 1999, Rabat, Morocco

**E Request for American Embassy Tel Aviv or AID**  
**Actions None**