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Title: Kala azar in Brazil: Immunology and genotypic characterization of viscertropic *Leishmania*.

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INTRODUCTION

Leishmaniasis is a parasitic disease of manifold forms affecting millions of people (1-3). The parasite, a unicellular flagellated promastigote, is transmitted by the bite of a female sandfly to a mammalian host where it infects macrophages and transforms into the aflagellated intracellular amastigote (1). Among the major types of disease present throughout the World are cutaneous leishmaniasis (CL), a localized self-curing form caused by *Leishmania major* or *L. tropica*, and visceral leishmaniasis (VL), a fatal form caused by *L. donovani* (1-3). Other less frequent, but severe and mutilating, clinical conditions also occur (1-3).

VL or kala azar is characterized by irregular fevers, progressive hepatosplenomegaly and anergy to parasite antigen. The objectives of this grant were to apply modern biochemical, immunological and molecular biological techniques to the study of this disease, specifically the development of efficient, reliable methods for the diagnosis and survey of VL in endemic foci.

SUMMARY

This project focused on the development of diagnostic techniques for leishmaniasis and the identification of *Leishmania* species responsible for disease. A serodiagnostic assay based on the recognition of specific parasite proteins, gp70 and dp72, was evaluated with serum from dogs and human patients. Competitive assays based on the inhibition of monoclonal antibodies or direct

assays based on the reaction with the proteins can be used for the early detection of disease and to follow the efficacy of drug treatment. Further biochemical studies showed that both proteins are found primarily in the cytoplasim and appear to contain lipid anchors. One protein, gp70, appears to be associated with DNA. Protection experiments in BALB/c mice with dp72 showed that T-cells are responsible for the reduced liver parasite burdens and that antibodies to this protein have no effect on disease progression. Evaluation of several endemic foci in Brazil using kDNA probes for *L. chagasi* and *L. braziliensis* implicated *Lutzomyia whitmani* and *Lu. longipalpis* in the transmission of *L. braziliensis*. Two local rodents, *Rattus r. alexandrinus* and *R. r. frugivurus* were identified as reservoir hosts. Specific primers were developed for the identification of *L. braziliensis* and *L. chagasi* by the polymerase chain reaction (PCR). Further analysis of PCR products by schizodeme analysis was shown to be useful for geographic analysis of individual strains.

RESULTS

A. SERODIAGNOSIS (Israel)

Work was carried out on the development of a serodiagnostic assay for canine visceral leishmaniasis. This assay is based on the reaction of serum with two proteins purified by affinity chromatography from *L. donovani*, gp70-2 and dp72 (4). Preliminary studies have shown that they are useful for the diagnosis of human visceral leishmaniasis (5).

Our studies showed that infected dogs produce antibodies to these proteins. Dog sera from Spain and Brazil were examined by immunofluoresence (IFA), direct and competitive enzyme-linked immunosorbent assay (ELISA). Results presented in table 1 show that the competitive ELISA (C-ELISA) using monoclonal antibodies (mAbs) to both proteins correctly identified 94.9% of the IFA positive dogs assayed, as compared to 87.2% identified by a direct ELISA on crude parasite antigen.

Fifteen negative sera from dogs were examined (Table 2). No false positives were obtained using the direct ELISA and 2/15 samples were incorrectly identified by the C-ELISA. These results show that antibodies to the proteins gp70 and dp72 are present in the serum

of animals with canine visceral leishmaniasis and that the C-ELISA can be used to determine the prevalence of VL in endemic foci.

A direct ELISA for canine visceral leishmaniasis on the pure proteins was established. The direct assay is easier and faster to use than the C-ELISA. Serum from 50 dogs in southern France was obtained and screened by ELISA on the pure proteins. Healthy dogs, and dogs with VL, *B. canis* or *E. canis* were tested. Using absorbance (405nm)=0.500 as the positive/negative cutoff, no false positives and 4/20 false negatives were found with dp72; 1/30 false positives and 3/20 false negatives were found with gp70 (figure 1). These results demonstrate the usefulness of a direct ELISA on pure parasite proteins for serodiagnosis.

Western blotting on pure proteins can be carried out at high serum concentrations, 1/20 dilution. At these dilution's serodiagnostic assays which utilize crude parasite antigens encounter problems due to nonspecific crossreactions with serum from other diseases. Western blotting on pure *L. donovani* antigens should allow the early detection of VL when antibody titers to crude antigen are low. Results using dp72 as antigen are shown in figure 2 for serum from *L. donovani*, *B. canis*, *E. canis* infected dogs and dogs without disease. The reaction with dp72 is very strong when sera from dogs with VL is examined. No reaction is visible with serum from the negative dogs or dogs infected with *B. canis*, and only a very weak reaction is visible with one *E. canis* infected dog. These same sera were examined by ELISA on crude and pure antigens at 1/1000 and 1/100 dilution's, respectively.

Kinetic studies using serum from dogs infected with either *L. donovani* amastigotes or promastigotes were carried out. These serum were examined for antibodies to both dp72 and gp70 at different times post-infection. Serum antibodies to the pure proteins were measured by western blotting at 1/20 and 1/100 serum dilution, respectively. The results are shown in figures 3 and 4. Antibodies to dp72 can be detected as early as 0.5 months after infection with amastigotes and as early as 2.5 months after infection with promastigotes. By 2.5 and 3.5 months all of the amastigote and promastigote infected dogs, respectively, show antibodies to dp72. A similar kinetics is observed when gp70 is used (figure 4). These results suggest that western blot analysis on pure antigens might be useful for the early dianosis of canine VL.

The rate of disappearance of serum antibodies to gp70 and dp72 in three Israeli children diagnosed and treated for VL was determined. It is frequently difficult to evaluate the success of drug therapy for VL without resorting to spleen or bone marrow tissue aspirates. Anti-parasite antibodies in VL patients generally remain positive for more than 6 months and are not useful indicators of successful treatment. A rapid loss of anti-gp70 or dp72 antibodies in patient serum following chemotherapy would be extremely helpful in evaluating the success of therapy. The serum antibody titers on crude *L. donovani* antigen of the patients following chemotherapy were measured by direct ELISA. Five months post-treatment the anti-parasite antibody levels (at 1/1000 dilution) have almost dropped to a normal positive to negative control absorbance ration (P/N ratio) < 3. The P/N ratios are 5.8 and 6.0 for the two patients, respectively (figure 5).

Reactions on the pure proteins by western blotting (1/100 serum dilution) parallel the direct ELISA and steadily decrease over time following chemotherapy (figure 6). The first two bleeds from patient 1 strongly reacted with gp70. By the third bleed (1 month post-treatment) a decrease in intensity is observed which steadily decreases and is essentially gone 1 to 1.5 months earlier than the direct ELISA. With dp72 serum antibody levels remain high for 2 months following drug treatment and then decline rapidly, disappearing by 22/9. Similar results are seen with patient 2. These results suggest that a ELISA on pure proteins may be useful for determining the effectiveness of chemotherapy.

B. BIOCHEMISTRY OF DIAGNOSTIC PROTEINS (Israel)

Both dp72 and gp70 show molecular weight (Mr) micro heterogeneity between isolates of *L. donovani*. Other leishmanial antigens, such as lipophosphoglycan, also show Mr variation during growth in culture. However, no change in the reaction pattern of mAbs D2 (anti-gp70) or D13 (anti-dp72) to the pure proteins was observed throughout the growth of promastigotes *in vitro* (logarithmic to stationary phases) or following heat-shock (28 to 37°C, data not shown).

Polyclonal antibodies to dp72 and gp70 were used for immune localization by transmission electron microscopy on promastigotes of *L. donovani*. Promastigotes were fixed in 2% glutaraldehyde and

embedded in LRgold. The two polyclonal antibodies were affinity purified from western blots of the pure proteins and were used for localization on thin sections. Experiments show that dp72 is associated primarily with internal membrane structures and the nuclear membrane. Gp70 is associated both with internal structures and the flagella, but both proteins are found in small amounts on the cell surface membrane (figure 7 a-c).

Lectin binding experiments showed that gp70 is glycosylated. However, no evidence for N- or O-glycosylation of dp72 was found. Neither tunicamycin, N-glycanase or O-glycanase affected the migration of metabolically labeled dp72 on polyacrylamide gels (data not shown). Treatment of pure dp72 with N-glycanase, O-glycanase, phosphodiesterase I or alkaline phosphatase in the presence of proteolytic inhibitors had no affected on the Mr of dp72 or the binding of mAb and polyclonal antibodies to the protein. Similar experiments with gp70 showed that treatment with phosphodiesterase I destroyed the epitope recognized by the mAb in western blots. Phosphodiesterase I cleaves diphosphate ester linkages in DNA, RNA and in mono- or poly(ADP)ribose. Treatment of gp70 with DNAse I lead to a loss in mAb binding while RNAse treatment had no affect on recognition by the mAb. Treatment with N-chlorosuccinimide (NCS), trypsin or SV8 protease also results in the loss of mAb binding to the pure protein (data not shown). These results indicate that gp70 exists as a protein - DNA complex.

The technique of phase partition in the detergent TX-114 has been used to study membrane proteins of *Leishmania* and other parasites. Preliminary dot blot experiments with TX-114 suggested that both gp70 and dp72 are anchored to the membrane by a lipid tail. Cleavage of the lipid anchor by phospholipase C (PLC) affects the phase into which the protein partitions. An inhibitor of PLC, $ZnCl_2$, was included in some of the samples. Samples were analyzed by SDS-PAGE and western blotting using mAbs specific for each protein (figure 8).

The inclusion of $ZnCl_2$ in the detergent lysis buffer drastically reduces the solubilization of dp72 (Panel A, Tot), as has been reported with other antigens. The solubilization buffers also contain a cocktail of proteolytic enzyme inhibitors. Interestingly, the small amount of dp72 which is solubilized partitions exclusively into the detergent phase (Det, +). No reactive antigen is present in the

aqueous phase under these conditions (Aq, +). In the absence of Zn (-) most of the dp72 is found in the aqueous phase (Aq, -), though a small amount of reactive antigen of similar Mr partitions into the detergent phase (Det, -). The Mr of the reactive antigen, solubilized in the presence of Zn, is greater than that found in the absence of Zn and the Mr micro-heterogeneity is also reduced.

The same samples were also analyzed for reactions with mAbs to gp70 (Panel B). The results were even more striking. In the absence of Zn (-) all of the gp70 partitions into the aqueous phase (Aq, -) and in the presence of the PLC inhibitor all of the protein partitions into the detergent phase (Det, +). Addition of Zn appears to have little affect on the efficiency of solubilzation of gp70 (Tot), however the Mr of the protein in the presence of the metal ion is higher. Furthermore, both proteins can be labeled with myristic acid (data not shown). The small weight antigen, 30 kDa, apparent in western blots of the solubilized parasites in the presence of Zn has been observed in affinity purified gp70 labeled with myristic acid or orthophosphate. Additional experiments are underway to substantiate the presence of a lipid anchor.

C. IMMUNOLOGY (Israel)

In preliminary experiments the protein dp72 protected BALB/c mice against a challenge by *L. donovani* promastigotes. Mice immunized with 12μg of the protein and *C. parvum* as adjuvant had an 85% reduction in parasite liver burdens (LPB). The kinetics of protection against *L. donovani* in mice immunized with dp72 (6μg/mouse) was examined. BALB/c mice are of the Lsh^s, $H\text{-}2^d$ genotype and are highly susceptible during the first 2-4 weeks of the disease, after which the parasite burden slowly decreases. Following challenge with 10^7 stationary-phase promastigotes, animals were sacrificed at 1, 20 and 108 days post-infection and the LPB determined microscopically from liver touch biopsies (figure 9). Examination of the LPB at day 1 showed no significant difference ($P=0.98$) between the parasite burden in the immunized mice and the non-immune control mice which only received adjuvant. These results were confirmed by limiting dilution assay. However, by day 20 the mice receiving dp72 showed a 60% reduction in the LPB compared to the control mice ($P<4.7\times10^{-7}$). The difference was even

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more pronounced in the mice sacrificed at 108 days, 78% lower ($P<5x10^{-6}$) in the immunized animals.

The identical LPB found at day 1 in the two groups indicates that the protection observed in the immunized mice at later times is not a result of differences in the percentage of promastigotes surviving immediately following injection. This implies that antibody mediated killing does not play any role in the protection observed, even though high titers of anti-dp72 (>1/1000) are present in the immunized mice. Indeed the passive transfer of hyper-immune sera to naive mice did not impart any protection when compared to non-treated naive mice (data not shown).

The lympho-proliferative response (LPR) was measured in mice immunized with dp72. *C. parvum* was used as adjuvant for only the first boost. These mice also received 10^7 irradiated *L. donovani* promastigotes as a final boost. Results of one typical experiment are shown in figure 10. A strong proliferative response (SI=46.2) to dp72 was found, peaking at 0.43μg/ml. No response to other pure parasite antigens such as the promastigote surface protease (PSP) or lipophosphoglycan were observed. A very weak LPR was observed to gp70, SI=0.9 and a weak consistent proliferation to phosphoglycan was also observed (SI=12.2). Polyclonal and mAbs to dp72 show no reaction with the lipophosphoglycan associated protein.

In vitro depletion experiments were carried out to identify the T-cell subset proliferating in culture to dp72. Spleen cells from mice immunized with only the pure antigen were stimulated with dp72 in the absence or presence of mAbs to the $CD4^+$, $CD8^+$ or both subsets (figure 11). Thymidine uptake showed that antibody to the $CD4^+$ T-cell subset eliminated proliferation *in vitro* to dp72, as did a mixture of anti-CD4 and -CD8 antibodies. Antibody to the $CD8^+$ T-cell subset did not inhibit proliferation.

Adoptive transfer to naive mice of antigen specific T-cell blasts was carried out. The blasts were purified on Percoll gradients after 5 days in culture with dp72 and expanded for an additional 3 days with IL-2. Twenty-four hours following transfer of the cells to the mice, the animals were challenged with 10^7 *L. donovani* promastigotes and sacrificed after an additional 18 days. Mice receiving the T-cell blasts showed a 57% reduction in their LPB compared to the control animals (figure 12).

In order to confirm the importance of T-cell mediated responses to dp72 in protection against VL, we examined the effect of *in vivo* depletion of CD4+ and CD8+ T-cells, and neutralization of γ-IFN on protection in vaccinated mice (figure 13). Immunized mice were treated with antibodies against the T-cells or γ-IFN pre- and post-challenge. Control immunized mice received injections of either IgG or PBS. Mice not immunized with dp72 were included as controls. Immunized mice treated with either IgG or PBS showed significantly lower LPB than non-immunized mice, 61 or 81% respectively. However, if mice immunized with dp72 were injected with antibodies to either the CD4+ and/or CD8+ T-cell subsets or to γ-IFN it reversed the protective effect of immunization.

D. DETECTION OF PARASITES IN ENDEMIC FOCI (Brazil).

Samples from vertebrate hosts and sandflies collected in different foci of leishmaniasis in Brazil were analyzed for *Leishmania*. Sandflies were collected at Baturite, a major focus of cutaneous leishmaniasis in the state of Ceara. Castro's aspirator was used to collect insects inside residences. Light traps mounted at 50 m from the houses were also used to obtain insects. The insects were identified by microscopy and prepared for hybridization.

Sandflies were digested with lysis buffer (10mM Tris-HCl, pH8.0, 10mM EDTA, 0.2% Triton X-100 and 100μg/ml protease K) at 50°C for 2 hr. The digest was applied to Genescreen Plus membranes using a dot-blot manifold and hybridized under stringent conditions with radio-labeled kDNA probes. The results are shown in table 3. Probes for *L. braziliensis* and *L. donovani* were employed.

Around 5% of the sandflies collected reacted with the *L. braziliensis* probe. The incidence of infection was highest in sandflies of the *Lu. whitmani* species. Surprisingly, the second most prevalent species which was positive for *L. braziliensis* was *Lu. longipalpis*, a known vector for *L. chagasi* in Brazil. Rehybridization of the material with the *L. chagasi* specific kDNA probe was negative for all the sandfly samples.

Domestic and peridomestic rodents were captured at Aquiraz, another focus of cutaneous leishmaniasis and processed for detection of *Leishmania* by hybridization. Table 4 shows the results obtained in these studies. Material from 32/56 of the rodents analyzed reacted with the *L. braziliensis* kDNA probe. The rodents with the

highest levels of infection, 10 and 21%, were *Rattus rattus alexandrinus* and *R. r. frugivurus*. Parasites were primarily found in the liver or skin of the infected rodent.

E. DEVELOPMENT OF A POLYMERASE CHAIN REACTION FOR THE DETECTION OF *L. CHAGASI* AND *L. BRAZILIENSIS*.

The development of a specific polymerase chain reaction (PCR) for the viscerotropic Leishmaniases will be based on the knowledge of minicircle sequences. By coupling the sensitivity of the PCR technique with the potential of schizodeme analysis of parasite subpopulations we should be able to develop an excellent procedure for the diagnosis and identification of *L. chagasi*. Total kDNA of *L. chagasi*, *L. infantum* and *L. donovani* were digested with the endonucleases Dra 1 and Pst I. The full length minicircles were cloned into the plasmid pBluescript. Data analysis of the sequences of two *L. chagasi* minicircles from different classes and one *L. donovani* minicircles are presented in figure 14.

Sequence alignment revealed conserved region(s), however no specific sequences for this group of *Leishmania* were found in the region shown. The alignment of other regions in the minicircle identified a variable region (data not shown).

Amplification of the variable region of the minicircle generates distinct sized products depending on the species used (figure 15). When hybridization of the amplified variable regions is carried out using a probe from the variable region of *L. infantum* a reaction is only seen with the homologous sequence from *L. infantum*. No hybridization to the amplified regions from the other species is observed (figure 16).

Digestion of the PCR amplified product with the restriction enzyme Hae III results in different patterns between the species (figure 17). Further analysis demonstrated that *L. chagasi* digests with Rsa I yielded reproducible profiles which could be grouped according to the geographical origin of the strain (figure 18).

F. REFERENCES.

1. Zuckerman, A and Lainson R (1977). In Parasitic Protozoa, ed. Kreier, JP, Academic Press, New York, Vol. 1, p57.

2. Adler, S (1964). In Advances in Parasitology, ed. Dawes, B, Vol. 2, p35.

3. Pearson, RD, Wheeler, DA, Harrison, LH and Kay HD (1983) Rev. Infect. Dis. 5: 907.

4. Jaffe, CL and Zalis, M (1987). Mol. Biochem. Parasitology 27: 53.

5. Jaffe, CL and Zalis, M (1988) J. Infectious Dis. 157:1212.

TRAINING AND COLLABORATION

During the four year period covered by this contract, three Brazilians participating in the project spent time in the Israeli laboratory. Dr. Ulisses Lopes the co-operating investigator visited for one week in July 1991 to discuss progress and future collaboration. Two of his students subsequently spent three month periods in Israel learning various biochemical and molecular biological techniques. Some of the results presented above are a direct outcome of these visits. Flavia C.A. Gomes spent Oct. 1991-Jan. 1992 and Julia R. Araripe spent May to Aug. 1993 in the laboratory of Dr. Charles L. Jaffe.

PUBLICATION SUPPORTED BY THIS CONTRACT

1. Rachamim, N and Jaffe, CL (1993). Pure protein from *Leishmania donovani* protects mice against both cutaneous and visceral leishmaniasis. **J. Immunol. 150**, 2322-2331.

2. Vasconcelos, IAB, Vasconcelos AW, Fe Filho, NM, Queiroz, RG, Santana, EW, Bozza, M, Sallenave, SM, Valim, C, David, JR and Lopes, UG (1994). The identity of *Leishmania* isolated from sand flies and vertebrate hosts in a major focus of cutaneous leishmaniasis in Baturite, Northeastern Brazil. **Am. J. Trop. Med. Hyg. 50**, 158-164.

3. Several other manuscripts are either in preparation or submitted.

TABLE #1

Comparison of Methods for Diagnosing Canine Visceral Leishmaniasis

DOGS POSITIVE BY IMMUNOFLUORESCENCE

Dog	D-ELISA (P/N ratio)*	Comp-ELISA (% inhibition) D2	D13	
unca	7.9	0	94	
6170L	6.9	62	46	
whiskey	7.0	93	77	
ens	3.7	0	79	
7683	11.3	97	94	
7788	13.5	93	100	
7500	16.6	56	92	
7685	7.8	66	82	
7701	1.0	59	86	
7686	1.0	2	1	
7709	3.2	90	90	
7684	15.1	95	98	
7687	12.9	90	91	
7691	23.1	89	100	
7498	12.4	87	100	
7794	1.0	33	4	
7692	8.8	61	100	
7702	10.4	74	94	
7799	3.5	52	90	
5740	9.9	93	92	
5744	7.0	23	84	
5817	16.2	81	88	
5815	21.1	88	92	
5784	23.1	98	96	
5733	20.3	91	92	
Serro	13.5	46	100	
7538	18.4	54	84	
7540	19.1	93	96	
7499	20.5	79	96	
7541	16.1	84	55	
7457	22.3	63	98	
7711	24.6	40	96	
7690	3.3	39	27	
5765	21.5	63	98	
7500	14.5	35	47	
7498	23.9	91	96	
7710	11.2	0	91	
7704	8.8	44	84	
7697	2.0	28	20	
D42	---	67	87	pos. control
TOTAL		33 POS.	35	POS.
ASSAYED -39	34 POSITIVE	COMBINED POSITIVE		37

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TABLE #2

Comparison of Methods for Diagnosing Canine Visceral Leishmaniasis

DOGS NEGATIVE BY IMMUNOFLUORESCENCE

Dog	D-ELISA (P/N ratio)*	Comp-ELISA (% inhibition) D2	D13	
S.perro	1.0	0	0	
4501	1.0	0	9	
7495	1.0	0	0	
7696	1.0	50	30	
7682	1.0	25	2	
7492	1.0	23	24	
7703	1.0	6	0	
7695	1.0	4	0	
7501	1.0	37	27	
7495	1.0	29	0	
7698	1.0	0	0	
7706	1.0	0	0	
7708	1.0	26	20	
7705	1.0	9	20	
7703	1.0	11	11	
D23	---	8	7	neg. control
TOTAL		13 NEG.	15	NEG.
ASSAYED -15	15 NEGATIVE	COMBINED NEGATIVE	13	

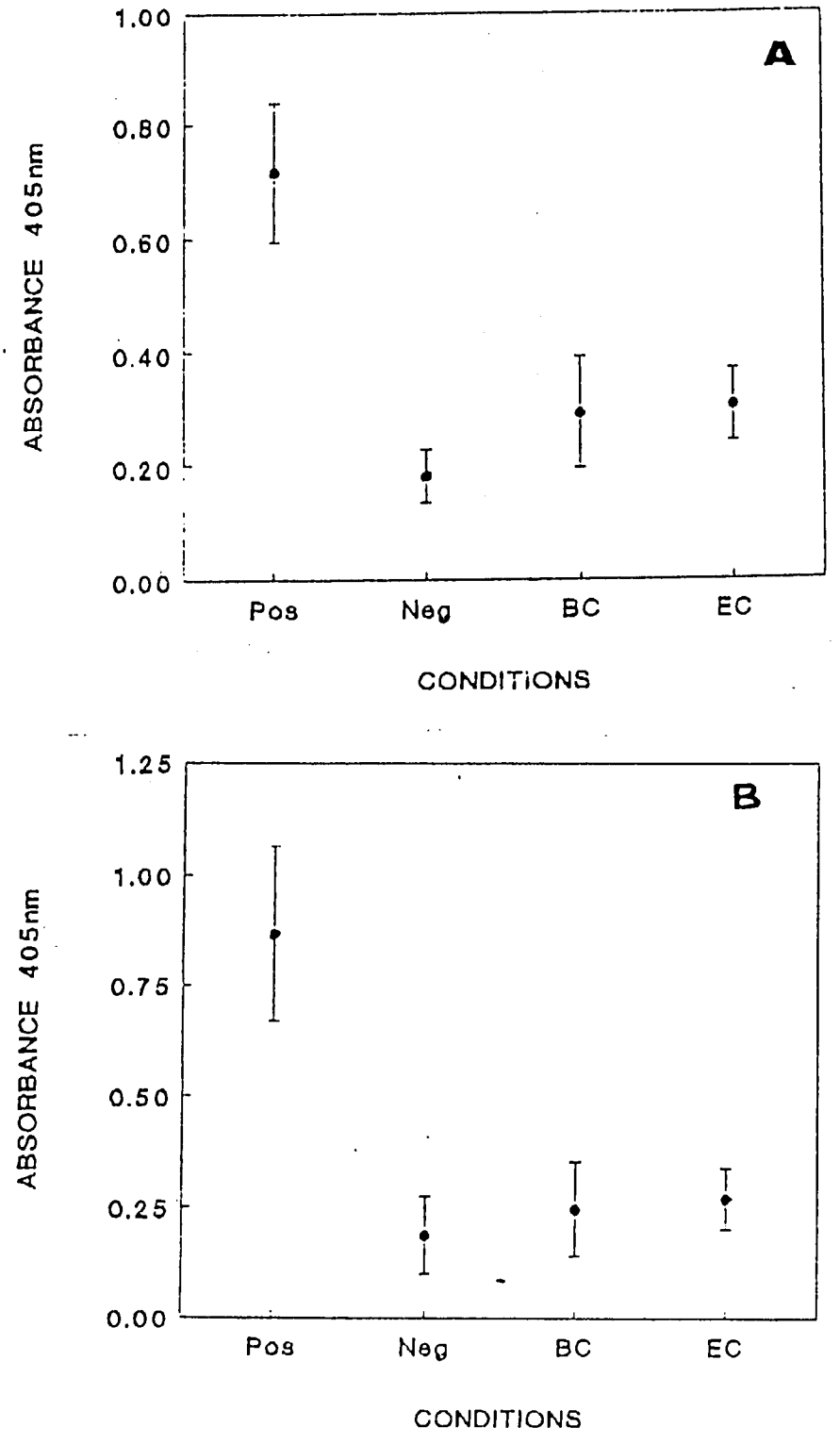

Figure 1. Direct ELISA on pure *Leishmania donovani* proteins using sera from dogs with different diseases. Pos - visceral leishmaniasis (n=20), Neg - normal controls (n=10), BC - *B. canis* (n=10) and EC - *E. canis* (n=10). Panel A - Protein dp72 and Panel B - Protein gp70-2. Each o shows the average value for each group. The standard errors bars represent the 99% confidence level.

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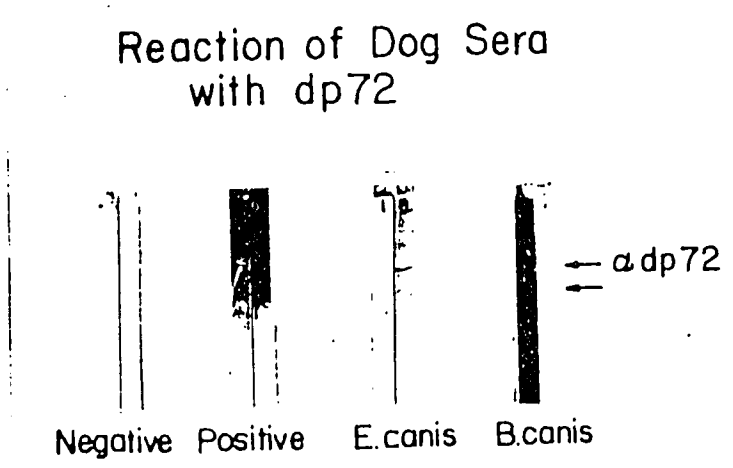

Figure 2. Western blot of sera from dogs with different diseases on pure Leishmania donovani dp72. The arrows show the position at which anti-dp72 control monoclonal antibodies bind.

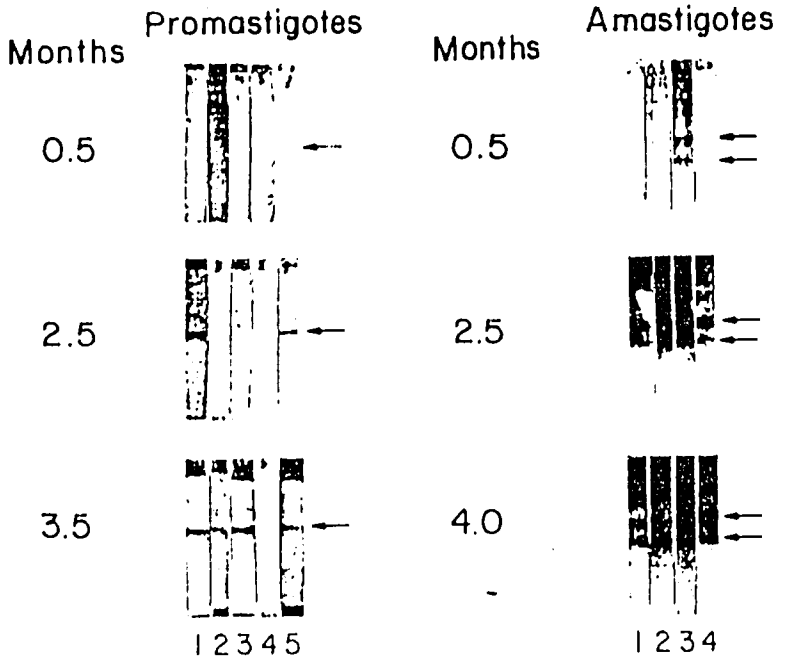

Figure 3. Development of anti-dp72 serum antibodies in dogs challenged with Leishmania donovani parasites. Protein (0.8 μg/gel) was separated by SDS-PAGE and transferred to nitrocellulose paper. The strips were reacted with sera at 1/20 dilutions and developed with protein A - peroxidase using 3,3'- diaminobenzidine.

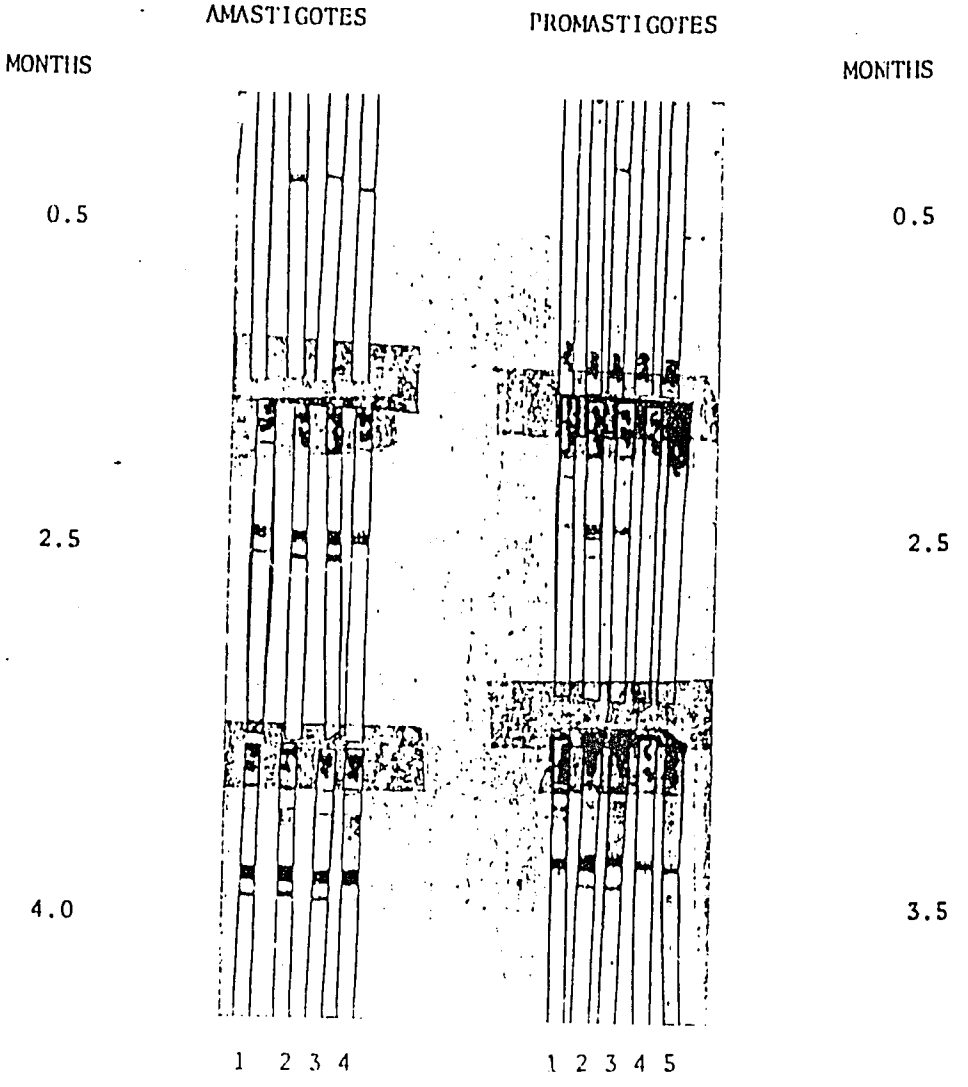

Figure 4. Development of anti-gp70-2 serum antibodies in dogs challenged with Leishmania donovani parasites. Protein (0.5 or 1 μg/gel) was separated by SDS-PAGE and transferred to nitrocellulose paper. The strips were reacted with sera at either 1/100 (amastigote) or 1/200 (promastigote) dilutions and developed as in figure 3.

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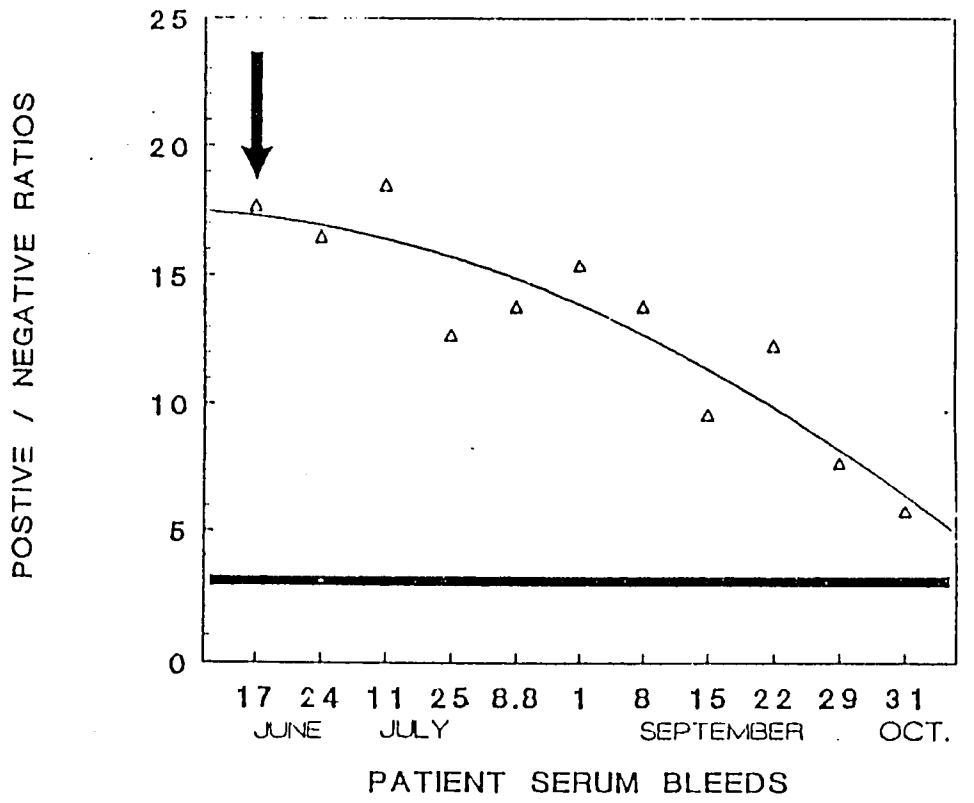

Figure 5. Change in anti-leishmanial antibody titers over time following drug treatment of patient with visceral leishmaniasis. Arrow shows the date at which a course of glucantime treatment was begun. Each point represents the ratio patient serum binding to negative control serum binding (O.D. 405nm) in an direct ELISA on crude L.donovani promastigote antigen at a 1/1000 dilution of the sera for the various dates that patient serum was obtained. Ratios > 3 (dark line) represent positive reactions.

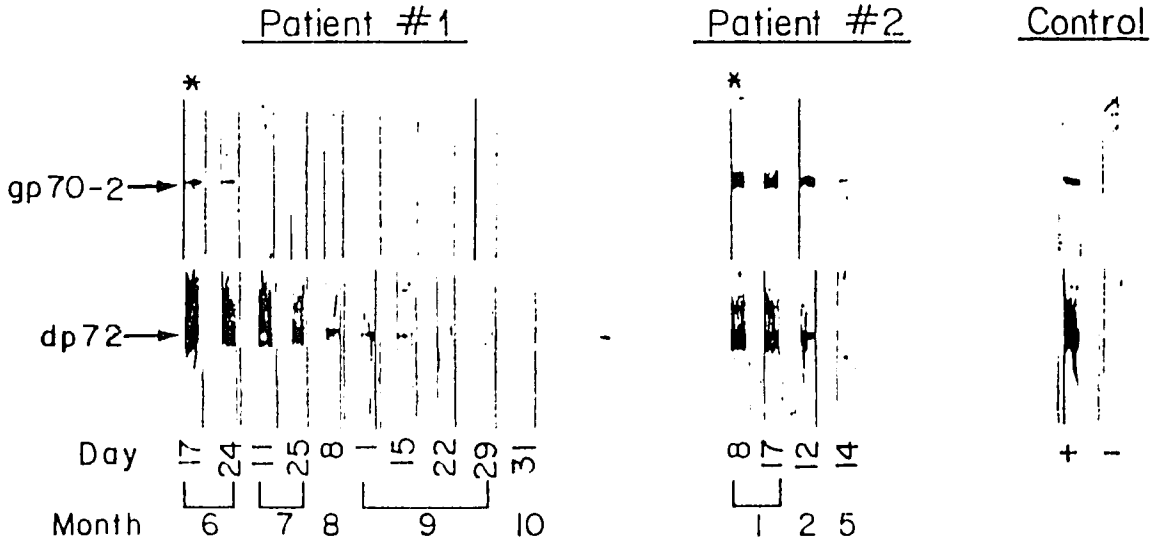

Figure 6. Western blot analysis of anti-dp72 and -gp70-2 antibody titers over time following drug treatment of patients with visceral leishmaniasis. The * marks the date at which a course of glucantime treatment was begun. Pure Leishmania donovani proteins (0.8 μg/gel) were separated by SDS-PAGE and transferred to nitrocellulose paper. Strips were incubated with 1/100 dilutions of patient serum from different dates and developed with protein A - peroxidase and 3,3'- diaminobenzidine. Control serum were also included: + positive for visceral leishmaniasis

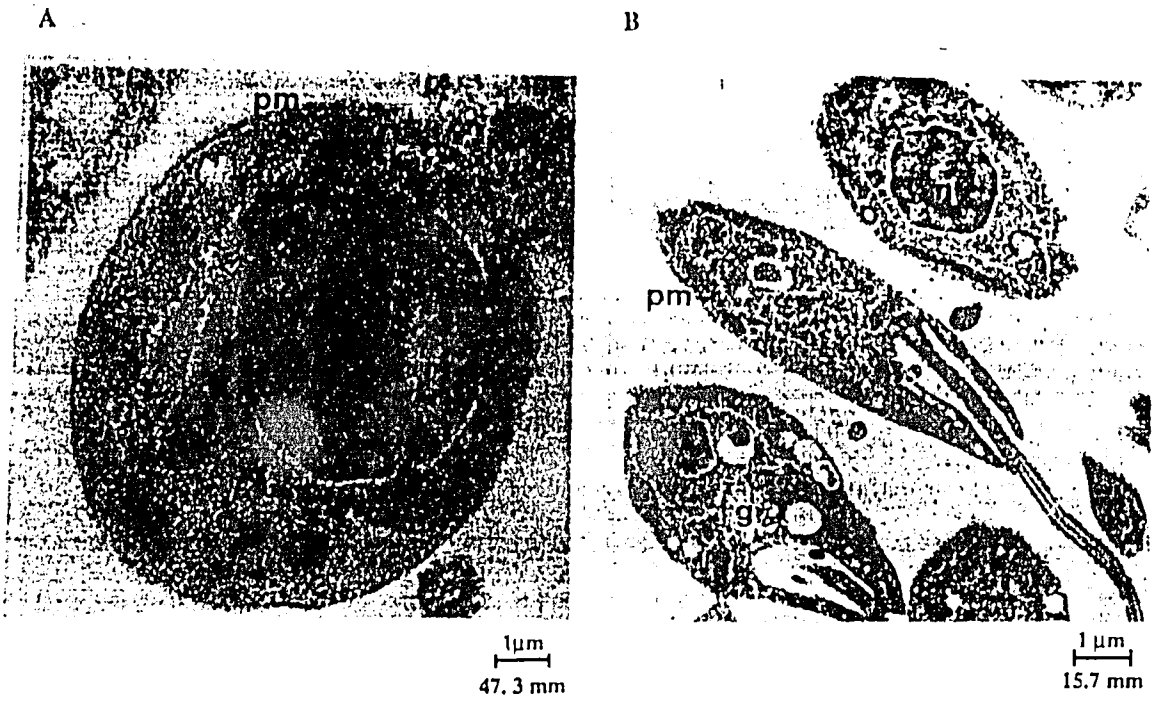

Figure 7. Immune localization of dp72 and gp70-2 in promastigotes of *Leishmania donovani.* Post-embedding, plates A and B. Thin sections were incubated first with affinity purified rabbit αdp72 (A, 1:50 dilution) or αgp70-2 (B, 1:50 dilution) and following washing with the second antibody, goat anti-rabbit collodial gold conjugates (15nm). For key see page 35.

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Pre-embedding, plate C. Parasites were incubated in rabbit αgp70-2 prior to fixation in glutaraldehyde. Thin sections were incubated in goat anti-rabbit collodial gold conjugates (5nm). er-endoplasmic reticulum, f-flagellum, g-golgi apparatus, k-kinetoplast, n-nucleus, pm-plasma membrane.

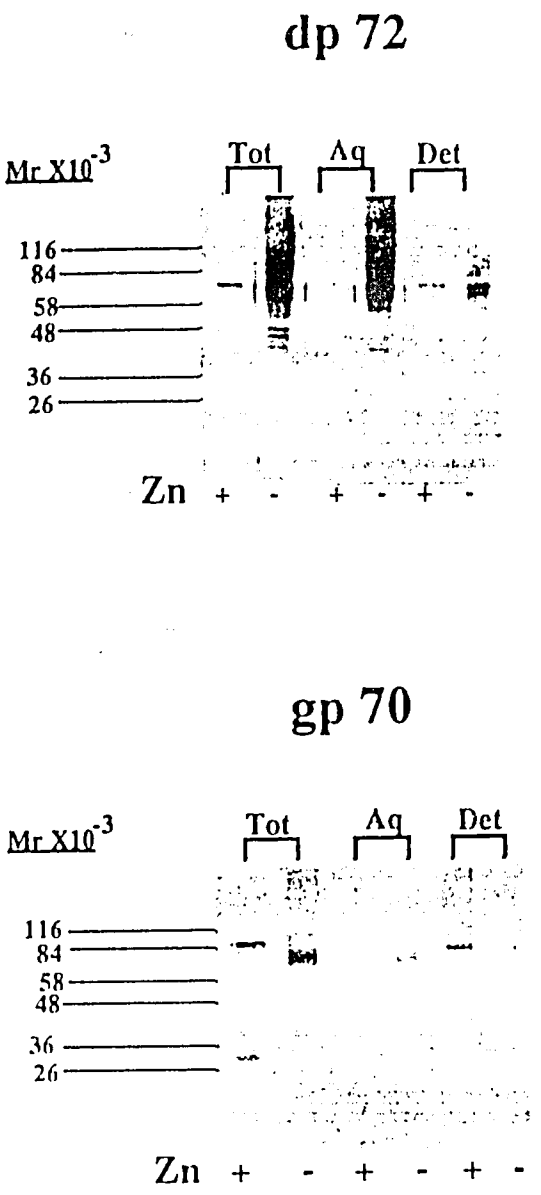


Figure 6 Analysis of dp72 and gp70-2 partitioning in TX-114 by SDS-PAGE and western blotting. Promastigotes (LV9) were solubilized with TX-114 (TOT) containing proteolytic inhibitors in the presence (+) or absence (-) of 5 mM $ZnCl_2$ Non-soluble material was removed by centrifugation and the supernatant was equilibrated at 30°C. The detergent (Det) and aqueous (Aq) phase were separated by centrifugation on sucrose gradients and diluted to identical volumes. Following SDS-PAGE and electrophoretic transfer the nitrocellulose was analyzed by probing either with Panel A, mAb D13 (dp72) or Panel B, mAb (gp-70-2).

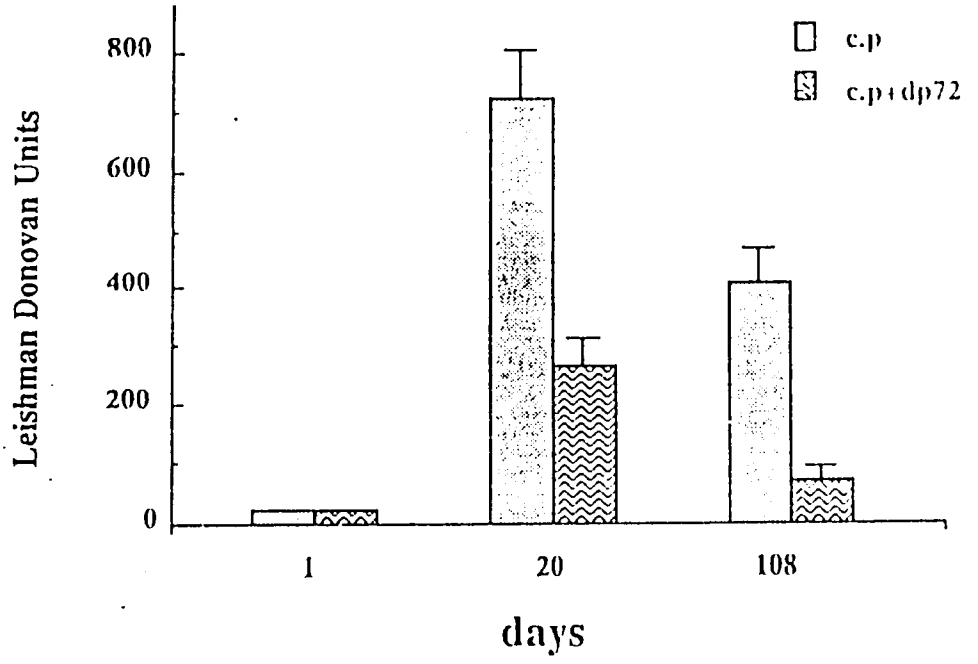


Figure 7. Kinetics of protection against L.donovani in mice immunized with dp72. BALB/c mice were immunized with dp72 (6ug total per mouse). Control mice received adjuvant alone. Mice were challenged i.v. with 10^7 promastigotes. At 1, 20 and 108 days, the mice were sacrificed and the Leishman Donovan Units (LDU) calculated from liver biopsies. The mean LDU±SE is shown (n=6)

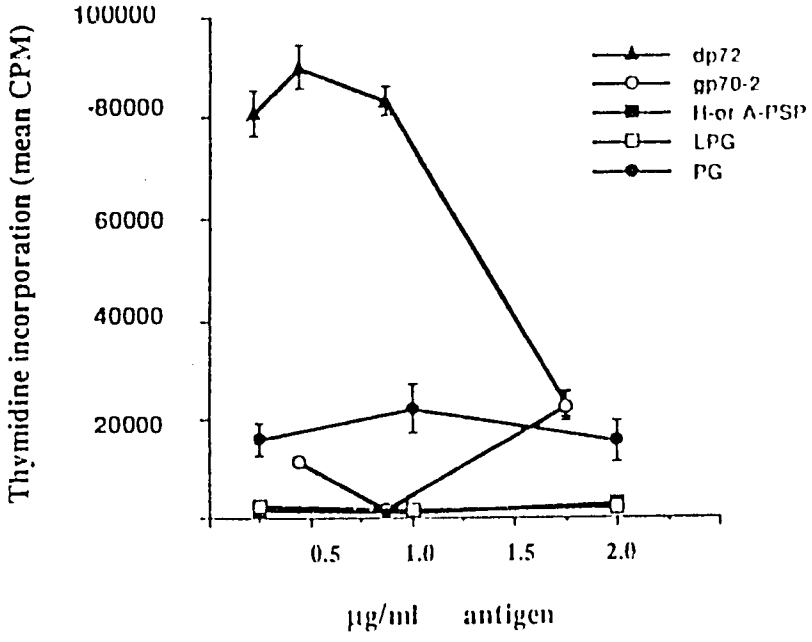


Figure10. Proliferative response of lymphocytes from dp72 immunized mice to pure antigens. Spleen cells from a mouse immunized with 7.5 ug dp72 and boosted with 10^7 irradiated promastigotes (100 kRads) were cultured with dp72, gp70-2, promastigote surface protease (H-hydrophilic and A-ampiphilic PSP), lipophosphoglycan (LPG) and phosphoglycan (PG). After four days the proliferation was determined by measuring [^{3}H]-thymidine incorporation for 18 h. Each point represents triplicate cultures ± SE. PSP was kindly provided by Drs. Bordier and Rivier from the University of Lausanne. LPG and PG were gifts from Dr. Handman, Walter and Elisa Hall Institute.

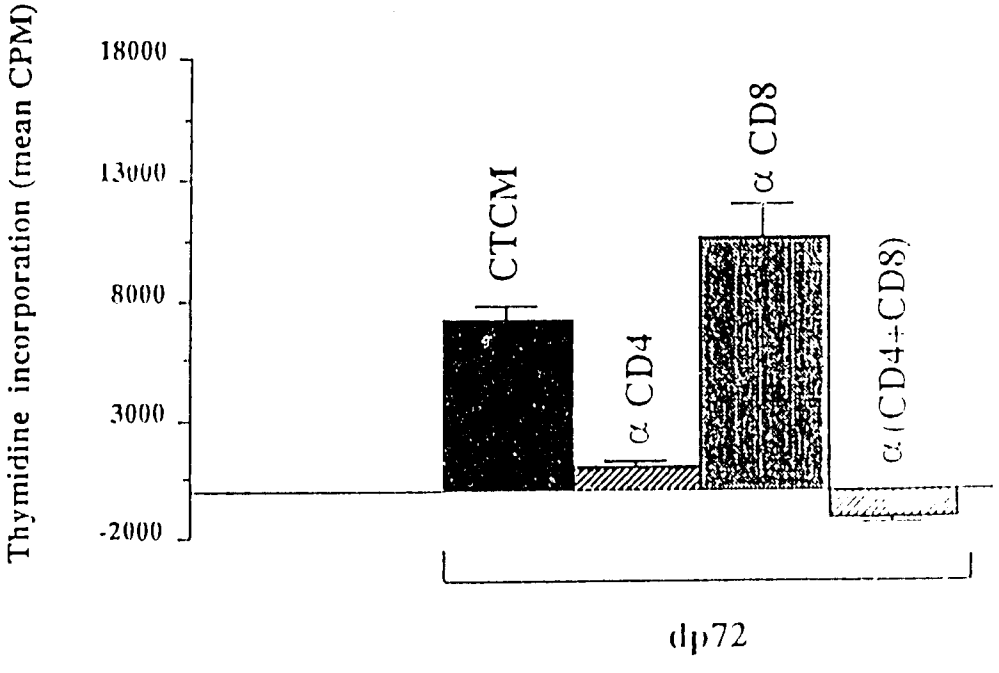


Figure11. Effect of T-cell subset depletion on proliferation to dp72. Mice immunized with dp72 (7.5 ug total per mouse) were sacrificed 8 d after the final boost. The spleen cells were incubated for 4 d with antigen and antibodies to CD4+, CD8+ or CD4+ and CD8+ T-cells. The proliferation was then determined by measuring [^{3}H]-thymidine incorporation for 18 h

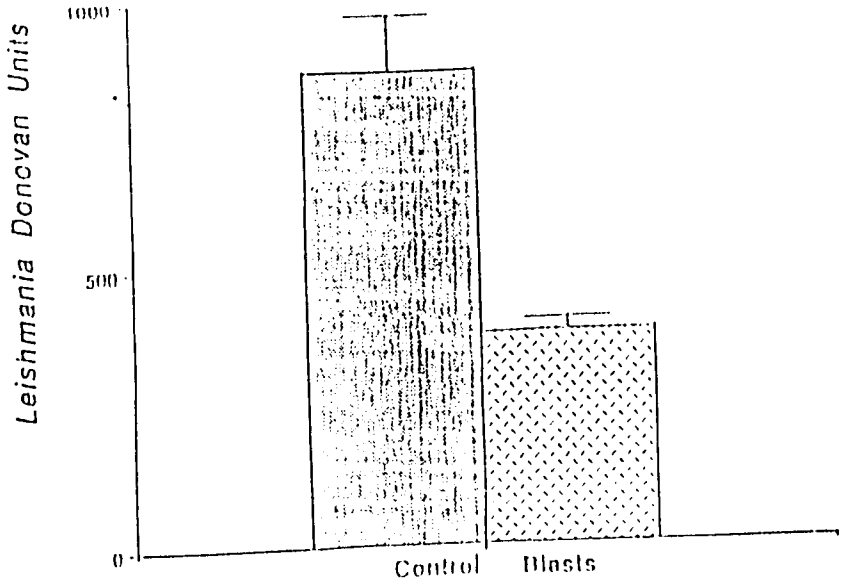


Figure 12. Effect of adoptive transfer of dp72 antigen-specific blasts to naive BALB/c mice on L. donovani infection. Spleen cells from immunized mice were incubated with dp72 for 5 days, purified on Percoll gradients and expanded with IL-2. Naive mice were injected, i.v., with 10^7 blasts/mouse and challenged 24h latter with 10^7 promastigotes i.v., ▨. Control mice, ■, were only challenged with promastigotes. After 18d the mice were sacrificed and the liver parasite burdens calculated from tissue biopsies.

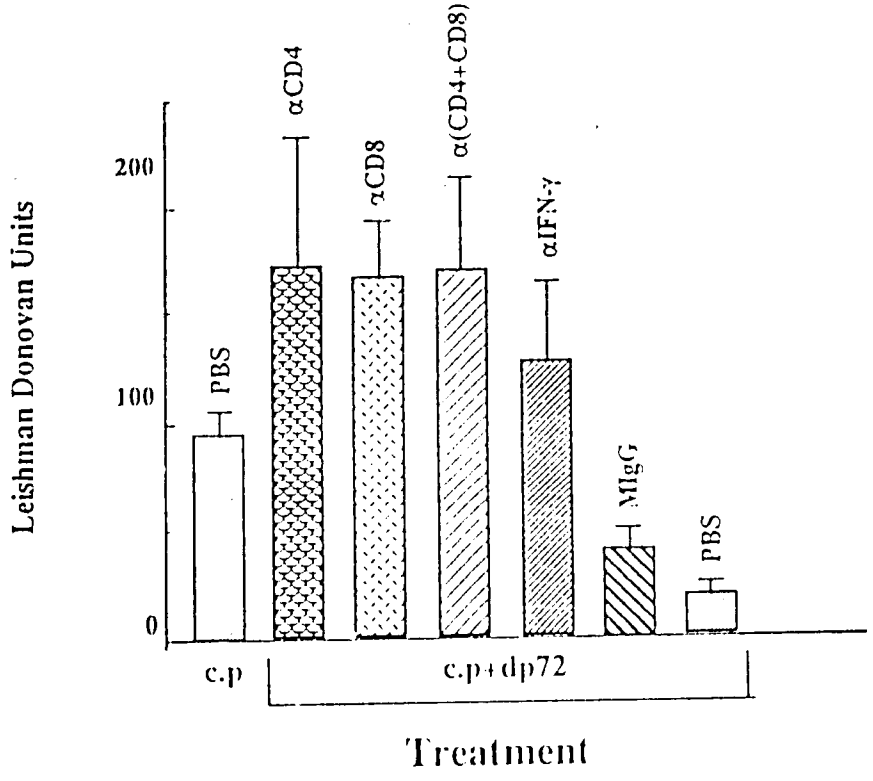


Figure 13. Passive transfer of antibodies to T-cell subsets or gamma-interferon (gIFN) and their affect on visceral leishmaniasis in BALB/c mice. Five days before challenge with promastigotes (10^7) of L.donovani a regime of injections of antibodies was begun. Immunized mice (6 ug total) received were treated with anti-CD4, -CD8, -CD4 plus CD8, -gIFN, IgG or PBS. Non immunized mice only received injections of PBS. At 18 d all the mice were sacrificed and the LDU calculated from liver biopsies. The mean LDU ± SE (n=6) is shown.

Table 3- Analysis of positivity in sandflies captured in Baturite ,State of Ceara, hybridized against the *L. braziliensis* probe .

species	number examined	number examined %	number positive sandflies	number positive/ species %
Lu. whitmani	21	13.1	3	14.3
Lu. migonei	50	31.3	1	2.0
Lu. wellcomei	4	2.5	–	–
Lu. shannoni	6	3.8	–	–
Lu. lenti	1	0.6	–	–
Lu. longipalpis	33	20.6	2	6.0
outros	11	6.9	–	–
not identified	34	21.2	2	5.9
total	160	100	8	–

The same samples were rehybridized against *L.chagasi* kDNA probe.None hibrydization was observed using this probe, supporting and confirming the *L.brasiliensis* infection of the positives

Table 4- Analysis of positivity by organs in rodents captured in the State of Ceará, Brazil, hibridized against the *L.brasiliensis* probe

organs	number of rodents positives	number of rodents positives %
skin/liver	16	28.6
liver	13	23.2
skin	03	5.3
skin/spleen	01	1.8
–	23	41.1
total	56	100

ALIGNMENT OF THE CONSERVED REGION OF LEISHMANIA MINICIRCLES

```
          1                                                  50
LEISH     AAAATTCCCAAACTTTTCTGGTCCTCTGGGTAGGGGCGTTCTGCGAAATC

MCLB      .....CGTACTC.CCGA.AT.-...........................
MCLG      .....BDTACHC.CCGA.AT.-......................--..AT
MCLAM     G.CCCG..........-...C-..GTG...G...........-.-...AT
MCLMA     CG..A.TT................CG......................T
MCLAE     T...A.T....G.AGG.AGTAG....C...............C.......
MCLC      C...A.................-..TC................T...-.
MCLD      C.......................T.A....................
MCLT      .....C..A..CT....AG.TC....A-..............C.....C.

         51                                                 100
LEISH     CGAAAAATGGCRTGCAGAAACCCCGTTCAAAAWTCGSCCAAAAA-TCGCS

MCLB      ...........A.A..................A...C......T-....G
MCLG      ...........A.A..................A..CC..G....-..A.G
MCLAM     G.C....AT.GG.............-...T.TTT-----------...GG
MCLMA     ..........-G................G.TTT.T.G..G....-.T..C
MCLAE     ........C.-A.A..................T.G.G.......-.-..C
MCLC      ..........-G........T...........A.G.C.A....T-G..GA
MCLD      G......AT.GG........T........TTTT.G.C..G....-.GC.-
MCLT      GA....T--..A....................A...G.......W....C

        101                                                 150
LEISH     ATTTTTGGGCTC-GGGGCGGAAAACTAGGGGTTGGTGTAAAATAGGGSGG

MCLB      ........C...-CCC.T.C.C..T...............T....T.G.C
MCLG      T.......C...-CCC.T.C.C..T...............T....T.G.C
MCLAM     GAA...C.....G..C.GT......G...................GC.
MCLMA     .........G..G.A.....G...T....................CC..
MCLAE     .....C......G.A....TC.......................CC..
MCLC      .AA.---.....C.............G...................C..
MCLD      .........TC.-.......G.....G...................CC.
MCLT      ......----..----.T.TG.......................GT.

        151                                                 200
LEISH     GGGGSTCCSGGGGTTKTGCTTTATKGGGKTGAATTTGTGCGTGKWAGCCG

MCLB      C.C.CA..CC..C.AGA.T.AG.ATT.ATAA..AG.....C.CTT..A..
MCLG      C.C.CA.HCC..C.DGA.Y.AGHABSHCG....A.A.-..C.CTT..AG.
MCLAM     .CT.C.T.G..CT.GT.T....T.GA..TC.CG.....TT.G.TAT.TAC
MCLMA     .T..G..GG..AA..AAAT.C.C.G...TG.CCGGGT..T...GGTCTG.
MCLAE     .T..AC.GG.AAT.CC.CGA..T.G..CC..GT.GGC..GAAAGTG...T
MCLC      .--.G.A.G....A.T.TGGG...TTT.GCC.GG.C.GC.TG.GACC.T.
MCLD      ..T.G.GGCT...A.GG....CCCT...C..G..-..GC.T .AA.C...
MCLT      ...CTC..C....HHT.C.BGG.AATC.GG.MY.YAT.TA.ACCGGBHAA
```

Figure 14. Sequencing alingment of the conserved regions of <u>Leishmania</u> minicircles. Minicircle sequences found in the Gene Bank and the sequences obtained in our laboratory were compared by the programs Lineup, GAP and Pileup.

FIGURES

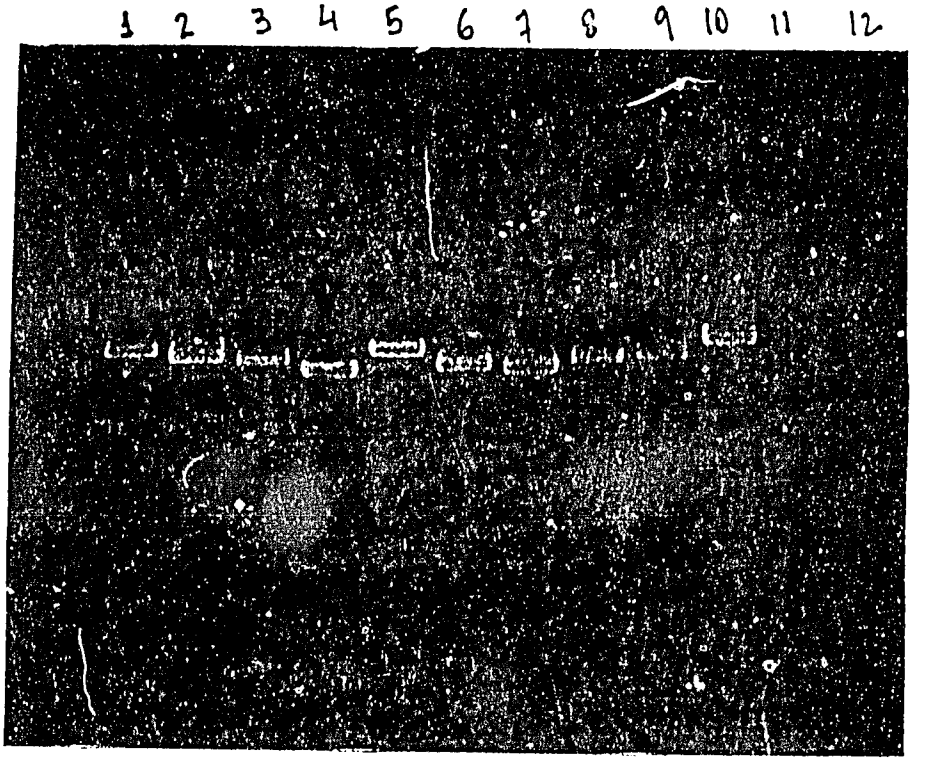
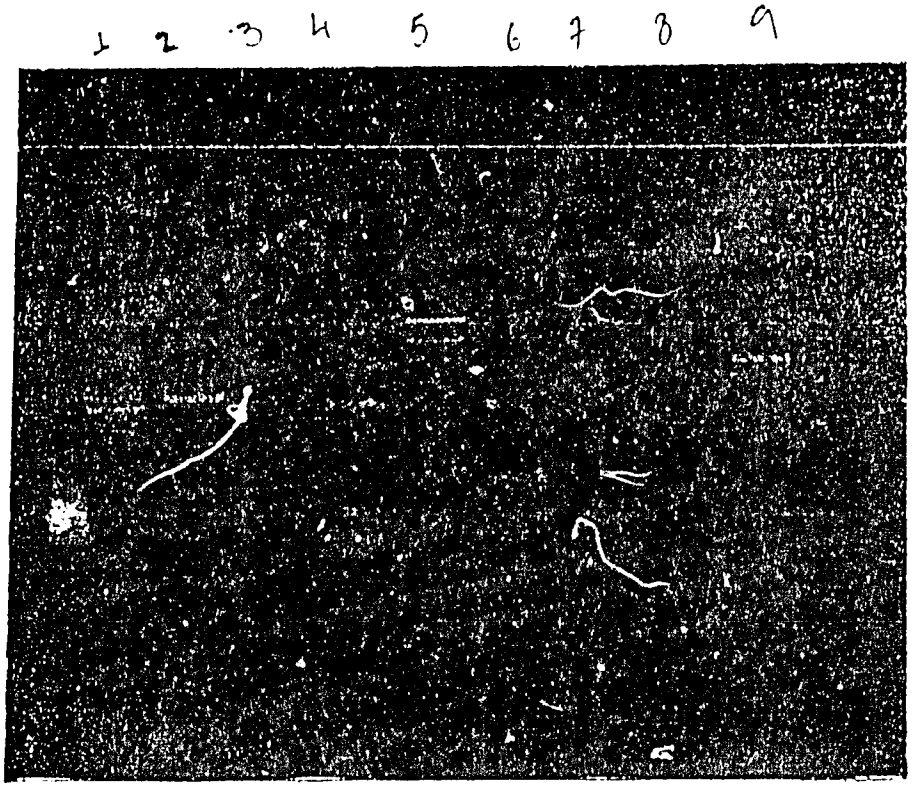


Figure 15. The variable region of different Leishmania isolates was amplified as previously described. Lane 1, molecular weight marker OX/Hae II; lane 2, L.donovani, lane 3, L. infatum, lane 4, L. chagasi, lane 5, L. major, lane 6, L. tropica, lane 7, L. amazonensis, lane 8, L. pifanoilane 9, L.braziliensis, lane 10, L. guyanensis, lane 11, L. tarentolae, lane 12,
negative control and lane 13 OX/Hae III

1 2 3 4 5 6 7 8 9 10 11 12

Figure 1b. Hybridization of the amplified variable regions. The same products listed in the Figure 1, were blotted and hybridized against the L.infantum product. Hybridization ocurred only with the homologous material, lane 2.



Figure|9. The amplified products of different Leishmania species were digested with Hae III. Lane 1, L.donovani; lane 2, L. chagasi; lane 3, L. major; lane 4, L. tropica; lane 5, OX/Hae III; lane 6, L.pifanoi;lane 7, L. braziliensis; lane 8, L. guyanensis and lane 9, L.tarentolae.

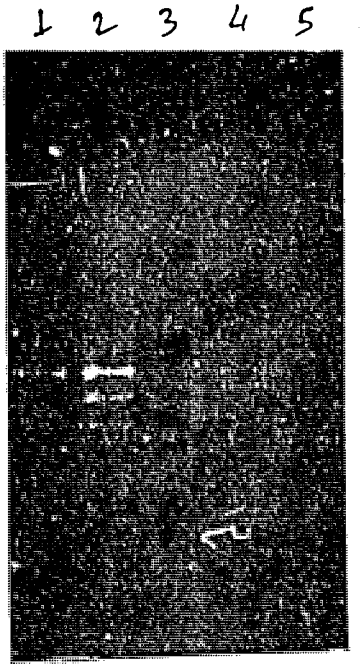


Figure 18. Schizodeme analysis of amplified variable regions of L.chagasi(state of Ceara, Br.),lane 1; L. chagasi (state of Rio de Janeiro, Br.), lane 2; L. chagasi (Tolima, Colômbia), lane 3; L. chagasi (Tolima Callejon, Colômbia), lane 4. Lane 5, ØX/Hae III. The PCR products were digested with Rsa I.

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THE IDENTITY OF *LEISHMANIA* ISOLATED FROM SAND FLIES AND VERTEBRATE HOSTS IN A MAJOR FOCUS OF CUTANEOUS LEISHMANIASIS IN BATURITE, NORTHEASTERN BRAZIL

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Abstract. During a field investigation carried out in Baturite, Brazil from 1989 to 1991, sand flies, sympatric rodents, domestic dogs and humans were surveyed for leishmaniasis. Twenty strains of *Leishmania* were isolated by in vitro culture from *Lutzomyia whitmani*, three strains were obtained from *Rattus rattus*, two strains from dogs, and five strains from humans. The isolates were characterized by isoenzyme electrophoresis by hybridization with kinetoplast DNA–specific probes. All the samples were identified as *L. (Viannia) braziliensis*. The importance of these results in the dynamics of the *Leishmania* infection in this focus is discussed.

The taxonomy of the genus *Leishmania* has been extensively modified.[1-3] Because of the complexity of the epidemiology and transmission of New World Leishmaniasis, the correct identification of the *Leishmania* populations, the phlebotomine vectors, and reservoir hosts is important in order to design effective control measures.

A major focus of cutaneous leishmaniasis in northeastern Brazil is in Baturite, a rural mountain area of the State of Ceara. However, no data are available on the identity of the *Leishmania* species present in this region. Moreover, little is known about the possible vectors and reservoir hosts in this area. Several species of *Lutzomyia* have been described in the Baturite area, and sympatric rodents have been reported to be infected with *Leishmania* in the State of Ceara.[4,5] As a first approach, we attempted to isolate flagellates from the putative *Leishmania* invertebrate and vertebrate hosts of the region, and then identify the species of the isolates.

MATERIALS AND METHODS

Strains and isolates

The isolates and the *Leishmania* reference strains used in this study are shown in Table 1. The World Health Organization (WHO) reference strains were donated by Dr. Ralph Lainson (Instituto Evandro Chagas, Belem, Brazil).

Isolation of the parasites

The primary isolation from humans was made by the in vitro cultivation of samples of skin biopsies or aspirates of enlarged lymph nodes. Rodents were killed and samples were taken from the liver, spleen, and skin. Biopsy specimens were also obtained from dogs. The samples from humans, dogs, and rodents were inoculated into blood agar medium with tissue culture media overlay and were examined for parasites twice a week for four weeks.[6] Samples of the some biopsies were triturated in saline and inoculated into golden hamsters when available. Female sand flies were dissected and examined; those found to contain flagellates were macerated in saline and inoculated into hamsters. Further isolation of parasites from infected hamsters was carried out by the inoculation of infected tissue into blood agar medium.

In vitro cultivation

The stocks were initially cultivated in blood agar base (Difco, Detroit, MI) culture medium as described elsewhere.[7,8] Promastigotes were cultivated until an optimal rate of growth was achieved. The parasites were then transferred to

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TABLE 1

Genotypic and phenotypic identification of the Leishmania *isolates obtained from vertebrate and invertebrate hosts from the Baturite area of Brazil from 1989 to 1991*

		Identification		
		Hybridization*		
Code	Host	*L.(V.) braziliensis* complex	*L. (L.) mexicana* complex	Isoenzyme†
H1389	Human‡	+	–	*L. (V.) braziliensis*
H1500	Human§	+	–	*L. (V.) braziliensis*
H1505	Human‡	+	–	*L. (V.) braziliensis*
H1511	Human§	+	–	*L. (V.) braziliensis*
H1512	Human§	+	–	*L. (V.) braziliensis*
R19407	Rodent	+	–	*L. (V.) braziliensis*
R19416	Rodent	+	–	*L. (V.) braziliensis*
R19418	Rodent	+	–	*L. (V.) braziliensis*
C35	Dog	+	–	*L. (V.) braziliensis*
C51	Dog	+	–	*L. (V.) braziliensis*
Lu600	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu612	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu613	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu631	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu690A	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu713C	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu727	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu735	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu1115	Phlebotomine	+	–	*L. (V.) braziliensis*
Lu1122	Phlebotomine	+	–	*L. (V.) braziliensis*

* The total kinetoplast DNA of the reference strains MHOM/BR/74, MHOM/BR/67/M2903, and IFLA/BR/68/PH8 was used as probes in the experiments.
† The results originated from the analysis of nine enzymes as described in the Materials and Methods.
‡ From lymph nodes.
§ From cutaneous lesions.

an enriched liquid medium, Schneider's *Drosophila* medium (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum, and incubated at 25°C. When the promastigotes were in the log phase of growth, they were harvested by centrifugation at 1,500 × *g* for 10 min at 4°C and washed twice in phosphate-buffered saline, pH 7.3. The final pellet was resuspended in lysis buffer containing 0.04 M NaCl, 0.01 M EDTA, and 0.01 M Tris, pH 8.0, for enzyme electrophoresis.

Enzyme electrophoresis

Electrophoresis was carried out in agarose gels as previously described.[8] Briefly, the agarose multiphore tank (#2117; LKB, Uppsala, Sweden) was connected to a refrigerator (LKB-ProductorAB) to regulate the temperature. A 1% agarose gel was prepared in the appropriate buffer with the final mixture heated to its boiling point. The agarose gel was spread over an electrophoresis bond film (Gelbond; FMC Bioproducts, Rockland, ME) placed horizontally and left to adhere over its entire surface. The buffers and stains used for the enzymes were as previously described.[8] The leishmanial extracts were run in many separate experiments and the order of the extracts was interchanged to allow side-by-side comparisons of the variants. All variants having mobilities similar enough to preclude consistent separation were scored conservatively as the same band. All isolates were studied with nine enzymes (Table 2).

Kinetoplast DNA (kDNA) hybridization assays

Approximately 10^4 promastigotes were harvested by centrifugation, washed in SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0), and dotted on nylon membranes (Gene Screen Plus; Dupont, Boston, MA). The membranes were soaked in solution containing 2 × SSC (5× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 100 μg/ml of Denhardt's solution (0.02% polyvinylpyrrolidone, 0.2% Ficoll, 0.02% bovine serum albumin), 50% formamide, and 100 μg/ml of denatured salmon sperm DNA at 42°C for 2 hr.[10]

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TABLE 2
Enzymes used in the identification of Leishmania *parasites*

Name	Enzyme Commission (EC) number	Abbreviation
Glucose phosphate isomerase	E.C.5.3.1.9	GPI
Nucleoside hydrolase	E.C.3.2.2.2	NH
Phosphoglucomutase	E.C.2.7.5.1	PGM
Glucose-6-phosphate dehydrogenase	E.C.1.1.1.49	G6PDH
Malic enzyme	E.C.1.1.1.40	ME
6-Phosphogluconate dehydrogenase	E.C.1.1.1.44	6PGDH
Proline dipeptidase	E.C.3.4.13.9	PEP D
Malate dehydrogenase	E.C.1.1.1.37	MDH
Isocitrate dehydrogenase	E.C.1.1.1.40	IDH

Approximately 100 ng of total kDNA of *L. (Viannia) braziliensis* MHOM/BR/68/M2903 and *L. (Leishmania) amazonensis* IFLA/BR/68/PH8 were either radiolabeled or used as controls in the hybridization experiments. In addition, a probe (pJL18) for a viscerotropic strain of *Leishmania* was also used in the experiments.[11] A typical hybridization reaction contained 1×10^5 cpm/ml of denatured kDNA probe and was carried out for 12 hr at 42°C. The membranes were washed with 0.1× SSC, 0.5% sodium dodecyl sulfate at 65°C.

RESULTS

Analysis of the electrophoretic migration of nine enzymes identified all isolates as *L. (V.) braziliensis* when compared with the reference strains. Figure 1 shows the enzymatic electrophoretic profiles of five isolates, selected among the strains from rodents, dogs, sand flies, and humans, which were identified in this study. The profile of four enzymes, malic enzyme, 6-phosphogluconate dehydrogenase, malate dehydrogenase, and glucose phosphate isomerase, could clearly discriminate the reference strains. These results were confirmed by the DNA hybridization method, as demonstrated in Figure 2. When used as a probe, the total kDNA of *L. (V.) braziliensis* recognized all the isolates as well as the total kDNA of *L. (L.) chagasi* (Figure 2A). The *L. (L.) amazonensis* kDNA probe did not react with any of the isolates, but it hybridized with the total kDNA of *L. (L.) chagasi*, as shown in Figure 2B. However, the specific minicircle clone pJL18 bound uniquely to *L. (L.) chagasi* (Figure 2C). We concluded from these results that the isolates from Baturite were *L. (V.) braziliensis*, since the other kDNA probes did not hybridize with them.

DISCUSSION

Cutaneous leishmaniasis is widespread in the New World and shows different ecologic patterns of transmission.[12,13] Several *Leishmania* species have been described as agents of human infections in Brazil.[14] In addition, different species may be found in the same focus, generating in some cases, mixed infections in humans hosts.[15,16]

We carried out an investigation in a major focus of cutaneous leishmaniasis in the State of Ceara, Brazil from 1989 to 1991. *Leishmania* isolates were obtained from humans, sand flies, dogs, and rodents. All the isolates were identified as *L. (V) braziliensis* by enzyme electrophoresis analysis of the nine enzymes used in the study. Furthermore, this result was confirmed by hybridization with specific kDNA probes. The total kDNA of *L. (V.) braziliensis* hybridized with the purified kDNA of *L. (L.) chagasi*. However, the real identity of the isolates could be demonstrated through the use of the cloned minicircle sequence pJL18 as a probe. This clone, which originated from a minicircle of an *L(L.) chagasi* reference strain, reacts solely with viscerotropic leishmanias.[11] Figure 2A clearly shows the binding of this probe only to the kDNA of *L. (L.) chagasi*, indicating that all the Baturite samples belong to the *Viannia* complex. The enzyme electrophoresis method is commonly used in the biochemical identification of *Leishmania* species and is the method recommended by WHO.[17] The application of the DNA hybridization technique in the detection and typ-

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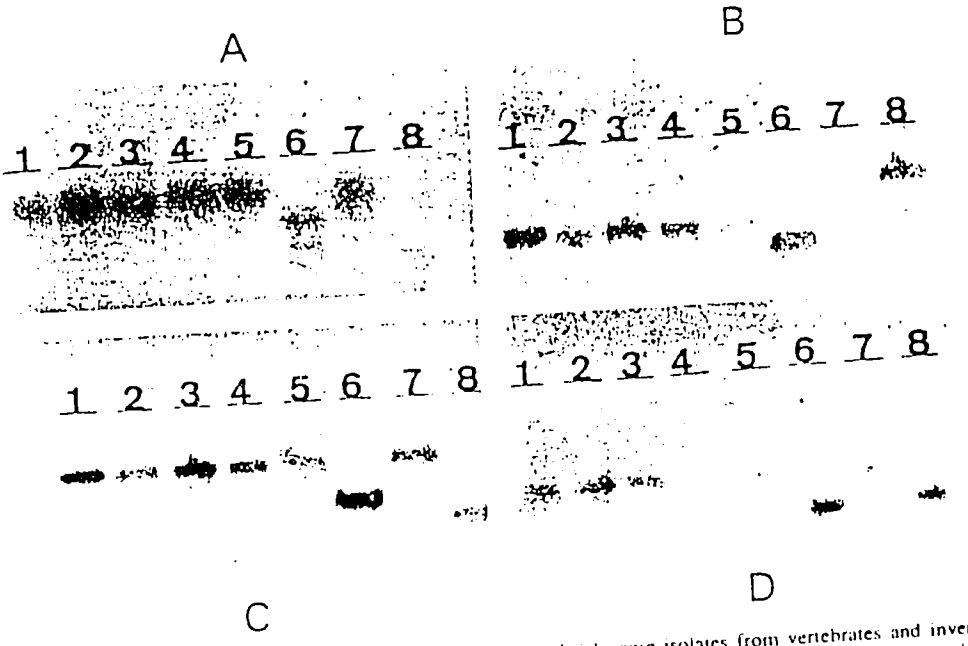

FIGURE 1. Enzyme electrophoresis showing representative *Leishmania* isolates from vertebrates and invertebrate hosts from Baturite, State of Ceara for the following enzymes: A, malate dehydrogenase; B, malic enzyme; C, 6-phosphogluconate dehydrogenase; D, glucose phosphate isomerase. The stocks tested were 1, C35; 2, R19407; 3, LU613; 4, H1511; 5, H1512; 6, IFLA/BR/68/PH8; 7, MHOM/BR/68/M2903; 8, MHOM/BR/74/PP75. Lanes 6–8 are reference strains.

ing of *Leishmania* isolates has been extensively reviewed.[18]

Many mammals have been described as hosts of *Leishmania*, and some of them are thought to play a role as reservoir hosts of dermotropic leishmanias.[3] In Brazil, silvatic rodents, as well as edentates and marsupials are reported to be the usual reservoir hosts of dermotropic leishmanias in the Amazonian basin, and these parasite isolates were identified by several methods.[19–21] Black rats (*Rattus rattus*) have been identified as the hosts of *Leishmania* in Brazil in the States of Ceara, Rio de Janeiro, and Para (Santos GPL and others, unpublished data).[3,22,24] Although most of these reports were based on the observation of amastigotes in tissues without any further characterization of the *Leishmania* species, these observations suggest that this commensal rodent is a participant in the transmission cycle of dermotropic leishmanias in the New World. Our results support this observation and suggest that sympatric rodents are hosts of *L. (V.) braziliensis* in the State of Ceara and could play a role as reservoirs of this parasite. Moreover, the presence of *Leishmania* infection in black rats may be more widespread in Brazil despite the absence of available data. In fact, the capture and the investigation of sympatric rats within cutaneous leishmaniasis sites is not usually done in field studies.

Dogs are major reservoirs of viscerotropic leishmanias in South America.[1,25] However, *L. (V.) braziliensis* infection in dogs has also been described by several investigators.[26,27] In some cases, dogs were among the domestic mammalian hosts of *Leishmania* in active foci of cutaneous leishmaniasis, where silvatic hosts were not found to be infected.[28,29] *Leishmania (V.) braziliensis* was identified in two of four isolates of dogs from Baturite. It is intriguing that canine infection by this parasite is found side-by-side with rodent infection, suggesting a domestic/peridomestic cycle of leishmaniasis transmission. The importance of dogs as reservoirs of *L. (V.) braziliensis* is still uncertain despite the previous finding of this parasite in this vertebrate.[22]

Our data also confirmed that *Lutzomyia whit-*

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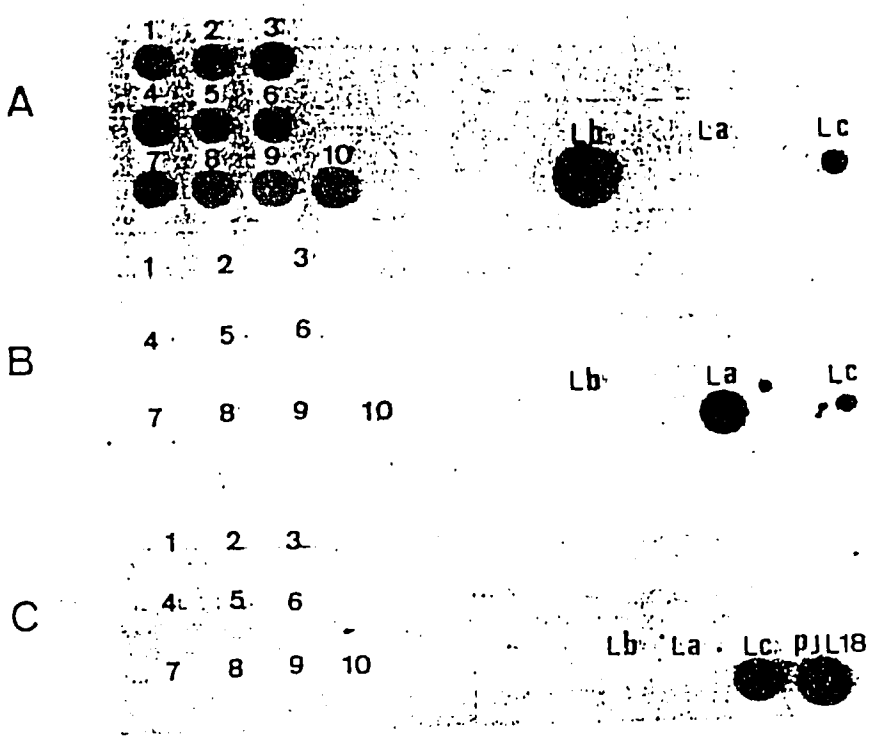

FIGURE 2. Kinetoplast DNA (kDNA) hybridization of 10 *Leishmania* isolates from Baturite. The isolates tested were 1, H1511; 2, H1512; 3, R19407; 4, 19416; 5, 19418; 6, C35; 7, C51; 8, Lu 600; 9, Lu 612; 10, Lu 631. Lb = 1 ng of total kDNA from the reference strain *L. braziliensis* MHOM/BR/68/M2903; La = 1 ng of total kDNA from *L. amazonensis* IFLA/BR/68/PH8; Lc = 1 ng of total kDNA from *L. chagasi* reference strain MHOM/BR/74/PP75; pJL18 = 1 ng of recombinant plasmid containing an *L. chagasi* minicircle insert. The membranes were hybridized with A, total kDNA of MHOM/BR/68/M2903, B, total kDNA of IFLA/BR/68/PH8, and C, viscerotropic *Leishmania*-specific probe pJL18.

mani can be a vector of *L. (V.) braziliensis* in the State of Ceara in northeastern Brazil. In addition, other phlebotomine species were also found to be naturally infected by this parasite in the Baturite region.[4] Further studies on the prevalence of infection in rodents and dogs and the ability of this parasite to infect sand flies will be required before these species can be implicated as natural reservoirs hosts. We also need to know the relative role of other sand fly species as vectors of *L. (V.) braziliensis*. This information is necessary for the design of control strategies for cutaneous leishmaniasis in this area.

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REFERENCES

1. Lainson R, Shaw JJ. 1972. Leishmaniasis in the New World: taxonomic problems. *Br Med Bull* 28: 44–48.
2. Chance ML. 1979. The identification of *Leishmania*. Taylor AER, Muller R, eds. *Problems in the Identification of Parasites and their Vectors*. Oxford: Blackwell Scientific Publication, 55–74.
3. Lainson R, Shaw JJ. 1987. Evolution, classification and geographical distribution. Peters W, Killick-Kendrick R, eds. *The Leishmaniasis in Biology and Medicine*. London: Academic Press, 1–128.
4. Queiroz RG, Vasconcelos IAB, Vasconcelos AWW, Sousa RN, Fe Filho NM, David JR. 1991. Natural infections in Phlebotomine sand flies of an endemic zone for American cutaneous leishmaniasis in Baturite hills, Ceara state, Northeast Brazil. *Mem Inst Oswaldo Cruz* 86: 256.
5. Queiroz RG, Vasconcelos AW, Vasconcelos IAB, Sousa RN, Pessoa FAC, Alencar JE, David JR. 1991. Phlebotomine sand fly (Diptera Psychodidae) fauna survey in an American cutaneous leishmaniasis (ACL) focus in Baturite, Ceara state, Northeast Brazil. *Parasitologia* 33: 159–167.
6. Shaw JJ, Lainson R. 1981. The in vitro cultivation of members of the *Leishmania braziliensis* complex. *Trans R Soc Trop Med Hyg* 75: 127.
7. Hendricks LD, Wood DE, Hadjuk ME. 1978. Haemoflagellates: commercially available liquid media for rapid cultivation. *Parasitology* 76: 309–316.
8. Momen H, Grimaldi G Jr, Pacheco RS, Jaffe CL, McMahon-Pratt D, Marzochi MCA. 1985. Brazilian *Leishmania* stocks phenotypically similar to *Leishmania major*. *Am J Trop Med Hyg* 34: 1076–1084.
9. Sales CA, Da Silva AR, Momen H. 1986. Enzyme typing and phenetic relationships in *Vibrio cholerae*. *Rev Bras Genet* 9: 407–419.
10. Wirth DF, McMahon-Pratt D. 1982. Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. *Proc Natl Acad Sci USA* 79: 6999–7003.
11. Lopes UG, Wirth DF. 1986. Identification of visceral *Leishmania* species with cloned sequences of kinetoplast DNA. *Mol Biochem Parasitol* 20: 77–84.
12. Lainson R. 1983. The American leishmaniasis: some observations on their ecology and epidemiology. *Trans R Soc Trop Med Hyg* 77: 569–596.
13. Shaw JJ, Lainson R. 1987. Ecology and epidemiology: New World. Peters W, Killick-Kendrick R, eds. *The Leishmaniasis in Biology and Medicine*. London: Academic Press, 291–363.
14. Grimaldi G Jr, Tesh RB, McMahon-Pratt D. 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. *Am J Trop Med Hyg* 41: 687–725.
15. Silveira FT, Lainson R, Shaw JJ, Ribeiro RSM. 1984. Leishmaniose cutanea na Amazonia. Registro do primeiro caso humano de infeccao mista, determinado por duas especies distintas de leishmanias: *Leishmania braziliensis* e *L. mexicana amazonensis*. *Rev Inst Med Trop Sao Paulo* 26: 27–275.
16. Oliveira Neto MP, Marzochi CA, Grimaldi G Jr, Pacheco RS, Toledo LS, Toledo LM, Momen H. 1986. Concurrent human infection with *Leishmania donovani* and *Leishmania braziliensis braziliensis*. *Ann Trop Med Parasitol* 80: 587–592.
17. World Health Organization. 1986. Control of the leishmaniases. *World Health Organ Tech Rep Ser* 793: 128–129.
18. Barker DC, Butcher J. 1984. The use of DNA probes in the identification of leishmanias: discrimination between isolates of the *Leishmania mexicana* and *L. braziliensis* complexes. *Trans R Soc Trop Med Hyg* 77: 285–297.
19. Lainson R, Shaw JJ, Povoa M. 1981. The importance of edentates (sloths and anteaters) as primary reservoirs of *Leishmania braziliensis guyanensis*, causative agent of pian bois in north Brazil. *Trans R Soc Trop Med Hyg* 75: 611–612.
20. Arias JR, Naiff RD, Miles MA, Souza AA. 1981. The opossum *Didelphis marsupialis* (Marsupialia:Didelphidae) as a reservoir host of *Leishmania braziliensis guyanensis* in the Amazon Basin of Brazil. *Trans R Soc Trop Med Hyg* 75: 537–541.
21. Lainson R, Shaw JJ, Ready PD, Miles MA, Povoa M. 1981. Leishmaniasis in Brazil: XVI. Isolation and identification of *Leishmania* species from sandflies, wild animals and man in north Para State, with particular reference to *L. braziliensis guyanensis* causative agent of "pian bois". *Trans R Soc Trop Med Hyg* 75: 530–536.
22. Vasconcelos IAB, Vasconcelos AW, Lopes UG, Santos ANM Jr, Abreu RMR, Lima JWO, Alencar JE. 1987. Reservoir host of *L. b. braziliensis* in a peculiar and coastal-sited focus of cutaneous leishmaniasis in Ceara state. *Mem Inst Oswaldo Cruz* 82: 200.
23. Alencar JE, Pessoa EP, Fontenele ZF. 1960. Infeccao natural de *Rattus alexandrinus* por *Leishmania* (provavelmente *L. braziliensis*) em zona endemica de leishmaniose tegumentar do estado do Ceara, Brasil. *Rev Inst Med Trop Sao Paulo* 2: 347–348.
24. Araujo-Filho NA, Coura JR, Reis VLL. 1981. Leishmaniose tegumentar americana na Ilha Grande, Rio de Janeiro. III. Reservatorios silvestres e comensais. *Rev Soc Bras Med Trop* 14: 153–161.
25. Chagas E, Cunha AM, Ferreira LC, Deane G, Guimaraes FN, Paumgartten ML, Sa B. 1938. Leishmaniose visceral Americana (Relatorio dos trabalhos realizados pela comissao encarregada do estudo da leishmaniose visceral American a

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35

em 1937). *Mem Inst Oswaldo Cruz 32*: 321–390.

26. Cuba Cuba CA, Miles MA, Vexenat A, Barker DC, McMahon-Pratt D, Butcher J, Barreto AC, Marsden PD, 1985. A focus of mucocutaneous leishmaniasis in the Tres Bracos, Bahia, Brazil: characterization and identification of *Leishmania* stocks isolated from man and dogs. *Trans R Soc Trop Med Hyg 79*: 500–507.

27. Falqueto A, Coura JR, Barros GC, Grimaldi G Jr, Sessa PA, Carias VR, de Jesus AC, de Alencar JT, 1986. Participation of the dog in the cycle of transmission of cutaneous leishmaniasis in the municipality of Viana, state of Espirito Santo, Brazil. *Mem Inst Oswaldo Cruz 81*: 115–116.

28. Rosa AC, Cuba CC, Vexenat A, Barreto AC, Marsden PD, 1988. Predominance of *Leishmania braziliensis braziliensis* in the regions of the Tres Bracos and Corte de Pedra, Bahia, Brazil. *Trans R Soc Trop Med Hyg 82*: 409–410.

29. Barreto AC, Cuba Cuba C, Vexenat JU, Rosa A, Marsden PD, Magalhaes AV, 1984. Caracteristicas epidemiologicas da leishmaniose tegumentar americana em uma regiao endemica do estado da Bahia. II. Leishmaniose canina. *Rev Soc Bras Med Trop 17*: 59–65.

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Pure Protein from *Leishmania donovani* Protects Mice against Both Cutaneous and Visceral Leishmaniasis[1]

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Abstract. A protein purified from *Leishmania donovani* promastigotes, dp72, was shown to partially protect BALB/c mice against a challenge by this parasite. Immunized mice infected i.v. with 10^7 *L. donovani* promastigotes showed a 0, 60, and 78% reduction in liver parasite burden compared with the control mice at each time point examined after challenge, 1, 20, and 108 days, respectively. Western blotting demonstrated that the sera from the immune mice, which reacted specifically with dp72 in lysates of *L. donovani*, cross-reacted with one major band in total homogenates of *Leishmania major* and *Leptomonas collosoma*. Lymphocyte proliferation to crude and pure parasite Ag was also examined in mice immunized with dp72. Strong proliferation was found at low concentrations of crude *L. donovani* Ag (0.5 μg/ml) and with pure dp72. Proliferation at higher concentrations to crude *L. major* and *L. collosoma* Ag was observed. Little or no reaction (stimulation index < 1.0) was seen with other pure leishmanial Ag, including gp70-2, the promastigote surface protease, and lipophosphoglycan. Depletion in vitro of the CD4+ T cell subset from immune spleen cells abolished proliferation to dp72, whereas depletion of CD8+ T cells enhanced proliferation to the pure Ag. Experiments in vivo showed that immunized mice treated with antibodies to either CD4+, CD8+, both T cell subsets, or to IFN-γ had larger LPB (178, 173, 176, and 130%, respectively) after challenge with *L. donovani* than nonimmunized controls. Mice immunized with dp72 but treated with either PBS or mouse Ig showed reduced LPB, 81 and 61%, respectively, compared with the nonimmunized animals. BALB/c mice immunized with dp72 were also protected against *L. major* which causes cutaneous leishmaniasis. Immunized mice infected with either 10^4 or 10^6 promastigotes did not develop lesions. Limiting dilution assays confirmed the protection. *Journal of Immunology*, 1993; 150: 2322.

The protozoan parasites *Leishmania* cause a spectrum of different human diseases. The three major forms include: CL,[3] mucocutaneous leishmaniasis, and VL (1–3). VL is a disease of high morbidity normally, associated with anergy to parasite Ag, hypergammaglobulinemia, hepatosplenomegaly, and death (1–3).

Little is known about the immunology of VL. Most studies have been carried out on CL in humans, or using mice as a model. From these studies, it is now clear that the course of CL is T cell dependent. This disease in susceptible and resistant mice can be altered by immunologic stratagems involving CD4+ cells. In vitro and in vivo evidence point to the role of several lymphokines, especially IFN-γ, IL-4, and TNF-α, in the outcome of the disease (4–6). Re-

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[3] Abbreviations used in this paper: CL, cutaneous leishmaniasis; CTCM, complete tissue culture medium; LDA, limiting dilution assay; LDU, Leishman-Donovan units; LPB, liver parasite burden; LPG, lipophosphoglycan; PSP, promastigote surface protease; T-PBS, PBS containing 0.05% Tween 20, pH 7.0; VL, visceral leishmaniasis; S.I., stimulation index; A-PSP, amphiphilic-PSP; H-PSP, hydrophilic-PSP; ANOVA, analysis of variance.

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parvum as an adjuvant per mouse. *C. parvum* was a gift from Dr. F. Y. Liew, Wellcome Research Laboratories, Beckenham, England. Control mice received adjuvant alone for the first boost and PBS for the second and third boosts. After 2 wk each mouse was given a second boost i.p of 1.5 μg pure dp72 without adjuvant and 2 wk later a third boost i.p of 1.5 μg dp72, for a total of 6 μg protein. Two weeks after the third boost all the mice were challenged with *L. donovani* (Khartoum), 10^7 promastigotes i.v per mouse, or *L. major* (Fredlin), 10^4 or 10^6 promastigotes s.c in the right rear footpad. Protection was evaluated in experiments with *L. donovani* by killing the mice and examining liver touch biopsies microscopically after staining with Diff-Quick. The number of amastigotes per 600 liver (or spleen) cell nuclei were counted, except for experiments ended after 1 day where amastigotes per 1200 cell nuclei were determined.

LDU were calculated (34) as follows: LDU = (number amastigotes/number liver nuclei) × weight of liver in milligrams. The specific time intervals used from infection to killing are given in the results. Protection against *L. major*, which causes CL, was evaluated by measuring footpad thickness at weekly intervals with a dial micrometer (L. S. Starrett Co., Athol, MA). The swelling of the infected footpad (millimeters) was compared to the contralateral uninfected footpad. In some cases, the number of parasites in either the infected footpad (challenge with *L. major*) or liver (challenge with *L. donovani*) was measured by LDA (35).

Depletion in vivo

Mice immunized as described above were injected 5 days before parasite challenge, daily, with supernatants containing mAb (20 μg/day/mouse) against the T cell surface Ag, $CD4^+$ (GK1.5), $CD8^+$ (H35), or both $CD4^+$ and $CD8^+$. Immunized mice were also treated in parallel with a mAb against IFN-γ (HB170) (20 μg/day/mouse), PBS, or mouse IgG (25 μg/mouse). Nonimmunized mice received injections of PBS. After challenge i.v. with 10^7 *L. donovani* promastigotes, the mice were injected every 2 to 3 days with the same antibodies. On day 20 the animals were killed and the amastigotes counted in the liver and spleen as described above. mAb GK1.5 (IgG2b), anti-CD4, was generously provided by Dr. F. W. Fitch, University of Chicago, Chicago, IL (36). mAb H35-17.2 (IgG2b), anti-CD8, was kindly provided by Dr. M. Pierres, INSERM-CNRS, Marseilles, France (37). The anti IFN-γ mAb, HB170 (IgG1), was obtained from the American Tissue Culture Collection, Rockville, MD.

Lymphocyte proliferation

Mice were immunized twice i.p., essentially as described above for the protection experiments. The only change was in the amount of dp72 used, 5 μg and 2.5 μg for the first and second boosts, respectively. In the case of proliferation with pure Ag the mice received a third boost of 10^7 irradiated (100 krad) *L. donovani* promastigotes. Spleen cells from immunized mice were adjusted to 5×10^6/ml in CTCM comprising 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 5×10^{-5} M 2-ME in RPMI 1640, supplemented with either 1% normal mouse serum or 5% FCS (Seromed lot 5708, Germany). Cells (100 μl) were incubated 4 days in CTCM with either 100 μl pure Ag (dp72, gp70-2, A-PSP, H-PSP, LPG, or phosphoglycan), or lysates (*L. donovani*, *L. major*, or *L. collosoma*). The concentrations of the various Ag are given in *Results*. The cells were then pulsed for 18 h with 1 μCi/well methyl-[^{3}H]TdR. (NEN Research Products, Boston, MA) and harvested onto glass fiber filters by means of a multiple automated sample harvester (Cambridge Technology, Cambridge, MA). Incorporation of [^{3}H]TdR was assessed in a beta counter.

For T cell depletion in vitro, spleen cells were incubated 4 days with dp72 and supernatants, diluted 1/10, containing either anti-CD4 (GK1.5), anti-CD8 (H35), or mixture of anti-(CD4 and CD8) mAb. The percentage of the $CD4^+$ and $CD8^+$ T cells remaining after this treatment was determined in similar experiments by FACS. The cultures were then pulsed for 18 h with methyl-[^{3}H]TdR (1 μCi/well) and processed as described above.

Statistical analysis

One way or two way with contrast ANOVA tests or Student's *t*-test were used to determine the statistic significance of differences found between parasite loads in various experiments and groups of mice.

Results

Analysis of antibodies produced in mice immunized with dp72

BALB/c mice, which are susceptible to both VL and CL, were immunized three times with dp72 (total 6 μg/mouse, i.p.). Dp72 was purified from an Ethiopian strain (LV9) of *L. donovani* and the purity of the Ag monitored by SDS-PAGE and silver staining (Fig. 1A). *C. parvum* was only used as adjuvant in the first boost (100 μg/mouse). This adjuvant was successfully employed in several protection studies against *Leishmania* (15, 19, 20, 29). Control mice received an equivalent amount of *C. parvum* in PBS. Ten days after the second boost, sera were obtained for analysis. Pooled sera, diluted 1/100, were examined by SDS-PAGE and Western blotting on total Ag lysates of *L. donovani* (LV9), *L. major* (Fredlin), and *L. collosoma*. Mice immunized with pure dp72 reacted strongly with one band, m.w. ≈ 70 kDa, in lysates of *L. donovani* out to dilutions of at

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cent experiments have shown that the final outcome of CL is related to the selective stimulation of the different CD4+ subsets, Th1 and Th2, although CD8+ T cells appear to influence disease outcome (4). These subsets can be distinguished by the different lymphokines. T cells belonging to the Th1 subset secrete IFN-γ and IL-2, whereas cells belonging to the Th2 subset produce IL-4, IL-5, IL-6, and IL-10 (7, 8). In general, adoptive transfer of clones belonging to the Th1 subset results in protection, whereas those belonging to the Th2 subset cause exacerbation of disease (4–6).

Studies in mice infected with VL show no evidence for the production of CD4+ Th2 restricted cytokines, IL-4, and IL-5, and no correlation between the overexpansion of the Th2 subset and the seriousness of infection (9). Indeed, recent evidence suggests that both CD4+ and CD8+ T cells are required for immunity to VL (10). However, a correlation between the serum levels of IL-4 or IgE and disease has been shown for human VL (11).

Vaccination for CL, which is self-healing, is believed to be possible. Certain subcellular fractions (12–17), as well as pure Ag (18–20) and avirulent parasites (3, 5), have been implicated as potential vaccine candidates. Few similar studies have been carried out with VL. However, several recent reports that asymptomatic VL, which self-cures, is present in at least 15% of the population infected by *Leishmania donovani* suggest that a vaccine should be possible for VL (21, 22). Similar reports have been made for canine VL (23). Early studies in animal models immunized with killed parasites have demonstrated that reductions in LPB can be achieved (24–26), although little examination of the specific Ag or immunologic mechanisms involved in this protection has been made. Recently, we purified two proteins from *L. donovani*, dp72 and gp70-2 (27). These proteins are specifically recognized by serum from patients or dogs with VL (27, 28). Preliminary experiments with BALB/c mice showed that immunization with dp72 can reduce LPB by 81% compared with the control mice (29). The protein dp72 is present in both stages of the parasite life cycle (promastigotes and amastigotes) and a related protein has been identified in other species of *Leishmania* (29). In this study we further investigate the protection of BALB/c mice immunized with dp72 against VL caused by *L. donovani*. In addition, we show that the pure *L. donovani* protein can also protect mice against a second species, *Leishmania major*, which causes CL.

Materials and Methods

Parasites

Promastigotes for Ag were grown in Schneider's *Drosophila* medium containing 10% FCS (Biolab, Jerusalem, lot 769) plus antibiotics (streptomycin, 0.1 mg/ml; penicillin 100 U/ml). Stationary phase parasites used to challenge mice were grown on blood agar (30). The isolates employed in these studies were obtained from Dr. L. Hendricks, Walter Reed Army Institute, Washington, DC (*L. major*, WR309); Dr. J. Keithly, Cornell Medical School, New York, NY (*L. donovani*, Khartoum); Dr. S. Reed, Issaquah Health Research Institute, Seattle, WA (*L. donovani chagasi*, Edmael); Dr. M. Hommel, Liverpool School of Tropical Medicine, Liverpool, UK (*L. donovani infantum*, LV9); Dr. D. Sacks, LPD, National Institutes of Health, Bethesda, MD (*L. major*, Fredlin), and Dr. S. Michaeli, Weizmann Institute of Science, Rehovot, Israel (*Leptomonas collosoma*).

Preparation of parasite Ag

Crude parasite Ag for Western blots, ELISA, or lymphocyte proliferation were prepared as described (29). The two *L. donovani* proteins, dp72 and gp70-2, were purified by affinity chromatography as previously reported (27) from crude promastigote membranes of either Edmael (MHOM/BR/00/A2) or LV9 (MHOM/ET/67/L82). Eluted material was analyzed by Western blotting and silver staining after SDS-PAGE (31). The hydrophilic and amphiphilic forms of PSP were prepared according to Reference 32 and were kindly provided to us by Drs. Bordier and Rivier from the University of Lausanne, Lausanne, Switzerland. LPG and PG were prepared by affinity chromatography (20) and were gifts from Dr. E. Handman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

SDS-PAGE and Western blotting

The reactions of pooled mouse serum were examined using total lysates from *L. donovani infantum* (LV9, 400 μg/gel), *L. major* (Fredlin), and *L. collosoma* (1200 μg/gel) as Ag. Proteins were separated on 10% gels by SDS-PAGE and electrophoretically transferred to 0.45 μm nitrocellulose (33). After quenching for 1 h in 50 mM PBS, pH 7.0, containing 0.3% Tween 20, membrane strips for each Ag were incubated 2 h at room temperature with pooled immune mouse sera, diluted 1/1000 in T-PBS. The strips were washed several times with T-PBS and probed (1 h at room temperature with [^{125}I]goat anti-mouse IgG (10 μCi in T-PBS; 2% FCS, New England Nuclear, Boston, MA). After additional washes with T-PBS the blots were dried and exposed for autoradiography.

Protein determination

Membrane and lysate protein concentration was determined using the Bio-Rad protein reagent (Richmond, CA). The amount of pure dp72 and gp70-2 was established by an Auro-Dye-binding assay (29).

Immunization and protection studies

Female BALB/c mice (Olac, Bicester, UK) were immunized i.p. with 3 μg pure dp72 and 100 μg *Corynebacterium*

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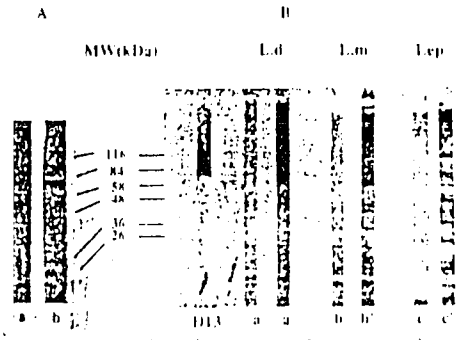

FIGURE 1. *A*, analysis by SDS-PAGE of pure dp72 from *L. donovani*. The protein was purified by affinity chromatography, separated by SDS-PAGE, and the gel silver was stained (27). *Lane a*, SDS-sample buffer, *lane b*, purified dp72. *B*, analysis by Western blotting of pooled sera from BALB/c mice immunized with pure dp72. Mice were immunized with dp72 (6 μg total plus 100 μg *C. parvum* as adjuvant/mouse). Control mice received adjuvant alone. Western blots were carried out on lysates of *L. donovani* (*L.d*), *L. major* (*L.m*) and *L. collosoma* (*Lep*), using 1/100 dilutions of pooled sera. As a positive control mAb D13 (diluted 1/4) against dp72 was included. *Lane D13*, mAb D13; *lanes a* to *c*, control mouse serum; *lanes a'* to *c'*, pooled sera from immunized mice.

least 1/1000. The mobility of this band is identical to dp72 detected in the same Western blot with a monoclonal or polyclonal antibody specific to this Ag (Fig. 1*B* and data not shown). Because the mAb to dp72 (D13) was previously found to cross-react with a lower m.w. protein in several other species of *Leishmania* (29), we examined the reaction of the anti-dp72 mouse sera on lysates of other *Kinetoplastidia*. The pooled mouse sera reacted with one major band around 60 or 100 kDa in lysates prepared from *L. major* or *Leptomonas*, respectively (Fig. 1*B*).

Some additional weak bands, m.w. < 45 kDa, were also seen with all the lysates (*lanes a'* to *c'*), but these appear to be nonspecific reactions and are also visible using the normal mouse sera (*lanes a* to *c*). Three times the amount of *L. major* and *Leptomonas* lysate was loaded on the gel in order to detect reactions with the pooled mouse sera produced to the pure *L. donovani* protein. No reaction was seen with lysates of *Crithidia fasciculata* (data not shown).

Proliferative response of lymphocytes from dp72-immunized mice

The proliferation of spleen cells from mice immunized with dp72 (7.5 μg total plus *C. parvum*, i.p.) to crude and pure Ag was assayed. Lymphocytes from these mice showed strong proliferation to crude freeze/thawed lysates of *L. donovani* and *L. major* (7962 and 9207 cpm, respectively, at 0.05 and 2.5 μg/ml of Ag), but only weak reactions with lysates prepared from *Leptomonas* (3102 cpm at 2.5 μg/ml of Ag), (Fig. 2). However, strong proliferation was still

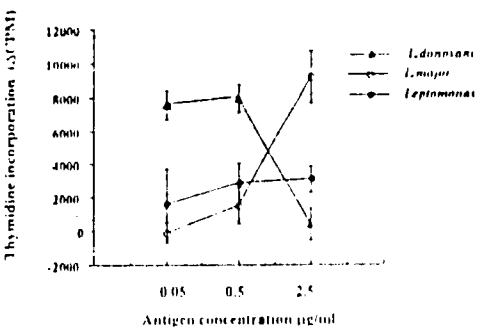

FIGURE 2. Proliferative response of lymphocytes from a dp72-immunized mice to lysates of *Leishmania* and other related species. Spleen cells from a BALB/c mouse immunized with dp72 (7.5 μg total plus 100 μg *C. parvum*/mouse) were cultured for 4 days with freeze/thawed extracts of *L. donovani* (Khartoum), *L. major* (Fredlin), or *L. collosoma*. The incorporation of methyl[^{3}H]TdR (1 μCi/well) after an additional 18 h was measured and the mean cpm of triplicate culture ± SE is shown.

observed when 50-fold less crude *L. donovani* Ag than for *L. major* was used. Lymphocyte proliferation in dp72-immunized mice that received a third boost of 10^7 irradiated *L. donovani* promastigotes i.v. was examined with pure Ag. Strong proliferation to pure dp72 (S.I. = 46.2) was observed, with the peak at 0.5 μg/ml Ag (Fig. 3). Weak reaction with gp70-2 (S.I. = 0.9) or no reaction was found with the Ag PSP and LPG at concentrations from 0.1 to 2.0 μg/ml Ag. Both the hydrophilic and amphiphilic form of PSP were examined. A weak proliferation (S.I. = 12.2) was consistently observed to phosphoglycan (Fig. 3) at all Ag concentrations examined (0.1 to 2.0 μg/ml).

The T cell subset responsible for proliferation to pure dp72 was characterized by antibody-mediated depletion in vitro. Lymphocytes from mice immunized with only dp72 (total 7.5 μg) were incubated in the presence of dp72, with or without antibodies against the T cell subsets $CD4^+$ (GK1.5), $CD8^+$ (H35), or both subsets. Thymidine uptake showed that proliferation to dp72 was inhibited by both anti-CD4 antibodies (86% inhibition) or a mixture of anti-CD4 and anti-CD8 antibodies (>100% inhibition). Addition of anti-CD8 antibodies alone consistently enhanced proliferation (150%) compared with the control cells (Fig. 4). Similar results were obtained using rat anti-CD4 or -CD8 IgM mAb plus C (data not shown).

Protection studies against VL

BALB/c mice are of the susceptible non-cure phenotype Lsh^r, H-2^d. When challenged with *L. donovani* amastigotes they show a rapid rise in LPB over the first 2 to 4 wk, after which the parasite burden remains more or less constant depending on the challenge dose used (38, 39). Two weeks after the final boost, mice (immunized and control) were

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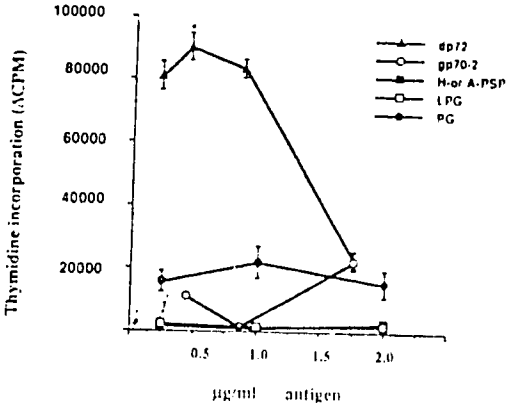

FIGURE 3. Proliferative response of cells taken from dp72-immunized mice to pure leishmanial Ag. Spleen cells from a BALB/c mouse immunized with dp72 (7.5 μg total plus 100 μg *C. parvum* and 10^7 irradiated *L. donovani* promastigotes, 100 krad), were cultured 4 days with the following pure Ag: dp72; gp70-2; H-PSP or A-PSP form; LPG and phosphoglycan. The proliferative response was determined by measuring methyl-[^{3}H]TdR incorporation (1 μCi/well) over 18 h. The mean cpm of triplicate cultures ± SE is shown.

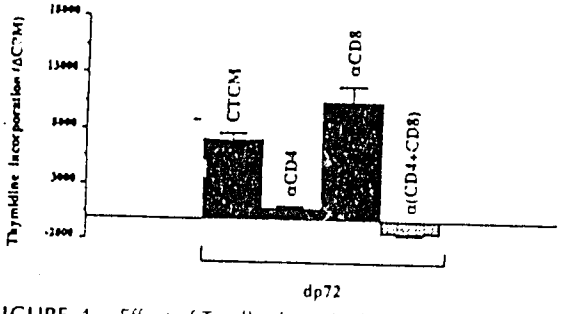

FIGURE 4. Effect of T cell subset depletion on proliferation to dp72. BALB/c mice immunized with dp72 (7.5 μg total plus 100 μg *C. parvum*/mouse) were killed 8 days after the final boost. The spleen cells were incubated for 4 days with Ag (CTCM) or Ag plus the following antibodies, anti-CD4 (GK1.5), anti-CD8 (H35), or anti-CD4 plus anti-CD8. The cells were pulsed with 1 μCi/well methyl-[^{3}H]TdR for 24 h and the incorporation was counted. The mean cpm of triplicate cultures ± SE is shown.

challenged with 10^7 *L. donovani* stationary phase promastigotes and the kinetics of protection evaluated by killing mice after 1, 20, and 108 days. Touch biopsies were made from each group and the mean number of amastigotes per liver was determined. Results are presented as LDU (34) in Figure 5. One day after the challenge both the immunized and the control group had the same LPB, 22.7 and 21.2 LDU, respectively ($p = 0.98$) (Fig. 5). However, by 20 days after the challenge the immunized mice showed a significantly lower LPB, 60% ($p < 4.7 \times 10^{-7}$), compared with the control group (Fig. 5). The difference became even more pronounced by 108 days after challenge where the parasite burden was 78% lower ($p < 5 \times 10^{-6}$) for the

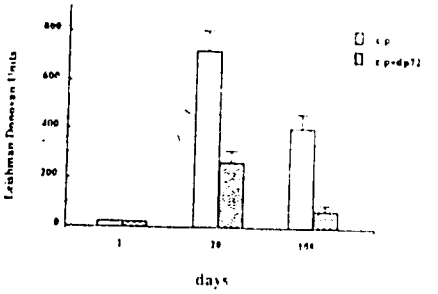

FIGURE 5. Kinetics of protection against *L. donovani* in mice immunized with dp72. BALB/c mice were immunized with dp72 (6 μg total plus 100 μg *C. parvum*/mouse). Control mice received adjuvant alone. Mice were challenged i.v. with 10^7 *L. donovani* promastigotes (Khartoum). At 1, 20, and 108 days, the mice were killed and the LDU calculated from liver biopsies (34). The mean LDU ± SE is shown ($n = 6$).

immunized group compared with the control mice (Fig. 5). Statistic analysis of the results by both the two-way ANOVA (p values given above) and contrast test showed that there was a significant difference both between the LPB in the control and immunized groups at each time point examined and also between the time course of disease development for each group ($p < 0.0001$).

Effect of antibody-mediated depletion and neutralization on VL in BALB/c mice

The T cell subsets responsible for protection against *L. donovani* in mice immunized with dp72 (total 6 μg) were characterized by injecting immunized mice daily with anti-CD4 (GK1.5), anti-CD8 (H35), or a mixture of both anti-CD4 and anti-CD8 antibodies. Treatment was initiated 5 days before parasite challenge and continued every 2 to 3 days until the experiment was terminated. Immunized animals receiving injections of either pure IgG (25 μg/mouse) or PBS were included.

Nonimmunized control mice, adjuvant only, were injected with PBS. Two weeks after the final boost the mice were challenged i.v. with 10^7 *L. donovani* (Khartoum) promastigotes and killed 20 days later. Significant protection was observed in the immunized mice that received injections of either IgG or PBS (61 and 81% reduction in the LPB, respectively) (Fig. 6) compared with the control nonimmunized mice (PBS). A significant difference (one-way ANOVA test at the 95% confidence level with ln of LDU) in the LPB was also found between dp72-immunized mice depleted of either the CD4$^+$, CD8$^+$ or both T cell subsets, and the dp72-immunized mice treated with either PBS or IgG (Fig. 6). No significant difference in the LPB was found between any of the T cell subset-depleted groups. Interestingly, immunized mice receiving injections of either anti-CD4, anti-CD8, or both anti-T cell subsets had greater LPB, (178, 173, and 175%, respectively) than the control

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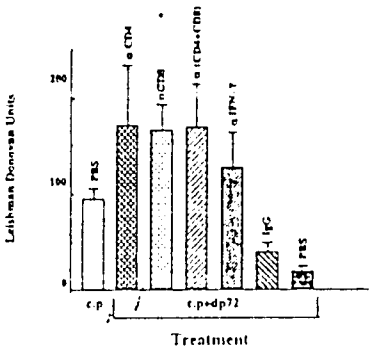

FIGURE 6. Passive transfer of antibodies to T cell subsets or IFN-γ and their affect on visceral leishmaniasis in BALB/c mice. Five days before challenge i.v. with 10^7 stationary phase *L. donovani* (Khartoum) promastigotes, a regime of antibody injections was begun. Immunized mice (as in Fig. 5) received either PBS, IgG, anti-CD4, -CD8, -CD4 plus -CD8 or anti-IFN-γ antibodies. Nonimmunized mice only received injections of PBS. At 18 days all the mice were killed and LDU were calculated from liver biopsies. The mean LDU ± SE ($n = 6$) is shown.

nonimmunized mice injected with PBS (Fig. 6).

In the same experiment the effect of antibodies to IFN-γ on the course of the disease in dp72-immunized mice was examined. IFN-γ was neutralized by injections of the antibody HB170 following the same protocol as described above. Neutralization of IFN-γ resulted in an increase in the LPB of the dp72-immunized mice (130%, compared with the nonimmunized group) (Fig. 6). The effect of IFN-γ neutralization was less pronounced than that seen after depletion of the $CD4^+$ or $CD8^+$ T cell subsets but was statistically significant (95% confidence level in the one-way ANOVA with ln of LDU) when compared with dp72-immunized mice receiving either PBS or IgG.

Pure dp72 from *L. donovani* protects against CL

Because both antibodies and lymphocytes from BALB/c mice immunized with the pure *L. donovani* protein, dp72, react to crude Ag from *L. major*, it was interesting to see whether immunization with dp72 can protect mice against CL. BALB/c mice immunized with dp72 (6 μg total) were challenged with *L. major* (Fredlin) stationary phase promastigotes (either 10^4 or 10^6 per mouse s.c. in the left rear foot). *C. parvum* was used as adjuvant for only the first boost. The ratio of infected to noninfected footpad thickness (millimeters) was measured at weekly intervals. The results are shown in Figure 7. Immunization with the *L. donovani* protein gave significant protection against a challenge by *L. major* at either dose. Statistic analysis of the mice challenged with 10^6 promastigotes showed a significant difference between the control and immunized group, $p < 0.04$ for 27 to 50 days and $p < 0.004$ for 57 to 70 days (two-way ANOVA with contrast). A significant difference

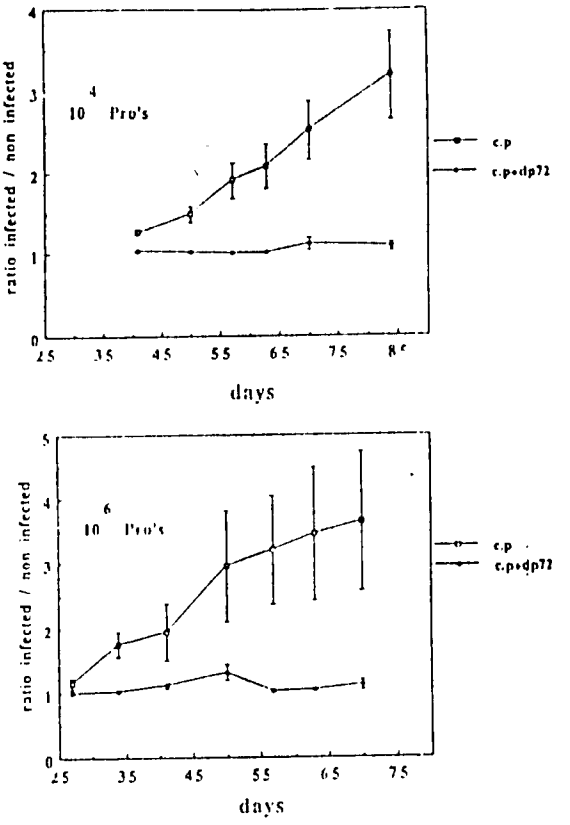

FIGURE 7. Protection of BALB/c mice immunized with the *L. donovani* protein dp72 against a challenge by *L. major*. Mice were immunized with dp72 (see Fig. 5). Control mice received only adjuvant. Mice were challenged, s.c., with either 10^4 or 10^6 stationary phase *L. major* (Fredlin) promastigotes in the left rear foot. The thickness of both footpads was measured (millimeters) and the ratio of infected to noninfected footpad ± SE is shown ($n = 6$).

($p < 0.01$, Student *t*-test for unequal variances) was also found for the control and immunized mice challenged with 10^4 promastigotes. No lesions were visible in either immunized group for up to 70 days (10^6 parasites) or 84 days (10^4 parasites) after infection, although a small swelling near the ankle was apparent at 50 days in the group receiving 10^6 parasites. At 70 days two mice each from the immunized and control groups challenged with 10^6 promastigotes were killed and the parasite load in the infected foot examined by LDA (35). Infected feet obtained from the control mice contained 2.1×10^6 more parasites than were found in the dp72-immunized mice, 1.3×10^6 parasites/foot vs 6.4×10^3 parasites/foot, respectively. Lesion size in the remaining immunized mice, both 10^4 and 10^6 parasite challenges, was monitored for more than 6 mo after challenge without detecting lesions (data not shown). At 6 mo the number of residual parasites remaining in the infected footpads from the immunized mice challenged with 10^4 and

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10^6 parasites was examined by LDA. No parasites were detected.

Discussion

In mice, the control of VL can be divided into two phases, innate and acquired immunity, which are under control of the Lsh and H-2 gene loci, respectively (1, 40). Naive BALB/c mice (Lsh^s, H-2^d) when challenged with *L. donovani* show a marked increase in parasite burden during the early phase of disease, reaching peak LPB at around 2 to 4 wk. During the latter chronic phase of disease, these mice slowly reduce their parasite burdens (10, 39, 41). Reduction of LPB may be partially dependent on the size of the parasite inoculum used for challenge (38, 39).

Protection against *L. donovani*, which causes VL, is thought, like CL, to be T cell mediated. Adoptive transfer of immune spleen cells to nude BALB/c or naive C57BL/6J mice imparts protection, whereas the depletion of T cells from naive BALB/c or C57BL/6J mice blocks acquisition of resistance (10, 42, 43). Transfer of B cells or immune serum has no effect on disease (42).

Unlike cutaneous disease caused by *L. major*, differential regulation of the CD4$^+$ T cell subsets, Th1 and Th2, does not appear to be involved in determining genetic control or vaccine-induced rate of cure for *L. donovani*. Kaye et al. (9) monitored lymphokine production at different times after challenge in naive *Lsh* congenic mice (C57/BL/10, Lsh^s H-2^b, and B10.L-Lsh, Lsh^r H-2^b) or MHC congenic noncuring mice (B10.D2/n, Lsh^s, H-2^d). Early in infection (14 days) all mouse strains produce about the same levels of IFN-γ, IL-2, and IL-3 after in vitro Ag-specific stimulation of their spleen cells. However, by 30 days noncuring mice already exhibit a marked decrease in the amounts of these cytokines, and by 50 days no cytokine production was detected. No evidence for either IL-4 or IL-5 production was found with any of the strains studied throughout the course of disease. The former cytokines are characteristic of Th1 cells, whereas the latter are typical of Th2 cells. IL-3 is produced by both T cell subsets (7, 8); therefore, the disappearance of cytokines characteristic of Th1 cells, rather than the expansion of cells belonging to the Th2 subset and concurrent overproduction of IL-4, appears to be associated with susceptibility to *L. donovani* in mice.

In an earlier report, we showed that immunization of BALB/c mice with dp72 reduces the peak LPB (18 days) by over 80% after challenge with *L. donovani* amastigotes (29). This protein is present in both stages of the parasite (29) and a significant reduction in peak parasite burdens (14 to 21 days) of immunized mice has been observed regardless of the leishmanial stage used for challenge (29, 44) (Fig. 5). Further studies, presented here, on the kinetics of infection show that the reduction observed in the parasite load at 18 to 21 days is not caused by differences in the initial parasite burden between the immunized and control mice. Measurements by both liver touch biopsy and LDA at 1 day after infection demonstrated that the parasite burdens were the same for both groups. Because LDA is based on transformation and growth of the intracellular parasites as promastigotes, equal numbers of viable amastigotes must have been present in the livers of both groups. Furthermore, this finding that the initial LPB were similar implies that mechanisms that neutralize parasites or block invasion, such as antibody-mediated C lysis or nonspecific activation of macrophages, do not play a role in the protection observed at 18 to 21 days, even though the immunized mice have high anti-dp72 antibody titers (>1:1000). Preliminary experiments have also shown that the passive transfer of hyperimmune sera to dp72 has no effect on LPB in naive mice (N. Rachamim and C. L. Jaffe, unpublished observations).

In another study (9) in which the kinetics of protection against VL were followed, no difference over the early phase of the disease (14 or 30 days) between the LPB of naive and immunized C57BL/10 mice was observed. However, neither serum antibody nor T cell responses to leishmanial Ag were observed in these immunized mice before challenge and the Ag fractions were administered without adjuvant. This is different from results presented here for mice immunized with dp72 plus adjuvant, which showed serum antibodies and lymphoproliferation to dp72 and significant protection at 18 to 21 days. In Kaye et al. (9) a decrease in the parasite load was apparent only much later in the disease course, at 50 days, for two Ag fractions, 38–45 and 79–85 kDa, tested.

Depletion experiments in vitro demonstrated that CD4$^+$ T cells specifically proliferate to dp72. Preliminary experiments have shown that adoptive transfer of these T cell blasts reduces LPB by 70% in naive BALB/c (N. Rachamim, J. A. Louis and C. L. Jaffe, unpublished results). T cells of this subpopulation can secrete large amounts of IFN-γ and IL-2 (7, 8), and we are presently analyzing the lymphokine profile of the dp72 Ag-specific T cells. Production of IFN-γ has been correlated with the ability of vaccinated and naive mice challenged with *L. donovani* to control and reduce parasite burdens both in vitro and in vivo (9, 45–48). Indeed, when mice immunized with dp72 were treated with anti-IFN-γ antibodies in vivo, the LPB at day 20 was similar to that of naive mice.

Although the adoptive transfer of CD4$^+$ cells from cured C57BL/10 or BALB/c mice alone has been shown to convey marked immunity to challenge in unprimed recipients (41, 49), these findings are in disagreement with reports that healing in BALB/c mice requires both CD4$^+$ and CD8$^+$ T cells, and that CD4$^+$ cells alone are insufficient to induce cure (10). Nude mice reconstituted with total spleen cells from naive BALB/c mice (nu/+) controlled their infections

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by 8 wk. However, mice receiving only CD4⁺- or CD8⁺-enriched spleen cells failed to eliminate the parasites. The requirement for both T cell subpopulations was further confirmed by showing that in vivo depletion of either subset prevented the resolution of the disease in naive mice (10). Protection in mice immunized with dp72 against *L. donovani* appears to involve both T cell subpopulations. Elimination of either the CD4⁺ or the CD8⁺ subset in vivo reversed the protective effect observed in immunized mice and resulted in LPB equivalent to those found when both subsets were depleted. In fact, these mice had parasite burdens higher than the nonimmunized PBS control mice, although not as great as nonimmunized mice depleted of their CD4⁺ or CD8⁺ T cell subsets. The latter mice show LPB approximately 2.5 times the PBS control (N. Rachamim and C. L. Jaffe, unpublished results).

Additional evidence for the role of CD8⁺ cells in the control of *L. donovani* infections comes from immunocytochemical studies in naive and cured BALB/c mice (50). The latter mice are resistant to reinfection by *L. donovani*. These studies showed that both CD4⁺ and CD8⁺ cells are prominent in the liver granulomas during the innate phase of disease. However, the CD4⁺ subset declines rapidly during resolution and only CD8⁺ lymphoid cells remain at a time when the intracellular parasites are being destroyed. Rechallenge of the cured mice was associated with a dramatic increase in CD8⁺ T cells.

Immunization with dp72 may prime immunologic responses so as to avoid the early induction of suppressor cells, either macrophages or T cells, that have been reported to appear during the first few weeks after the infection of BALB/c mice (51, 52). The rapid proliferation and influx into liver granulomas after parasite challenge of dp72-specific CD4⁺ T cells that secrete IFN-γ and IL-2 could enhance both the protective and functional activity of CD8⁺ cells. Indeed, IFN-γ and IL-2 have been shown to enhance the effectiveness of cytotoxic T and NK cells and induce IFN-γ production by CD8⁺ cells (53, 54). Secretion of IFN-γ by these latter cells would complement the production of IFN-γ by the CD4⁺ subpopulation resulting in the higher levels of IFN-γ that may be required to trigger effective leishmanicidal mechanisms (55). In addition, direct contact-mediated killing of intracellular amastigotes via cytotoxic CD8⁺ T cells has been shown to play an important role in host defense against other parasites, such *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Plasmodium yoelii* (56–58). Recently, a role for cytotoxic CD8⁺ cells in host defense against *L. major* has also been demonstrated (59, 60).

Cross-specific protection in humans after cure and reinfection with different species of *Leishmania* appears to be rare and has been documented in only a few cases between related species that cause CL, i.e., *Leishmania tropica* and *L. major* (2). In the mouse model several examples of cross-species protection have been reported between species that cause CL, or between *L. donovani* and *L. major* using either crude Ag or irradiated promastigotes (12, 14, 17, 61).

Dp72 is not related to the PSP, a major surface glycoprotein that is found in all species of *Leishmania* and both stages of the parasite. Polyclonal antibodies to dp72 do not recognize PSP (29) and lymphocytes from mice immunized with dp72 do not proliferate to PSP (Fig. 3). However, serum antibodies and lymphocytes from mice immunized with dp72 do recognize a cross-reactive Ag in crude lysates of *L. major* (Figs. 1 and 2), indicating that both B and T cell epitopes are shared between the *L. donovani* and *L. major* proteins. The *L. major* protein, 60 kDa, has the same m.w. as an Ag recognized in *L. major* lysates by mAb D13 against dp72 (29). Preliminary results showed that PBL from patients with South American CL proliferate specifically to dp72 (62). These results suggest that priming with the *L. donovani* protein might induce immunologic responses, perhaps protective, when later challenged with other species of *Leishmania*. Indeed when BALB/c mice, which are highly susceptible to *L. major*, were immunized with dp72 and challenged with this parasite, no lesions developed in the immunized mice over the course of this study, up to 6 mo. The protection was also confirmed by LDA. This paper represents the first report of a pure protein that protects against both VL and CL. Additional studies using this protein will help us define the mechanisms involved in disease and the appropriate immunologic responses required for the induction of a protective response in the mouse model system. Hopefully we will be able to extrapolate these findings to vaccine development for human disease.

Acknowledgments

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References

1. Peters, W., and R. Killick-Kendrick, eds. 1987. *The Leishmaniasis in Biology and Medicine*, Vol. 1 and 2. Academic Press, London, p. 941.
2. Mauel, J., and R. Behin. 1982. Leishmaniasis: immunity, immunopathology and immunodiagnosis. In *Immunity of parasitic infections*. S. Cohen and K. S. Warren, eds. Blackwell Scientific Publ., Oxford, England, p. 206.
3. Jaffe, C. L., and C. L. Greenblatt. 1991. Vaccine development against the intracellular parasite *Leishmania*. In *Vaccines and Immunotherapy*. S. J. Cryz, Jr., ed. Pergamon Press, London, p. 38.
4. Muller, I., T. Pedrazzini, J. P. Farrell, and J. Louis. 1989. T cell responses and immunity to experimental infection with *Leishmania major*. *Annu. Rev. Immunol.* 7:561.

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5. Titus, R. G., C. M. Theodos, P. B. Kimsey, A. Shankar, L. Hall, M. McGurn, and L. Povinelli. 1992. Role of T cells in immunity to the intracellular pathogen, *Leishmania major*. *Subcell. Biochem. 18:99.*
6. Scott, P., E. Pearce, A. W. Cheever, R. L. Coffman, and A. Sher. 1989. Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. *Immunol. Rev. 112:161.*
7. Mosmann, T. R., and R. L. Coffmann. 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol. 46:111.*
8. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
9. Kaye, P. M., A. J. Curry, and J. M. Blackwell. 1991. Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. *J. Immunol. 146:2763.*
10. Stern, J. J., M. J. Oca, B. Y. Rubin, S. L. Anderson, and H. W. Murray. 1988. Role of L3T4+ and Lyt-2+ cells in experimental visceral leishmaniasis. *J. Immunol. 140:3971.*
11. Zwingenberger, K., G. Harms, C. Pedrosa, S. Omena, B. Sandkamp, and S. Neifer. 1990. Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon-γ production. *Clin. Immunol. Immunopathol. 57:242.*
12. Howard, J. G., S. Nicklin, C. Hale, and F. Y. Liew. 1982. Prophylactic immunization against experimental leishmaniasis. I. Protection induced in mice genetically vulnerable to fatal *Leishmania tropica* infection. *J. Immunol. 129:2206.*
13. Barral-Netto, M., S. G. Reed, M. Sadigursky, and G. Sonnenfeld. 1987. Specific immunization of mice against *Leishmania mexicana amazonensis* using solubilized promastigotes. *Clin. Exp. Immunol. 67:11.*
14. Howard, J. G., F. Y. Liew, C. Hale, and S. Nicklin. 1984. Prophylactic immunization against experimental leishmaniasis. II. Further characterization of the protective immunity against fatal *Leishmania tropica* infection induced by irradiated promastigotes. *J. Immunol. 132:450.*
15. Scott, P., E. Pearce, P. Natovitz, and A. Sher. 1987. Vaccination against leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. *J. Immunol. 139:221.*
16. Scott, P., E. Pearce, P. Natovitz, and A. Sher. 1987. Vaccination against cutaneous leishmaniasis in a murine model. II. Immunologic properties of protective and nonprotective subfractions of a soluble promastigote extract. *J. Immunol. 139:3118.*
17. Frommel, D., B. W. Ogunkolade, I. Vouldoukis, and L. Monjour. 1988. Vaccine-induced immunity against cutaneous leishmaniasis in BALB/c mice. *Infect. Immun. 56:843.*
18. Russell, D. G., and J. Alexander. 1988. Effective immunization against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. *J. Immunol. 140:1274.*
19. Champsi, J., and D. McMahon-Pratt. 1988. Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infect. Immun. 52:3272.*
20. Handman, E., and G. F. Mitchell. 1985. Immunization with *Leishmania* receptor for macrophages protects mice against cutaneous leishmaniasis. *Proc. Natl. Acad. Sci. USA 82:5910.*
21. Jan, A., J. M. Leimett, and H-J. Diesfeld. 1986. Seroepidemiological study on kala-azar in Baringo District, Kenya. *J. Trop. Med. Hyg. 89:91.*
22. Badaro, R., T. C. Jones, E. M. Carvalho, D. Sampaio, S. G. Reed, A. Barral, R. Teixeira, and W. D. Johnson, Jr. 1986. New perspectives on a subclinical form of visceral leishmaniasis. *J. Infect. Dis. 154:1003.*
23. Pozio, E., L. Gradoni, S. Bettini, and M. Gramiccia. 1981. Leishmaniasis in Tuscany (Italy) VI. Canine leishmaniasis in the focus of Monte S. gentario (Grosseto). *Acta Trop. (Basel) 38:383.*
24. Holbrook, T. W., J. A. Cook, and B. W. Parker. 1981. Immunization against *Leishmania donovani* glucan as an adjuvant with killed promastigotes. *Am. J. Trop. Med. Hyg. 30:762.*
25. Jarecki-Black, J. C., E. R. James, J. W. Kirshtein, J. D. Kirshtein, and A. B. Glassman. 1986. *Leishmania donovani*: immunization against infection as a function of parasite growth phase. *Am. J. Trop. Med. Hyg. 35:1117.*
26. O'Daly, C. J. A., and Z. Cabrera. 1986. Immunization of hamster with TLCK-killed parasites induced protection against *Leishmania* infection. *Acta Trop. (Basel) 43:225.*
27. Jaffe, C. L., and M. Zalis. 1988. Purification of two *Leishmania donovani* membrane proteins recognized by sera from patients with visceral leishmaniasis. *Mol. Biochem. Parasitol. 27:53.*
28. Jaffe, C. L., and M. Zalis. 1988. Use of purified parasite proteins from *Leishmania donovani* for the rapid serodiagnosis of visceral leishmaniasis. *J. Infect. Dis. 157:1212.*
29. Jaffe, C. L., N. Rachamim, and R. Sarfstein. 1990. Characterization of two proteins from *Leishmania donovani* and their use for vaccination against visceral leishmaniasis. *J. Immunol. 144:699.*
30. Jaffe, C. L., G. Grimaldi, and D. McMahon-Pratt. 1984. The cultivation and cloning of *Leishmania*. In *Genes and antigens of parasites*. C.M. Morel, ed. UNDP/World Bank/WHO, Rio de Janeiro, p. 47.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:680.*
32. Bouvier, J., R. J. Etges, and C. Bordier. 1985. Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes. *J. Biol. Chem. 260:15504.*
33. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:4350.*
34. Stauber, L. A. 1955. Leishmaniasis in the hamster. In *Some physiological aspects and consequences of parasitism*. W. H. Cole, ed. Rutgers University Press, New Brunswick, NJ, p. 76.
35. Titus, R. G., M. Marchand, T. Boon, and J. A. Louis. 1985. A limiting dilution assay for quantifying *Leishmania major* in tissue of infected mice. *Parasite Immunol. 7:545.*
36. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierres, J. Quintas, M. R. Lohen, M. Pierres, and F. W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated

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L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol. 131:2445.*

37. Sarmiento, M., A. L. Glasebrook, and F. W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt-2 antigen block T cell-mediated cytolysis in the absence of complement. *J. Immunol. 125:2665.*

38. Ulczak, O. M., and J. M. Blackwell. 1983. Immunoregulation of genetically controlled acquired responses to *Leishmania donovani* infection in mice: the effects of parasite dose, cyclophosphamide, and sublethal irradiation. *Parasite Immunol. 5:449.*

39. Reiner, N. E. 1982. Host-parasite relationship in murine leishmaniasis: pathophysiological and immunological changes. *Infect. Immun. 38:1223.*

40. Blackwell, J. M., B. Roberts, and J. Alexander. 1985. Response of BALB/c mice to leishmanial infection. *Curr. Top. Microbiol. Immunol. 122:97.*

41. Holaday, B., M. D. Sadick, and R. D. Pearson. 1988. Isolation of protective T cells from BALB/cJ mice chronically infected with *Leishmania donovani*. *J. Immunol. 141:2132.*

42. Reizai, H. R., J. Farrell, and E. L. Soulsby. 1980. Immunological responses of *L. donovani* infection in mice and significance of T cell resistance to experimental leishmaniasis. *Clin. Exp. Immunol. 40:508.*

43. Skov, C. B., and D. W. Twohy. 1974. Cellular immunity to *Leishmania donovani*. I. The effect of T cell depletion on resistance to *L. donovani* in mice. *J. Immunol. 113:2004.*

44. White, A. C., Jr., and D. McMahon-Pratt. 1990. Prophylactic immunization against experimental *Leishmania donovani* infection by use of a purified protein vaccine. *J. Infect. Dis. 161:1313.*

45. Murray, H. W., J. J. Stern, K. Welte, B. Y. Rubin, S. M. Carriero, and C. F. Nathan. 1987. Experimental visceral leishmaniasis: production of interleukin 2 and interferon-γ, tissue immune reaction, and response to treatment with interleukin 2 and interferon-γ. *J. Immunol. 138:2290.*

46. Murry, H. W., G. L. Spitalny, and C. F. Nathan. 1985. Activation of mouse peritoneal macrophages in vitro and in vivo by interferon-γ. *J. Immunol. 134:1619.*

47. Hoover, D. L., C. A. Nacy, and M. S. Meltzer. 1985. Human monocyte activation for cytotoxicity against intracellular *Leishmania donovani* amastigotes: induction of microbiocidal activity by interferon-γ. *Cell Immunol. 94:5005.*

48. Squires, K. E., R. D. Schreiber, M. J. McElrath, B. Y. Rubin, S. L. Anderson, and H. W. Murray. 1989. Experimental visceral leishmaniasis: role of endogenous IFN-γ in host defense and tissue granulomatous response. *J. Immunol. 143:4244.*

49. Ulczak, O. M., E. Ghadirian, E. Skamene, J. M. Blackwell, and P. A. L. Kongshavn. 1989. Characterization of protective T cells in the acquired response to *Leishmania donovani* in genetically determined cure ($H\text{-}2^b$) and noncure ($H\text{-}2^d$) mouse strains. *Infect. Immun. 57:2892.*

50. McElrath, M. J., H. W. Murray, and Z. A. Cohn. 1988. The dynamics of granuloma formation in experimental visceral leishmaniasis. *J. Exp. Med. 167:1927.*

51. Blackwell, J. M., and O. M. Ulczak. 1984. Immunoregulation of genetically controlled acquired responses to *Leishmania donovani* infection in mice: demonstration and characterization of suppressor T cells in noncure mice. *Infect. Immun. 44:97.*

52. Murray, H. W., S. M. Carriero, and D. M. Donelly. 1986. Presence of a macrophage-mediated suppressor cell mechanism during cell-mediated immune response in experimental visceral leishmaniasis. *Infect. Immun. 54:487.*

53. Shalaby, M. R., L. P. Svedersky, P. A. McKay, B. S. Finkle, and M. A. Palladino, Jr. 1985. In vivo augmentation of natural killer-activity by combined treatment with recombinant gamma interferon and interleukin-2. *J. Interferon Res. 5:571.*

54. Gazzinelli, R. T., F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher. 1991. Synergistic role of $CD4^+$ and $CD8^+$ T lymphocytes in IFN-γ product on and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J. Immunol. 146:286.*

55. Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy. 1990. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-γ-stimulated macrophages by induction of tumor necrosis factor-α. *J. Immunol. 145:4290.*

56. Weiss, W. R., M. Sedegah, R. L. Beaudoin, L. W. Miller, and M. F. Good. 1988. $CD8^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc. Natl. Acad. Sci. USA 85:573.*

57. Tarleton, R. L. 1990. Depletion of $CD8^+$ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. *J. Immunol. 144:717.*

58. Hakim, F. T., R. T. Gazzinelli, E. Denkers, S. Hieny, G. M. Shearer, and A. Sher. 1991. $CD8^+$ T cells from mice vaccinated against *Toxoplasma gondii* are cytotoxic for parasite-infected or antigen-pulsed host cells. *J. Immunol. 147:2310.*

59. Louis, J. A., F. Conceicao-Silva, I. Muller, U. Fruth, and P. Romero. 1992. Analysis of the role of specific-$CD4^+$ T cells in resistance and susceptibility of mice to primary and secondary infections with *Leishmania major*. *J. Cell. Biochem. 16A:109.*

60. Hill, J. O., M. Awwad, and R. J. North. 1989. Elimination of $CD4^+$ suppressor T cells from susceptible BALB/c mice releases $CD8^+$ T lymphocytes to mediate protective immunity against *Leishmania*. *J. Exp. Med. 169:1819.*

61. Alexander, J. 1982. A radioattenuated *Leishmania major* vaccine markedly increases the resistance of CBA mice to subsequent infection with *Leishmania mexicana mexicana*. *Trans. R. Soc. Trop. Med. Hyg. 76:646.*

62. Zaiss, M. G., A. Schubach, M. Oliveira Neto, D. A. Costa, C. Jaffe, U. G. Lopes, and M. A. Barcinski. 1988. The cellular immune response of patients with cutaneous leishmaniasis to leishmanial antigen with different molecular weights. *Mem. Inst. Oswaldo Cruz 83:117.*

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