An Experimental Model for Canine Visceral Leishmaniasis

Pedro Abranches*, Gabriela Santos-Gomes*, Nurit Rachamim+, Lenea Campino*, Lionel F. Schnur#, and Charles L. Jaffe+

*Disciplina de Protozoologia, Instituto de Higiene e Medicina Tropical Lisbon, Portugal, +Department of Biophysics and MacArthur Center for Molecular Biology of Tropical Diseases, Weizmann Institute of Science, Rehovot and #Department of Parasitology, Kuvin Center, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Running Title: Canine Visceral Leishmaniasis

Key words: Leishmania donovani, dogs, model system, dp72, gp70-2

Correspondence: Dr. Charles L. Jaffe, Dept. of Biophysics, Weizmann Institute of Science, Rehovot 76100 Israel.

Tel. 972-8-343103 FAX. 972-8-468256.

Summary Seven mixed-breed dogs were challenged with either promastigotes or amastigotes of Leishmania donovani infantum strains recently isolated from naturally infected dogs. Different routes and numbers of parasites were utilized and each dog was monitored for at least 1 year post-infection. Anti-parasite specific antibody levels were measured by enzyme-linked immunosorbence, immunofluorescence, crossed-immune electrophoresis and western blotting on crude antigen. Western blotting on two pure parasite proteins, dp72 and gp70-2, was also done. Mitogenic and antigen-specific stimulation of peripheral blood lymphocytes was monitored; and the haematological, clinical and parasitological parameters measured. Dogs challenged with amastigotes exhibited a more pronounced humoral response to leishmanial antigens. Only in one case was strong antigen-specific proliferation detected. Clinical signs of disease, including hypergammaglobulinemia, enlarged lymph nodes and the presence of parasites, were also more apparent in the dogs challenged with amastigotes. None of the seven dogs died. Serum antibodies to leishmanial antigens were apparent between 1.5 to 3 months following challenge and correlated with the appearance of enlarged lymph nodes, hypergammaglobulinemia and the presence of parasites in tissue biopsies. Serum antibodies remained chronically high in these dogs throughout the period of the study. Only one dog (1/3) challenged intravenously with promastigotes and the dog challenged intradermally with amastigotes produced transient antibody responses to leishmanial antigen.

Introduction

Visceral leishmaniasis (VL) is a fatal human disease caused primarily by the Leishmania donovani sensu lato (Pearson et al. 1983, Peters & Killick-Kendrick 1987). This disease is typified by immune suppression, irregular fevers, hypergammaglobulinemia, hepatosplenomegaly and anemia. In many areas of the New and Old World, dogs and wild canids are the main reservoir hosts (Peters & Killick-Kendrick 1987). The prevalence of canine VL in the Mediterranean Region varies between 10 - 37% (Bettini & Gradoni 1986). Several epidemiological studies of canine VL have been carried out in this region (Pozio et al. 1981, Abranches et al. 1983, Abranches et al. 1987). While no direct correlation between the presence of human VL and the incidence of canine disease has been demonstrated (Bettini & Gradoni 1986), the elimination of infected dogs in hyperendemic areas of Brazil has been correlated with a decreased prevalence of disease in humans (Alencar 1961). Similar results have also been reported in China (Zhi-bio 1988). Together, these results suggest that infected dogs represent a medically important active reservoir for parasite transmission to humans. Furthermore, the presence of latent infections of up to a year or more in dogs would be important in maintaining long term presence of VL in specific regions (Bettini & Gradoni 1986).

Many of the symptoms of canine VL are similar to those of human VL (Bettini & Gradoni 1986, Peters & Killick-Kendrick 1987). In addition, depilation, onychogryphosis and emaciation are typical symptoms found in infected dogs (Adler 1936). Relatively few immunological studies of canine VL have been undertaken, even though dogs are important reservoir hosts and may serve as a good experimental model for human VL. Existing

studies have primarily examined chronically infected dogs collected from the field. Common findings include circulating immune complexes, autoantibodies and hypergammaglobulinemia (Vitu, Sanchis & Giauffret 1973, Brandonisio et al. 1985). Recently, the number of T-cells and their function in infected dogs was shown to be reduced compared to normal dogs (Brandonisio et al. 1989); and monocytes were reported to display lower phagocytic ability than cells recovered from normal dogs (Brandonisio et al. 1986).

In this study, we present findings on the establishment of an experimental model for canine visceral leishmaniasis, caused by L.d. infantum. We examined several different protocols for infection, and followed the clinical and parasitological development of the disease for up to 17 months following infection. Humoral and cell-mediated immunological parameters were also examined.

A vaccine against canine VL should help reduce the incidence of human disease, and would permit greater flexibility than would be acceptable in developing a similar vaccine for humans. The results presented here are a groundwork for studies on vaccine development.

Materials and methods

ANIMALS AND PARASITES

Mixed-breed dogs (1 - 2 months old) were obtained from several areas in Lisbon, Portugal. This region is endemic for canine visceral leishmaniasis (Abranches et al. 1983). The dogs were maintained under observation for 6 to 12 months and tested by IFA prior to inclusion in the study. At 6 - 12 months of age, seven dogs were challenged, six intravenously and one intradermally, with fresh isolates of Leishmania

donovani infantum. The parasites were isolated from infected dogs in the highly endemic Setubal Peninsula, located south of Lisbon (Abranches et al. 1986). Virulent strains were maintained either as amastigotes in hamsters or following transformation to promastigotes on NNN medium. The isolates used in these studies, IMT-154 and IMT-167, were typed by enzymes analysis and shown to be of the zymodeme MON-1 (LON 49) and their excreted factor subserotype was B_2, typical of all L.d. infantum strains isolated from this region (Abranches et al. 1986).

Infections by the i.v. route were carried out with either amastigotes (10^{10-11} parasites/kg) or stationary phase promastigotes (10^{7-8} parasites/kg). Amastigotes were isolated from the spleens of infected hamsters (IMT-154) or dogs (IMT-167). Promastigotes were maintained in culture from no more than 5 passages after transformation from amastigotes. The i.d. challenge was carried out with amastigotes (10^{11} parasites/kg) isolated from an infected hamster spleen.

CLINICAL AND IMMUNOLOGICAL EXAMINATION

At approximately monthly intervals, each dog was weighed, examined clinically for signs of disease, including popliteal lymph node size, onychogryphosis, depilation, ulceration and weight loss. Peripheral blood was taken for hematological and immunological analysis. Total erythrocytes, leukcocytes and hemoglobin were determined by an automated counter and hemoglobinometer (Model S5 Coulter Electronics, Hialeah, Fl). Total protein and albumin were measured by the Monarch 2000 Autoanalyser (Instrumentation Laboratory, Lexington, U.S.A.). Electrophoresis of serum proteins was carried out on cellulose acetate in Tris-barital buffer (pH 7.2), using Helena Process 24 equipment (Beaumont, TX). Parasite specific antibody titers were determined by

indirect immunofluorescence (IFA, Abranches, et al. 1983), direct enzyme-linked immunosorbent assay (ELISA, Jaffe et al. 1988c) and crossed immunoelectrophoresis (CIE, modified from Monjour et al, 1978). Local strains L.d. infantum were used for both the IFA (MCAN/PT/81/IMT 82) and CIE (MHOM/PT/88/IMT 151), and a Sudanese L.donovani (MHOM/SU/00/Khartoum) for ELISA.

Parasitological examinations on selected dogs were carried out at the times indicated by popliteal lymph node tissue biopsy. Preparations stained with Giemsa's stain were examined by microscopy and material was cultured for parasites in NNN medium.

WESTERN BLOTTING

Identification of parasite antigens recognized by the serum antibodies was carried out following the separation of total promastigote antigens by sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (Lammeli 1970, Towbin, Staehelin & Gordon 1979). Incubation of nitrocellulose paper with the serum samples was carried out using a fixed serum dilution (1/1000), exactly as previously described (Jaffe et al. 1990b). Kinetics of the appearance of serum antibodies against two pure L.donovani proteins, dp72 and gp70-2, was measured by western blotting carried out on the pure proteins. The proteins were purified and, after SDS-PAGE (0.8μg/gel), transferred to nitrocellulose paper as already described (Jaffe & Zalis 1988a). Strips were incubated with different serum samples (1/100 dilution in 0.5% Tween-20 containing 0.3% fetal calf serum). Following removal of excess antibody by washing (3 times for 15 minutes) the blots were incubated together with a protein A - horseradish peroxidase conjugate (1/2000 dilution in 0.5% Tween-20 containing fetal calf serum, 30 min) and

developed after removal of excess conjugate with the substrate 3,3'-diaminobenzidene (30mg/ml).

LYMPHOCYTE PROLIFERATION

Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-Hypaque by centrifugation for 25 min at 400xg. Blood samples were routinely kept on ice, while in transit from Portugal to Israel, but not for more than 36 h before separation. Mitogenic stimulation was carried out in 96-well flat bottom microtiter plates containing 10^5 cells/well. Several concentrations (10, 5 and 1μg/ml) of Concanavalin A (Con A) and Phytohemagglutinin (PHA) were examined. After 3 d at 37^{o}C in a 5% CO_2 incubator, [^{3}H]-thymidine (1μCi/well) was added for 24 h. The plates were harvested, using a PhD cell harvester (Cambridge Technology, Inc., Cambridge, MA) . Thymidine incorporation was counted following addition of scintillation fluid in a Tri-Carb liquid scintillation analyzer (Packard Instruments Co., Downers Grove, IL.). Antigen-specific stimulation was examined, using total L.donovani lysates from two different strains, L.d. donovani (Khartoum) and L.d. donovani (MHOM/ET/67/LV9), at 10, 5 and 1μg/well antigen. PBMC (10^5/well) were stimulated with parasite antigen for 6 d. [^{3}H]-thymidine (1μCi/well) was added for 24 h. The cells were harvested and counted as above.

Results

CLINICAL AND HEMATOLOGICAL PARAMETERS IN INFECTED DOGS

Seven dogs were challenged with local Portuguese strains of L.d. infantum recently isolated from dogs with natural infections. All the

strains used belonged to zymodeme MON-1 (LON-49) and were typical EF subserotype B_2 strains (Schnur 1982, unpublished data). Intravenous (iv) injection was used in six dogs, as this route gives highly reproducible infections in other models of VL, the hamster and mouse. Only 1/3 of the dogs challenged with promastigotes (No. 10) showed positive serological conversion for anti-leishmanial antibodies. Dog No. 10 was 12 months old at the time of challenge and received the greatest number of promastigotes/kg body weight. By the end of the second month following infection, this dog developed a transient antibody response against leishmanial antigen, detected by all three techniques (Figure 1a). At this point the IFA titer reached 1/512, the ELISA positive/negative (P/N) ratio was 3.3, and parasite antigen was immunoprecipitated by CIE. ELISA was carried out at a standard dilution of 1/1000. Positive reactions for IFA and ELISA are $\geq$1/128 and $\geq$3.0, respectively (Abranches et al, 1983, Jaffe et al. 1988). All the clinical and hematological parameters displayed by dog 10 were within normal limits. This dog remained serologically positive for 6.5 months by both IFA and ELISA, and was still positive by CIE until the ninth month. Peak IFA titers, 1/512, and ELISA P/N ratios, 7.4, were observed at 2.5 - 3.5 months and again at 5.5 months post-infection. No clinical signs of disease were observed at any time and the lymph nodes were normal. The remaining dogs, challenged at 6 months of age, received 10-fold fewer parasites/kg, did not convert serologically and showed no clinical or parasitological signs of infection.

Infections with amastigotes were more successful. The three dogs Nos. 7, 9 and 11 showed anti-leishmanial antibodies within 3 months post-infection, regardless of the diagnostic method employed (Figure 1b, 1c and data not shown). CIE consistently gave the earliest positive

diagnosis among the three methods employed to measure anti-leishmanial antibodies. Dog No. 9 (Figure 1b) converted serologically by 1.5 months post-infection (IFA titer 1/256) and reached a peak titer at 2 months (1/4096). A similar trend was observed by ELISA with peak reactions at 4 months (P/N >28). Serum antibodies to leishmanial antigens decreased dramatically between the fifth to seventh months post-infection, but remained positive (IFA titer between 1/128 - 1/512 and ELISA P/N ratio between 9.0 - 15.8) throughout the course of this study. Dog No. 11 showed a pronounced three peak pattern in antibody titers (data not shown). The first peak by IFA (1/1024) was apparent at 2.5 months post-infection and disappeared by 7.5 months. This was followed by a second peak at 9 months (1/1024) and a third at 16 months (1/4096). A similar pattern was also seen by ELISA. Dog No. 7 showed the greatest response to infection by leishmanial amastigotes (Figure 1c). This dog had converted to seropositive by 3.5 months post-challenge (IFA titer 1/128, ELISA P/N ratio >31.6)). The IFA titers remained consistently high (1/512 to 1/2048) throughout the course of this study, > 17 months. The ELISA P/N ratio showed a peak in antibody levels during months 3.5 - 5.5 and then remained stable at 11 ± 3 for the rest of the study. This was the only dog positive by parasitological examination; however dog 11 was not tested for the presence of parasites.

Dog No. 13 was the only one infected via the intradermal route. Amastigotes were used (10^{11}/kg body weight), since this stage of the parasite is more virulent than cultured promastigotes. A transient appearance of anti-leishmanial antibodies was observed from 3 to 6 months following infection (Figure 1d). However, no clinical signs of infection were observed.

Clinical and hematological examination of dogs 7, 9 and 11 correlated

well with anti-leishmanial antibody levels. Lymph node size was largest during peak antibody titers (Figure 1b-c), but remained abnormal throughout most of this study. During the first peak in antibody titers, 3 to 6 months post-infection, both the total serum protein (Figure 2a) and globulins (data not shown) was elevated compared to normal values. This finding coincided with an inversion in the albumin/globulin ratio (Figure 2b). During this period abnormalities were also observed in the values found for serum haemoglobin, peripheral blood erythrocytes and leucocytes, which were low compared to normal values (Figure 3 and data not shown). Serum protein electrophoretic profiles for each sample examined also reflect this abnormality (data not shown). By eight months post-infection all of the hematological parameters measured had returned to normal, even though the antibody titers remained positive. Dogs challenged with promastigotes did not show any clinical or hematological evidence of disease (Figure 2, 3 and data not shown).

Only in dog No. 7 were parasites demonstrated in tissue biopsies. Amastigotes were seen in smears, and promastigotes in cultures of lymph nodes on three separate occasions between 4 - 8 months post-infection.

LYMPHOCYTE PROLIFERATION

Mitogenic and antigen specific proliferation of PBMC from normal and infected dogs was examined twice. Infected dogs were between 7 and 18 months post-challenge when tested. Concentrations of 1, 5 and 10 μg protein/ml were used for mitogenic proliferation with Con A and/or PHA; and for parasite specific proliferation with particulate antigen from either L.d. donovani or L.d. infantum. The results with Con A at 10 - 15 months post-infection are summarized in Table 1. Similar results were obtained with PHA (data not shown). Compared to normal dogs, none of the

infected dogs showed suppression of mitogenic stimulation by either concanavalin A or PHA at any time examined (Table and data not shown). Even at eight or nine months post-infection, the earliest time examined for the amastigote-challenged dogs (nos. 9 and 11), the stimulation indexes (SI) were normal or elevated (31.0 and 129.2, respectively) compared to the control dogs (average - 29.9, n=4). This may mean that these dogs are clinically normal at eight months for many parameters, but not for anti-parasite antibody titers, popliteal lymph node size and the electrophoretic profiles of serum gamma globulins.

Antigen-specific proliferation was also examined using two different subspecies of Leishmania donovani. The results obtained with both strains were essentially the same and are shown in Figure 4 for L.d. donovani (10μg protein). The dogs challenged i.v. with amastigotes had the highest SI, with dog 9 responding the strongest to leishmanial antigen (SI = 7.6). Dogs, 7 and 11, showed a weak proliferative responses (SI = 2.3 and 2.45). Dogs challenged with either promastigotes or amastigotes, i.d., showed very weak or no proliferation compared to the four control dogs examined. Interestingly, dog 10 which had a transitory anti-parasite antibody response showed no lymphocyte proliferation when tested with the same antigen. No change in the SI to promastigote antigen was observed over the three month interval between the two tests. The strongest SI was found with 10μg parasite antigen.

PARASITE ANTIGENS RECOGNIZED BY SERUM ANTIBODIES FROM INFECTED DOGS

Western blotting, using post-infection dog serum obtained at different times, was used to identify the parasite antigens recognized by serum antibodies. Three parasite antigens were used: crude promastigote membranes, pure dp72 and pure gp70-2. The last two antigens proved

useful for the diagnosis of human VL and one of them, dp72, was shown to protect BALB/c mice against a challenge by L.d. donovani (ref). Results are shown for dogs No. 7, 10 and 13. IFA, ELISA, lymph node size and tissue biopsy results for these dogs are given in Figure 1. When antibody reaction (1/1000 dilution) on crude promastigote antigen was examined, multiple bands were recognized by each serum, ranging in molecular weight from less than 26,000 to over 116,000. As might be expected, maximum reactivity was directly correlated with peak antibody titers observed by IFA and ELISA (Figure 1). Reactions with bands at 30 kDa, 45 kDa, between 58-84 kDa and at the tracking dye are strong and appear early in the course of infection. Serum from all dogs examined with anti-leishmanial antibodies recognize these antigens. The disappearance of antibodies against these antigens parallels the IFA and ELISA results. The pure proteins dp72 and gp70-2 are recognized very early in the course of infection. By using a 1/100 serum dilution antibodies to these proteins can be detected one to two months prior to serum antibodies against crude antigens. Crude antigen can not be examined at a 1/100 serum dilution. At this concentration a high background due to non-specific binding and cross-reactions with sera from other diseases is observed with crude antigen (Jaffe & Zalis 1988b, data not shown). Disappearance of anti-dp72 and -gp70-2 antibodies parallels the disappearance of anti-leishmanial antibodies seen by the other assays.

Discussion

Successful vaccination against canine leishmaniasis would reduce the incidence of human visceral disease, in regions where human and canine

disease are prevalent and where infected dogs serve as a reservoir of human disease. To date, only one study on the vaccination of dogs against visceral leishmaniasis has been carried out (Dunan et al. 1989). That field study, done in southern France, employed LiF2, an L. donovani antigen shown to protect inbred mouse strains against a challenge with L. major, a cause of human and rodent cutaneous leishmaniasis. Immunization with LiF2 resulted in a significantly higher incidence of disease in the vaccinated group, compared to the control group which received a placebo. This demonstrates the importance of screening candidate antigens in a laboratory dog model, if possible, prior to field testing.

Few attempts have been made to establish a model for canine leishmaniasis (Mansour, Stauber & McCoy 1970, Keenan et al. 1984a, 1984b; Dubreuil, Vidor & Moreau 1990). We found that amastigotes were more infective than promastigotes, corroborating a recent report by Dubreuil et al. (1990). Here we show that dogs infected with amastigotes, intravenously, exhibit many of the same symptoms observed in chronic canine leishmaniasis. These dogs had elevated total serum proteins and gamma-globulins, and an inverted albumin/globulin ratio. All the dogs developed a moderate leukopenia and a normochromic, normocytic anemia. High levels of anti-leishmanial antibodies were present and the serum protein electrophoretic pattern was typical of visceral leishmaniasis, showing elevated α_1-, α_2-, β- and gamma-globulin fractions. Many of these symptoms are similar to those found in human visceral leishmaniasis.

None of the dogs infected artificially in this study died, even though death from infection is common under natural conditions, where 88% of dogs with symptoms of disease die (Pozio et al. 1981). Allusions

exist in the literature to a connection between diet and severity of the disease (Adler, 1936). Furthermore, approximately 50 - 60% of the seropositive dogs in epidemiological studies are negative when tissue biopsies are examined for the presence of parasites by microscopy and culturing (Pozio et al. 1981, Abranches et al. 1983). Amastigotes have been identified in parasite 'negative' dogs by hybridization with kinetoplast DNA probes (Evans et al. 1990), which might be more sensitive than examining Giemsa stained slides and culturing. These seropositive, but parasite 'negative' dogs represent a pool of asymptomatic carriers, of which 30 - 40% eventually progress to active infections (Pozio et al. 1981). The reasons for this dichotomy of response are unclear, but probably reflect genetic background, nutritional and immunological status of hosts; degree of repeated introduction of promastigotes by vectors, sandfly vector factors and leishmanial parasite virulence. In fact, more consistent infections were achieved in inbred dogs, German Shepherds (Keenan et al. 1984a, 1984b).

In our study the 'acute phase' of the disease lasted several months, after which, many of the clinical symptoms, including albumin/globulin ratio, returned to normal. However, the anti-leishmanial antibody levels and serum protein electrophorectic pattern remained abnormal through out the course of the study suggesting a persistent latent infection. Return to overt clinical normality (Monsour et al. 1970, Kontos 1986), but persistence of immunological abnormality (Dubreuil et al. 1990) have been recorded. In drug treated human patients, anti-leishmanial titers return to normal with in 3 - 12 months following cure (Jahn & Diesfeld 1983 and unpublished data).

Immunosuppression of mitogenic proliferation in the infected dogs, compared to the uninfected controls, was not observed. This is similar

to what has been observed in patients with active human visceral leishmaniasis (Peters & Killick-Kendrick 1987). Likewise, the antigen-specific responses in the dogs infected with either promastigotes or amastigotes appear to be depressed, similar to active human disease. Only one dog challenged with amastigotes showed strong proliferation, SI - 7.8. All of the other infected dogs demonstrated either very weak or no antigen-specific proliferation. In the case of the promastigote-infected dogs, especially those where no antibody response was observed, this may be due to the lack of sensitization and/or loss of memory cells over the long period, 10 to 15 months, which passed between the initial infection and the proliferative assay. For amastigote-infected dogs, suppression may be a result of a persistent latent infection, which is also reflected in the other abnormal responses observed. In humans with active visceral leishmaniasis, weak antigen-specific proliferative responses were only detected 2 to 6 months following cure (Sacks et al. 1987).

The pattern of parasite antigens recognized by serum antibodies from the infected dogs is similar to that seen with sera obtained from naturally infected dogs (unpublished data). The primary antibody response is directed against antigens whose molecular weights fall into four categories (tracking dye, 30.000, 45.000 and 58 - 84.000). These antigens are the major parasite components recognized and that elicit the strongest reactions, regardless of the stage or route used for infection. In all cases, an antibody response to parasite challenge is already apparent by 2.5 to 3 months post-challenge.

Responses to pure L. donovani proteins, dp72 and gp70-2, were also examined. Both proteins are specifically recognized by sera from patients with human visceral leishmaniasis and are also diagnostic of

canine visceral leishmaniasis (Jaffe & Zalis 1988b and unpublished data). Reactions against these proteins could be detected up to two months earlier than specific reactions with crude antigen. This was due the fact that false crossreactions at high serum concentrations on the pure proteins were minimal (Jaffe & Zalis 1988b and data unpublished). Peak titers against these proteins coincided with the peak total anti-parasite antibody response. However, anti-dp72 antibodies disappeared, in the case of dogs challenged with amastigotes i.v, at a faster rate than either anti-gp70-2 or total anti-leishmanial antibodies. Immunization with one of these proteins, dp72, partially protects mice against a challenge by L. d. donovani. These mice have antibodies to dp72 and show antigen specific T-cell proliferation (Jaffe, Rachamim & Sarfstein 1990a, Jaffe, Rachamim & Sarfstein 1991). The mechanism of protection in mice has not been elucidated, but the transfer to naive mice of antigen-specific T-cell blasts reduces liver parasite burden by 50% (unpublished data). However, host-specific immunological responses will require that putative vaccine candidates be tested in natural host models of visceral leishmaniasis, such as the dog, before their true efficacy can be ascertained.

Acknowledgements

Supported by the U.S.-Israel CDR Program Grant No. DPE-5544-G-SS-6020-00, Office of the Science Advisor, U.S. Agency for International Development, the John and Catherine T. MacArthur Foundation and the Instituto Nacional de Investigacao cientifica - CDIP, Portugal. CLJ would like to thank Dalia Goldenberg for her expert technical assistance.

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LEGENDS TO FIGURES.

Figure 1. Progress of visceral leishmaniasis in dogs infected with Leishmania donovani infantum. Dogs were infected with either amastigotes or promastigotes from recently isolated Portuguese strains as described in Materials and Methods. At each time point indicated a sample of peripheral blood was taken to measure anti-parasite antibodies by an immunofluorescence assay (IFA, o--o--o) and an enzyme-linked immunosorbent assay (ELISA, -□-□-□-). For IFA and ELISA, titers $\geq$1/128 or Positive/Negative (P/N) serum ratios $\geq$3 respectively, are considered positive. The negative cut off P/N = 3 for ELISA is indicated on the graphs. At selected points, biopsy samples were taken from the popliteal lymph nodes for parasitological diagnosis. The size of the popliteal lymph node is indicated on the graph: N - normal (0-3 mm), M - medium (4-7 mm) and L - large (>7 mm). Panels a. Dog No. 10, infected with promastigotes by the intravenous route; b. Dog No. 9, infected with amastigotes by the intravenous route; c. Dog No. 7, infected with amastigotes by the intravenous route and d. Dog No. 13, infected with amastigotes by the intradermal route.

Figure 2. Changes in canine serum protein and albumin/globulin ratio, following infection with Leishmania donovani infantum. Dogs were infected intravenously with either promastigotes (o) or amastigotes (▲) as described in the section on Material and Methods. Average value for infected dogs (n = 3) receiving promastigotes or amastigotes is given. Dotted lines show the fluctuation range found for normal dogs over the course of this study. Panel A. Total protein. Panel B. Albumin / gamma-globulin ratio.

Figure 3. Change in canine peripheral blood leucocytes following in infection with Leishmania donovani infantum. Dogs were infected intravenously with either promastigotes (o) or amastigotes (▲) as described in the Materials and Methods. The average value for infected dogs (n -3 ± standard error) is given. The dotted lines represent the range of fluctuation found for control dogs over the course of this study.

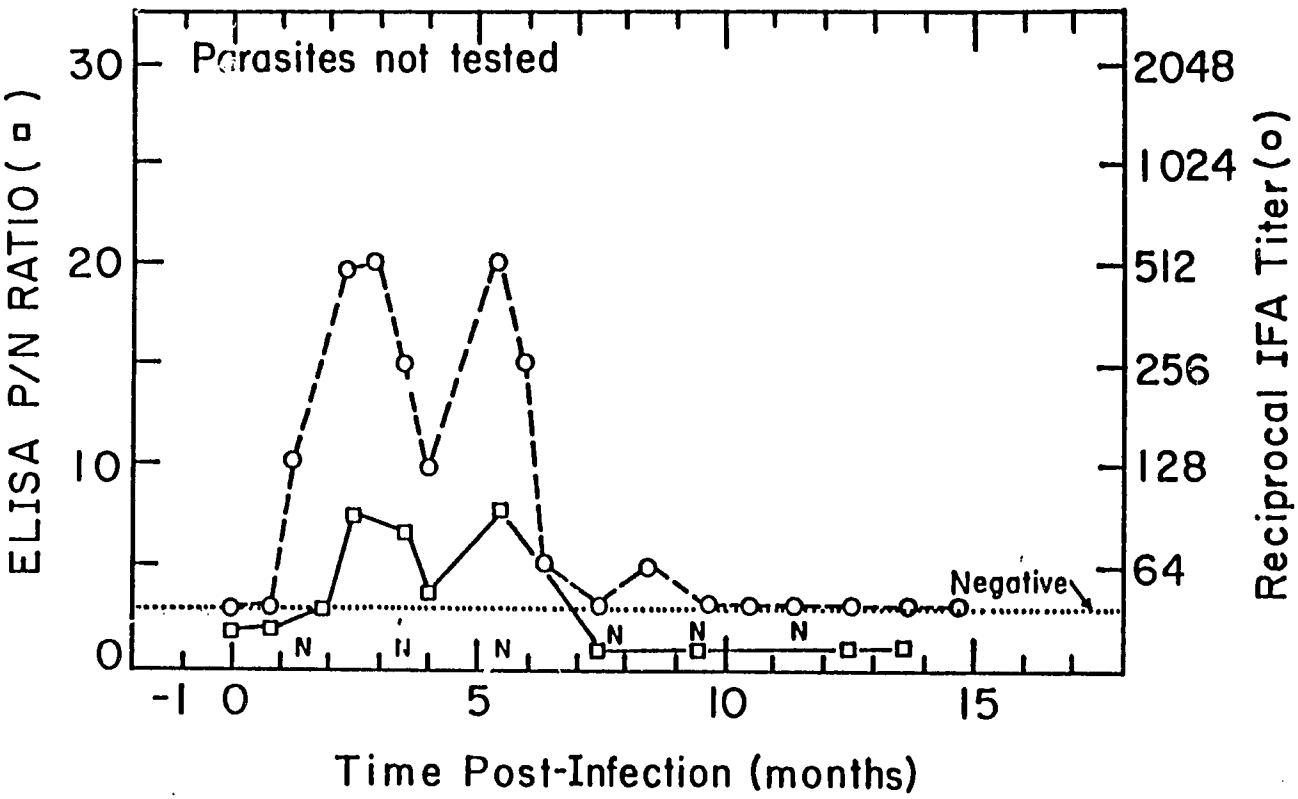
Figure 4. Antigen - specific proliferation of peripheral blood mononuclear cells (PMBC) to particulate leishmanial antigen. PMBC were stimulated in the presence or absence of L. donovani donovani (10μg/ml protein) antigen and the incorporation of ^{3}H-thymidine was determined as described in Material and Methods. Experiments were carried out in quadruplicate. The delta cpm (cpm in the presence of antigen - cpm in the absence of antigen) for each dog is presented. The number of the dog followed by the month assayed in brackets (# M) is given for each point. Negative - control dogs (n - 4), Amas - amastigote challenged dogs, and Pro - promastigote challenged dogs.

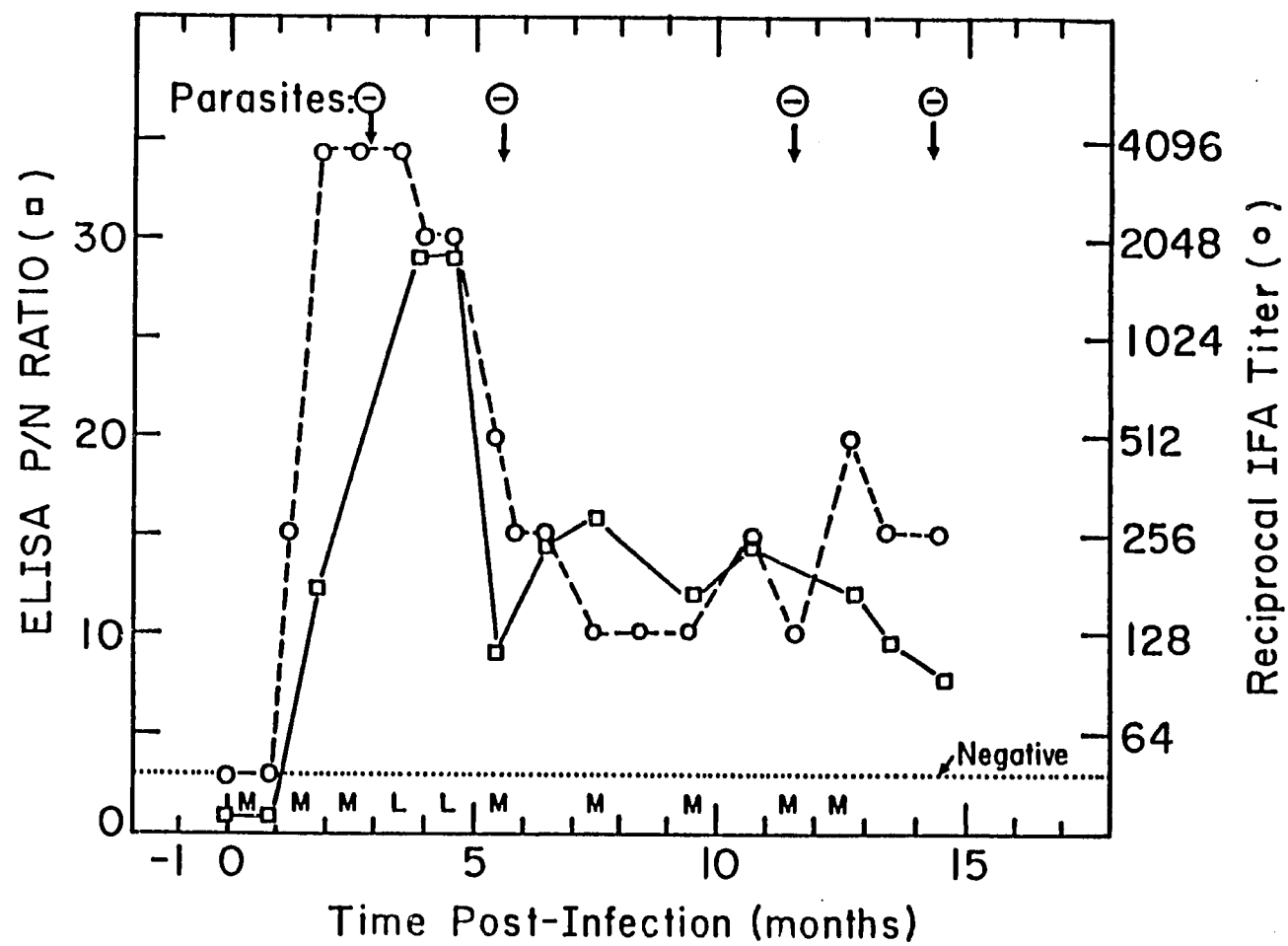
Figure 5. Recognition of leishmanial antigens by antibodies from dogs challenged with Leishmania donovani infantum. Western blots following SDS-PAGE were probed with infected dogs sera taken at monthly intervals over the period of this study. Either crude L. donovani membranes (350 μg/gel), pure dp72 (0.8μg/gel) or pure gp70-2 (0.8μg/gel) were used as antigen. Sera were diluted to 1/1000 for the crude and 1/100 for the pure protein antigens. Panel A. Dog No. 7, amastigotes via the intravenous route and negative control dog sera. Panel B. Dog No. 10, promastigotes via the intravenous route and dog No. 13, amastigotes via the intradermal route.

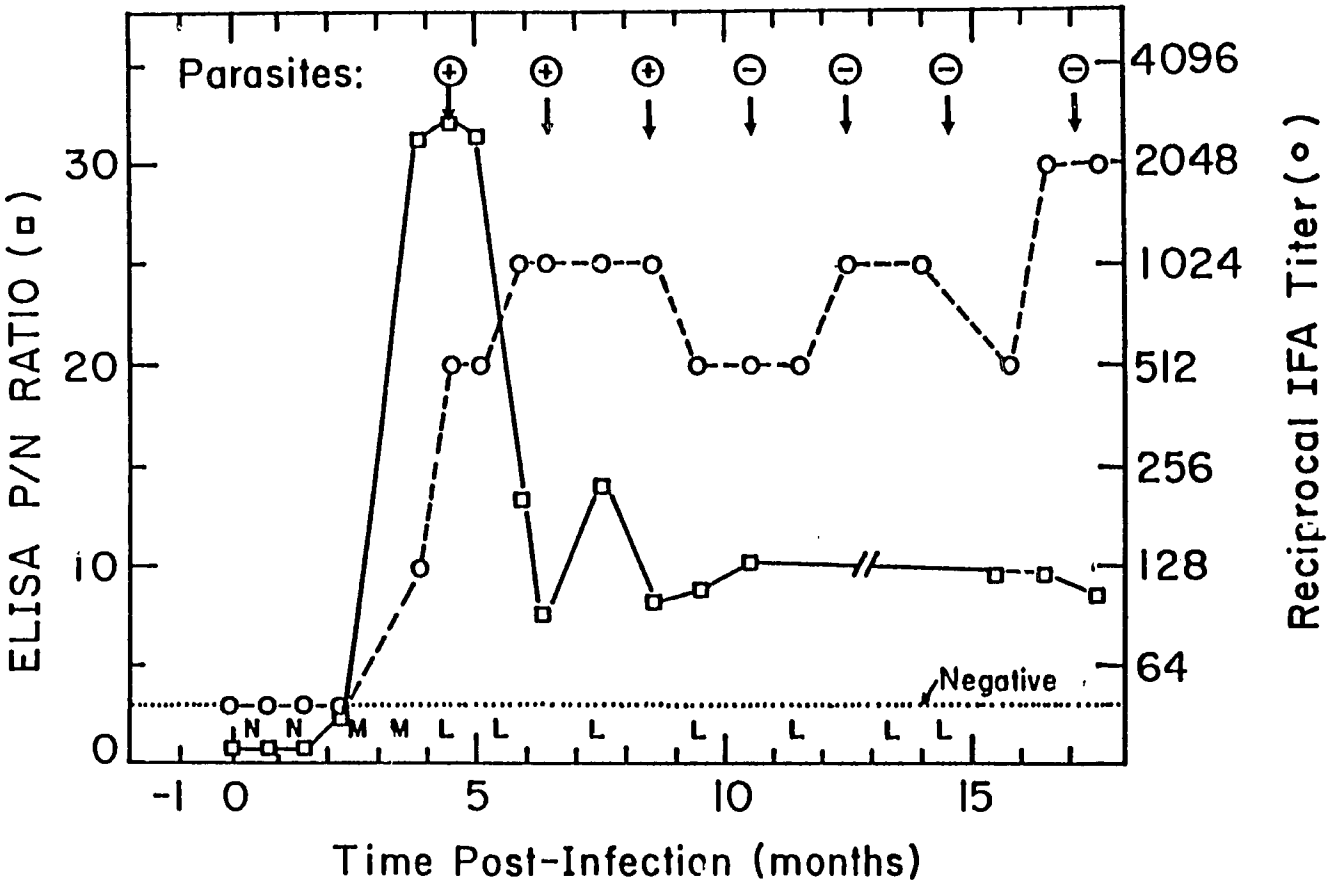
Table 1. Mitogenic Proliferation to Concanavalin A of PBMC from Normal and Infected Dogs

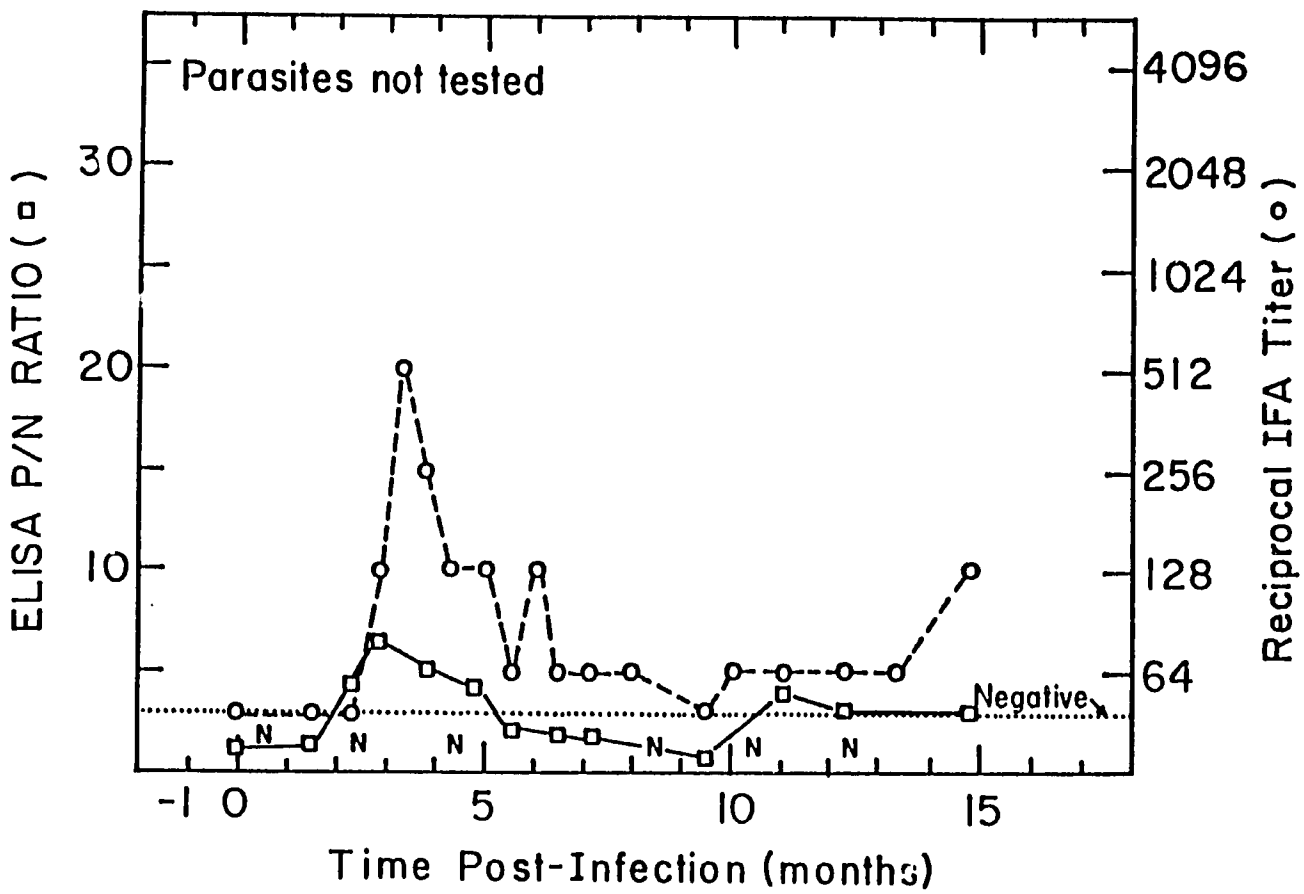
Dog #	Months Infected	Months postinfection	Concanavalin A (SI*) 10μg	5μg	1μg
3	-	n.a.	n.t.	32.1	n.t.
5	Pro	15	22.0	21.0	10.4
6	-	n.a.	70.1	71.2	33.1
7	Amas	12	44.5	50.6	13.1
9	Amas	11	42.0	33.3	12.0
10	Pro	10	n.t.	62.3	23.6
11	Amas	12	80.2	70.6	34.9
12	Pro	15	85.4	83.1	37.7
13	Amas, i.d.	11	49.4	49.5	19.5
14	-	n.a.	29.9	24.8	7.7
17	-	n.a.	15.7	12.3	4.9

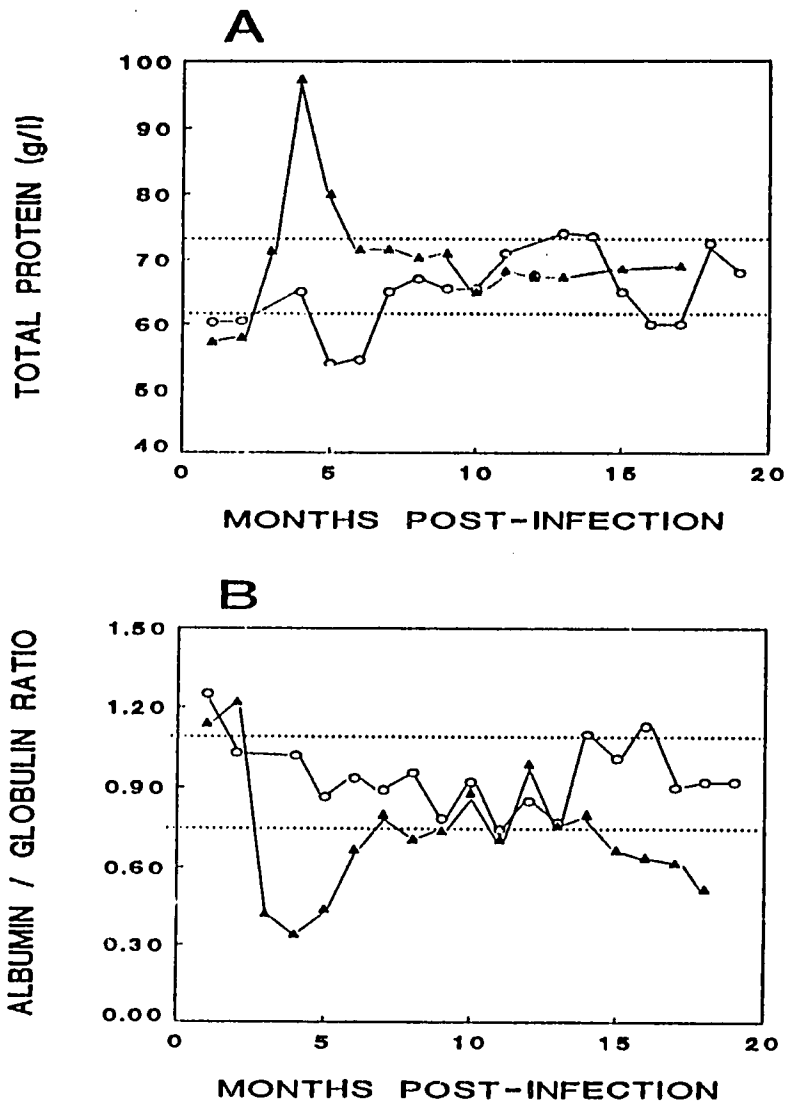
*SI - stimulation index, n.a. - not applicable, n.t. - not tested, Pro - promatigote, Amas - amastigote, i.d. - intradermal.

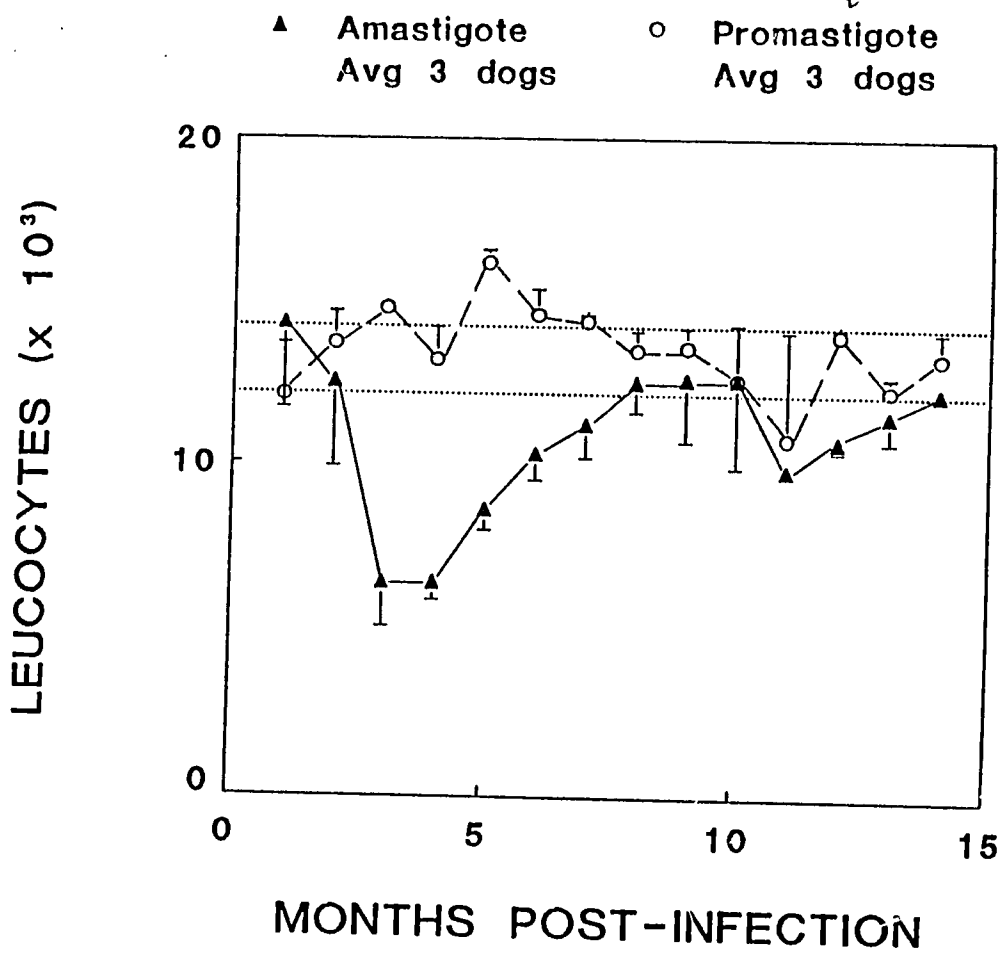

Parasites not tested
ELISA P/N RATIO (□)
Reciprocal IFA Titer (○)
30
20
10
0
2048
1024
512
256
128
64
Negative
N
N
N
N
N
N
-1
0
5
10
15
Time Post-Infection (months)

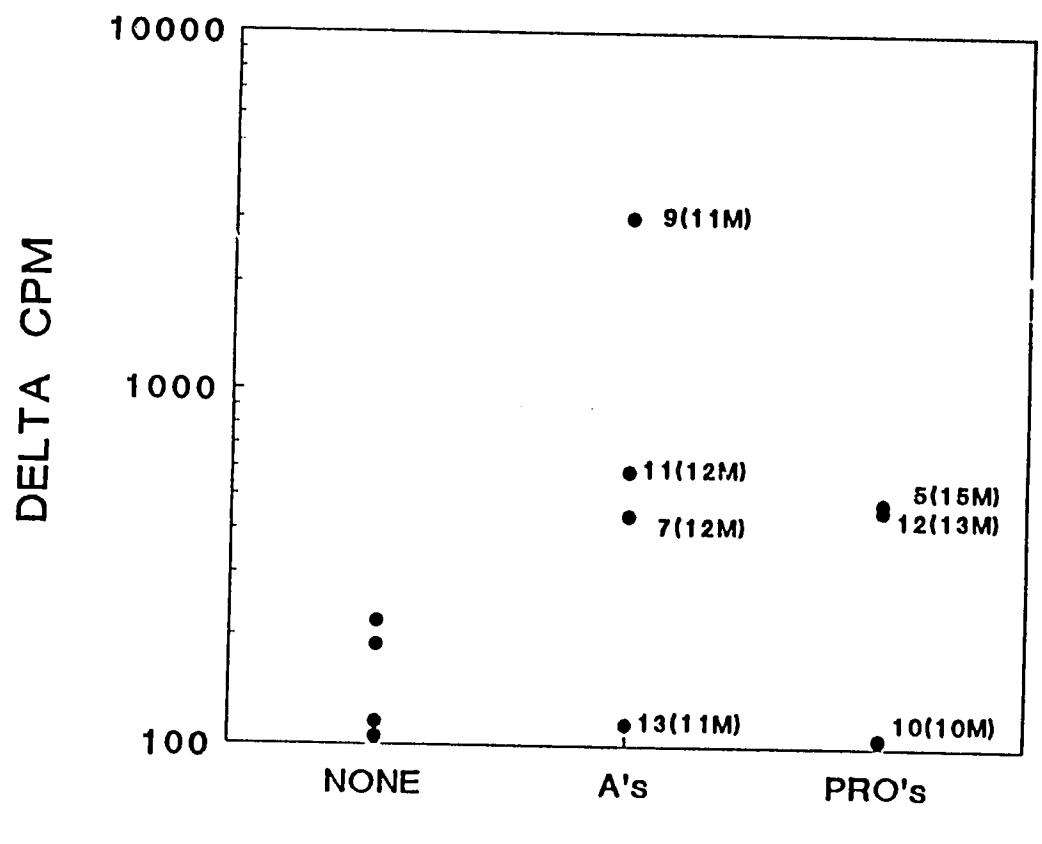

Parasites:
ELISA P/N RATIO (□)
Reciprocal IFA Titer (○)
30
20
10
0
4096
2048
1024
512
256
128
64
Negative
M
M
M
L
L
M
M
M
M
M
-1
0
5
10
15
Time Post-Infection (months)


Parasites:
ELISA P/N RATIO (□)
Reciprocal IFA Titer (○)
Negative
N N M M L L L L L L L
-1 0 5 10 15
Time Post-Infection (months)

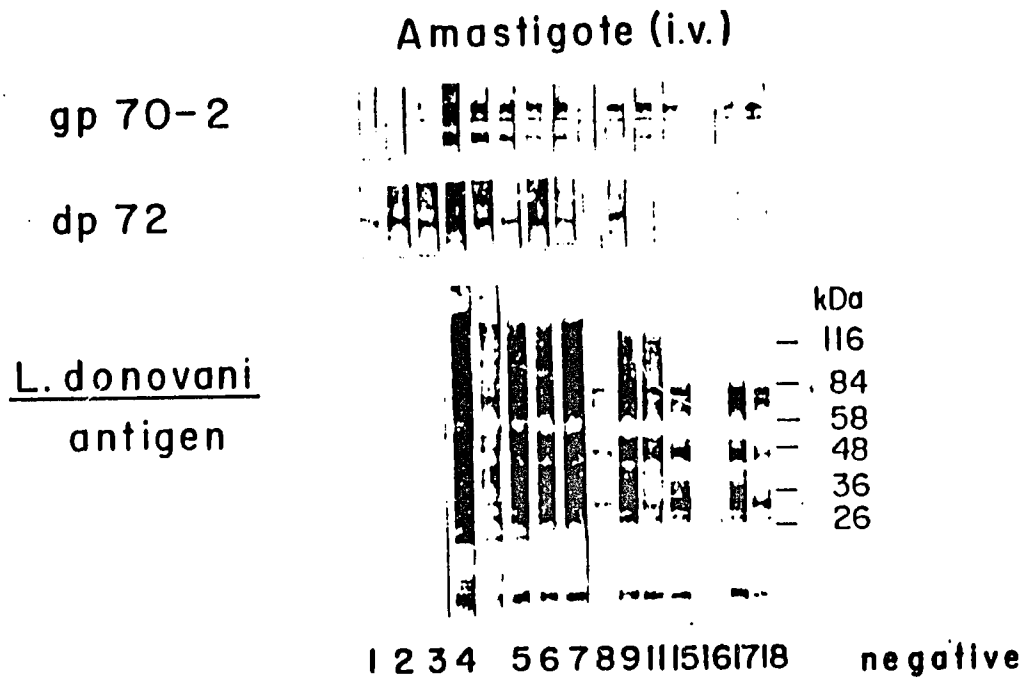

Parasites not tested
ELISA P/N RATIO (□)
Reciprocal IFA Titer (○)
Negative
N N N N N N
-1 0 5 10 15
Time Post-Infection (months)


A
TOTAL PROTEIN (g/l)
100
90
80
70
60
50
40
0
5
10
15
20
MONTHS POST-INFECTION
B
ALBUMIN / GLOBULIN RATIO
1.50
1.20
0.90
0.60
0.30
0.00
0
5
10
15
20
MONTHS POST-INFECTION

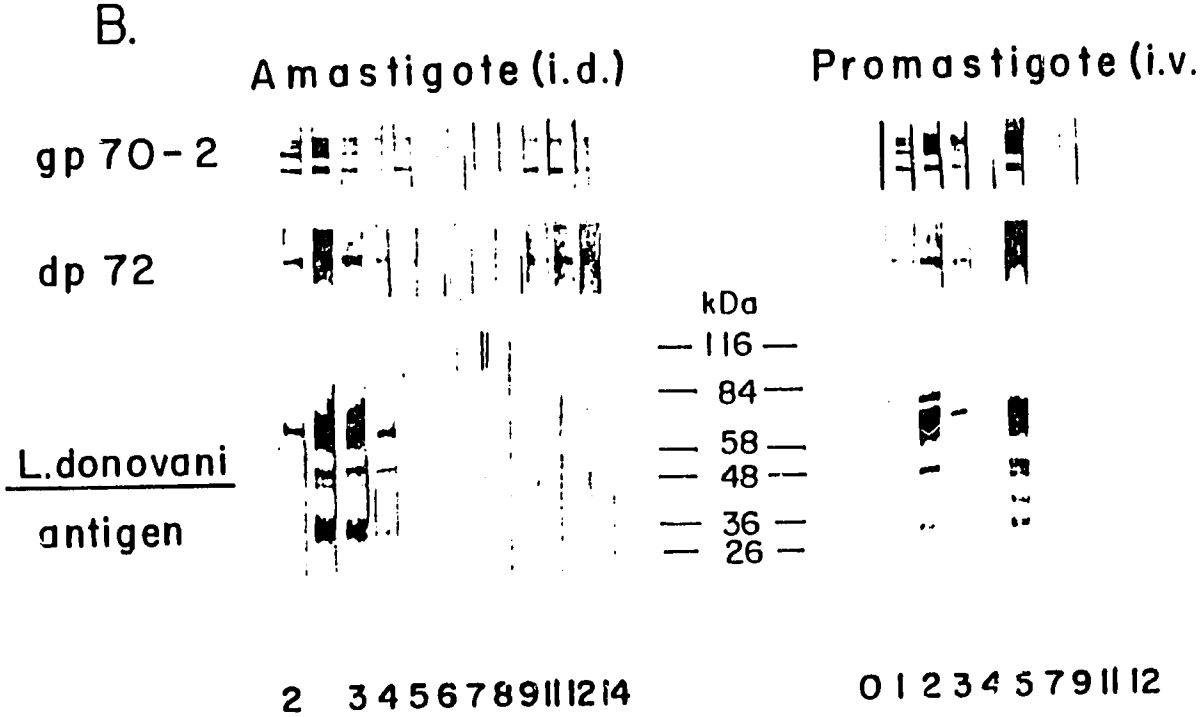

▲ Amastigote
Avg 3 dogs
○ Promastigote
Avg 3 dogs
LEUCOCYTES (x 10³)
20
10
0
0
5
10
15
MONTHS POST-INFECTION


DELTA CPM
10000
1000
100
9(11M)
11(12M)
7(12M)
5(15M)
12(13M)
13(11M)
10(10M)
NONE
A's
PRO's
PARASITE CHALLENGE

A.


Amastigote (i.v.)
gp 70-2
dp 72
L. donovani
antigen
kDa
116
84
58
48
36
26
1 2 3 4 5 6 7 8 9 11 15 16 17 18
negative
Time Post-Infection (mon.)

B.


Amastigote (i.d.)
Promastigote (i.v.)
gp 70-2
dp 72
kDa
116
84
58
48
36
26
L. donovani
antigen
2 3 4 5 6 7 8 9 11 12 14
0 1 2 3 4 5 7 9 11 12
Time Post-Infection (mon.)