PARTICULATE ANTIGENS OF *TRYPANOSOMA CRUZI* AND FOUR *LEISHMANIA* SPP.: STUDY OF BINDING SPECIFICITIES OF MONOCLONAL AND POLYCLONAL ANTIBODIES

By

ABIODUN OLADAYO ADIBI

Bachelor of Science
Southwest Missouri State University
Springfield, Missouri
1975

Master of Science
East Tennessee State University
Johnson City, Tennessee
1978

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1985

Thesis
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Thesis Approved:

Joseph Paul Fox
Thesis Adviser

John T. Homer

S. A. Ewing

Helen E. Jordan

Mark R. Sanborn

Norman N. Durham
Dean of the Graduate College

1233527

ACKNOWLEDGMENTS

I would like to express my profound gratitude to Dr. J. C. Fox, my major adviser, without whom this work would have being a dream. His guidance, advice and unceasing help are greatly appreciated. I am thankful to Dr. S. A. Ewing for the time spent editing this work and for all kinds of help he rendered during my stay at Oklahoma State University.

My gratitude also goes to Dr. J. T. Homer for the timely comments and for his help with fluorescent microscopy. I am grateful to Dr. Helen Jordan for serving on my thesis committee and for the knowledge I gained from my contact with her in the classroom.

Special thanks are due to Dr. Mark Sanborn and Dr. Timothy Yoshino for their guidance during the early stage of the monoclonal antibody study. I am also thankful to Dr. A. Confer and the staff of Animal Respiratory Disease Laboratory for their cooperation and help during the course of my studies. I wish to express my gratitude to Dr. Jim Wright for his help with the computer analyses of data.

I am greatly indebted to my parents, Chief and Mrs. Nathaniel A. Adibi and to my sisters and brother for their support and encouragement throughout my stay away from home. My special appreciation goes to my wife, Leanne, and my two sons, Olamide and Oyekola for their patience, support and understanding.

This research was partially funded by a grant from the College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma.

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CHAPTER I

INTRODUCTION

Some of the early literature on leishmaniasis reported serological cross-reaction between different species of *Leishmania* and also between *Leishmania* and *Trypanosoma cruzi*. Most studies demonstrating cross-reactions were done using soluble antigens obtained as supernatants from disrupted and centrifuged organisms; however, undisrupted and disrupted organisms prepared without centrifugation were also used as antigens. It has been theorized that the major source of cross-reactions resides in the soluble proteins present in most antigen preparations. It is postulated for the purpose of this study that particulate antigens might diminish the tendency for serological cross reactions.

The objective of this study was to examine serologic cross-reactions between several species of *Leishmania* and *Trypanosoma cruzi* using washed particulate antigens prepared from each of these organisms. Specific antiserum to each species was produced in rabbits. Particulate antigens from a *Leishmania* sp. isolated from a dog from Oklahoma (OKD-11) were used to immunize BALB/c mice. Spleen cells from these mice were used in

hybridoma and monoclonal antibody production. If particulate antigens are less cross-reactive than soluble ones as is postulated, the hybridomas would generate clones with a high frequency of monoclonal antibodies specific for the species which served as the source of the immunizing antigens.

Life Cycle and Morphology of *Leishmania*

The Order Kinetoplastida (Phylum Sarcomastigophora, Subphylum Mastigophora) contains the Family Trypanosomatidae which contains the genera *Leishmania* Ross, 1903, *Trypanosoma* Gruby, 1843, *Leptomonas* Kent, 1881, *Herpetomonas* Kent, 1881, *Crithidia* Leger, 1902 and *Phytomonas* Donovan, 1909 (Manwell, 1961; Thomson and Robertson, 1928). The life cycles of *Leishmania* species requires two different hosts - a vertebrate host and an insect vector; the latter is responsible for transmission of the organisms from one vertebrate host to another. Biological transmission of *Leishmania* is believed to be by phlebotomine sandflies; these include different species of the genera *Phlebotomus* Rondani, 1840 and *Lutzomyia* Franca, 1924 (Chandler and Read, 1961; Lainson and Shaw, 1978). McKenzie (1984) demonstrated transtadial transmission of *Leishmania* sp. by *Rhipicephalus sanguineus* (Latreille, 1806), an ixodid tick.

The mammalian hosts which have been recorded for

Leishmania are two-toed sloths (Gentile et al., 1981; Christensen and Vasquez, 1982),forest rodents including the genera *Oryzomys*, *Proechimys*, *Heteromys* and *Otolylomys*, porcupines, dogs, foxes, marsupials and human beings; hosts in which infections resulted in pathological disorders were considered to be unusual hosts (Lainson and Shaw, 1978).

Two morphologically distinct forms are found at different points in the life cycle of *Leishmania*; these two forms were formerly called leishmania and leptomonad but were renamed amastigotes and promastigotes, respectively by Hoare and Wallace (1966). The amastigotes which were also referred to as Leishman-Donovan (L-D) bodies is the definitive stage of *Leishmania*; they are present in the vertebrate host. Amastigotes are normally ovoid and each possess a rudimentary flagellum; they parasitize the cells of the reticuloendothelial system (RES) where reproduction occurs by binary fission. Amastigotes measure approximately 1.5-3.0 μm x 3.0-6.5 μm depending on the species (Gardener et al., 1977; Garnham and Lewis, 1959; Lainson and Shaw, 1978; Sanyal and SenGupta, 1967). The amastigotes are ingested from the skin or peripheral blood when female arthropod vectors feed on infected vertebrate hosts. Within the guts of the vectors, amastigotes transform to promastigotes.

The promastigotes measure 1.5-3.0 um x 16.0-40.0 um (Lainson and Shaw, 1978). Promastigotes normally have

a free flagellum arising from a kinetosome located at the extreme anterior end of the cell. A kinetoplast is situated very near and posterior to the kinetosome; the kinetoplast contains mitochondrial deoxyriobonucleic acid (DNA) (Aleman, 1969; Chatterjee and SenGupta, 1970). Promastigotes occur extracellularly in the midgut of the insect vectors where they multiply by binary fission and may attach to the wall of the midgut. The promastigotes eventually migrate to the pharynx and proboscis where they attach until they find access to new vertebrate hosts when female vectors take blood meals.

After entering the definitive hosts, the promastigotes are phagocytized by the cells of the RES where the promastigotes undergo morphological changes to become amastigotes. Although amastigotes and promastigotes have certain antigens in common, each form is believed to have stage-specific antigens (Handman and Curtis, 1982; McMahon-Pratt and David, 1982 and Fong and Chang, 1982).

Host Susceptibility

Guinea pigs have been shown to be susceptible to *L. enriettii*, whereas humaans are not. Likewise, mice are susceptible to *L. donovani* and not *L. enriettii*. Bradley (1974) demonstrated that host susceptibility to leishmanial organisms has a genetic basis associated with hematopoietic cell. Howard et al.

(1980) pointed out that susceptibility was due to an autosomal, partially dominant gene. Pinder (1984) demonstrated that certain sex-associated facators potentiated resistance to _T. congolense_ infection in female BALB/c mice; genetic control of resistance to _T. congolense_ was reported to be a recessive trait controlled by a single gene.

Susceptibility of the host to leishmaniasis may differ according to the route of inoculation. Leishmanial-resistant C57BL/6 mice remained resistant when the mice were given intradermal injections with _L. tropica_ promastigotes. On the other hand, intravenous injections with the same organism resulted in non-healing infections similar to those in genetically susceptible BALB/c mice (Scott and Farrell, 1982).

Leishmaniasis in Unusual Hosts

Three principal types of leishmaniasis are recognized worldwide in human beings: visceral, mucocutaneous and cutaneous forms. Visceral leishmaniasis known locally as "Dum-Dum fever" or "kala-azar" is caused by infection with _Leishmania donovani_. Infected human beings usually exhibit hepatosplenomegaly. Visceral leishmaniasis occurs in the tropical Americas, near the shores of Mediterranean Sea, India and in equatorial Africa. In the Mediterranean region, leishmaniasis occurs primarily in children, and there is also a high prevalence

in dogs; the etiologic agent in the Mediterranean region is L. infantum. Lainson and Shaw (1978),citing Deane et al. and Cunha and Chagas, expressed the view that visceral leishmaniasis of the New World should be referred to as American leishmaniasis; it was stated that the etiologic agent was L. chagasi. Explanation for this view was that the diseases in the New World was zoonotic as opposed to visceral leishma- niasis in India which was anthroponotic. They suggested that the latter disease should be called kala-azar, a hindu name shich best described the pathological condition seen in the human disease in India.

Mucocutaneous leishmanisis is variously known as chiclero ulcer, espundia or uta depending on the geographicla area. It is caused primarily by L. braziliensis and is characteristically associated with degeneration of cartilaginous and soft tissues of the nose, ear, lips, palate and portions of the upper respiratory tract (Chandler and Read, 1961).

Cutaneous leishmaniasis, also known as "oriental sore" and "Jericho boil" is caused by L. tropica. This infection is generally confined to the skin (Chandler and Read, 1961). Garnham and Humphrey (1969) wrote about two forms of cutaneous leishmaniasis: "moist" and the "dry" forms. The "moist" form of the disease is caused by L. tropica major; it is of zoonotic origin and is less chronic than the "dry" form which is caused by

L. tropica minor. The latter is believed to be of human or canine origin. Another form of cutaneous leishmaniasis is caused by the *Leishmania mexicana* complex. Normal hosts of these organisms are forest rodents and opssums (Lainson and Shaw, 1969 and 1972). Infections of human beings lead to single and mild cutaneous lesions. Occasionally, diffuse cutaneous leishmaniasis results from infection (Lainson and Shaw, 1973).

Host Immunologic Reacation to Leishmaniasis

Host immune responses to leishmaniasis are characterized by either a strong delayed hypersensitivity as in cutaneous diseases or a weak to strong humoral antibody response typical of visceral infections. The latter is rater non-speicific for parsite species (Bray, 1972; Clinton et al., 1969; Heyneman, 1971; Manson-Bahr, 1961). According to Heyneman and Welch (1980), the course of infection in cutaneous leishmaniasis will be determined by the status of the host's cell-mediated immune response which may be classified relatively as inadequate, adequate or excessive. An adequate cellular response usually leads to complete healing of lesions with subsequent protection against further infection. When the cellular response is inadequate or lacking, diffuse cutaneous leishmaniasis may occur as a result of metastasis of the organisms. On the other hand, if the cell-mediated immune response is strong, a condition known as "lupoid" or "recidiva"

leishmaniasis ensues; this condition is characterized by nonulcerated lymphoid nodules which form at the edges of the primary lesions. Observation of experimental infections in BALB/c mice by Mitchell et al. (1981) and in guinea pigs by Poulter and Pearce (1980) support the hypothesis that the lack of an adequate cell-mediated immune response may lead to the condition of diffuse cutaneous leishmaniasis. Poulter and Pearce (1980) showed that guinea-pigs infected with L. enriettii developed T-cell mediated immune responses from the second to the seventh week post inoculation with leishmanial antigens after which there was a steady decay of cellular response followed by metastasis of the lesions. They attributed the decay of T-cell mediated immunity to a lack of microbicidal capacity of macrophages resulting from a suppression of certain macrophage effector functions. This conclusion was arrived at because guinea-pigs which were infected with Listeria monocytogenes at the sites of metastatic lesions induced by L. enriettii showed suppressed resistance to the L. monocytogenes.

Susceptibility to L. tropica infection is genetically regulated in BALB/c mice and results from a non-specific immunodepression of the host (Mitchell et al., 1981; Scott and Farrell, 1981 and 1982). Indomethazin, a prostaglandin synthase inhibitor, alleviated the immunodepression in BALB/c mice (Scott

and Farrell, 1981); this group hypothesized that the immunodepression in infected BALB/c mice might be due to increased production of prostaglandin. Cellular immunosuppression in human beings suffering from visceral leishmaniasis was reported by May et al. (1983). The immunosuppression appeared to be both specific and nonspecific in nature in that there was no responses to leishmanial antigens nor to other antigens. However, immunosuppression to the nonspecific antigens was less consistent in the patients.

Host immunological responses during the early stages of visceral leishmaniasis is predominantly humoral which often leads to a nonspecific hypergammaglobulinemia. With treatment of leishmaniasis or with spontaneous recovery, the level of immunoglobulin decreased and eventually a measurable cell-mediated immunity could be detected (Manson-Bahr, 1961). Rezai et al. (1978) described some of the immunologic features of some untreated patients with visceral leishmaniasis. The majority of them had increased numbers of B-cells and elevated immunoglobulin G (IgG) levels. There was also a decrease of T-cells in the peripheral blood. Veress et al. (1977) also reported histologic examinations of spleen and lymph nodes from patients with visceral leishmaniasis; they observed depletion of small lymphocytes and plasma cell hyperplasia. These observations on T-cell populations may offer explanation

for lack of delayed hypersensitivity in visceral leishmaniasis. In another study, Carvalho et al. (1981) concluded that the lesions of visceral leishmaniasis were associated with impaired T-cell function. This conclusion was reached following observations that lymphocytes from untreated patients were not stimulated by *L. chagasi* antigens or phytohemagglutinin (PHA), whereas those from treated patients were stimulated. A probable explanation for the lymphocyte impairment was provided by Londner et al. (1983) and Wyler (1982). They found that the lack of lymphocyte transformation in the pretherapy lymphocytes was due to a factor excreted by *Leishmania*.

Serodiagnostic Tests in Leishmaniasis

Various serodiagnostic methods have been used for diagnosis of leishmaniasis including direct agglutination (Allain and Kagan, 1975), Ouchterlony double radial immunodiffusion (Bray and Lainson, 1966), fluorescent antibody test (Duxbury and Sadun, 1964; Mishra and Ludha, 1975; Behforouz et al., 1976) and an enzyme-linked immunosorbent assay (Voller et al., 1975; Hommel et al., 1978; Luzio et al., 1979; Anthony et al., 1980). Of these tests, indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA) are the most commonly used tests. A comparison of the ELISA and IFA tests by Edrissian and Darabian (1979) showed that the ELISA was more sensitive than the slide IFA test; however,

the IFA test was more specific.

According to Mansueto et al. (1980), useful serodiagnostic tests must be sensitive, specific and easy to perform. Both IFA and ELISA tests generally fit these criteria. However, all the available tests lack a high degree of specificity. Perhaps, IFA and ELISA tests could be made more specific through the use of specific monoclonal antibodies.

Monoclonal antibodies are used primarily for diagnostic reagents, vaccine design and production and analysis of antigens (Antczak, 1982). Hybridoma technology has been used to study various parasitic protozoa including _Toxoplasma gondii_ (Sethi et al., 1980). Johnson et al. (1983) produced two monoclonal antibodies which conferred total protection in mice against challenge with a moderately virulent strain of _T. gondii_. Freeman et al. (1980) produced merozoite-specific monoclonal antibodies to _Plasmodium yoelii_; passive transfer of these monoclonal antibodies into _P. yoelii_-infected mice prevented parasitemia and death of the mice. Similarly, Yoshida et al. (1980) produced monoclonal antibodies which bound specifically to surface membrane protein of _P. berghei_; when sporozoites were incubated with the antibodies, they were no longer infective.

Hybridoma technology has been used to produce monoclonal antibodies to several species of _Leishmania_

and *Trypanosoma*. As a result of their work with surface antigens of a *Trypanosoma* sp., Pearson et al. (1981) reported that monoclonal antibodies were ideal probes for studying antigenic sites. A study published by Lyon et al. (1981) also used monoclonal antibodies to characterize the surface coat glycoprotein from *T. rhodesiense*. In other studies, monoclonal antibodies were described that differentiated between *L. mexicana* and *L. braziliensis* (McMahon-Pratt and David, 1981), *T. rangeli* and *T. cruzi* (Anthony et al., 1981), *L. braziliensis* and *T. cruzi* (Constantine and Anthony, 1983), promastigote-specific antigens (McMahon-Pratt and David, 1982) and epimastigote-specific antigens (Snary et al., 1981).

Immunotherapy and Leishmaniasis

Induction of protective immunity against leishmaniasis in guinea-pigs was attempted by Lemma and Cole (1974) using irradiated promastigotes of *L. enriettii*. They observed that vaccination with the irradiated organisms had no protective value. Recently, Beacham et al. (1982) documented successful vaccination against mucocutaneous leishmaniasis in African white-tailed rats. These rats failed to develop ulcers at the sites of challenge; their spleen lymphocytes also exhibited vigorous response to *in vitro* challenge with homologous antigens. Similarly, Green et al. (1983)

observed that lymphocytes from subjects vaccinated with *L. tropica major* promastigotes responded to low concentration of antigens while lymphocyte responses of the control subjects were depressed. Cook and Holbrook (1983) and Holbrook and Cook (1983) observed that when glucan was used as an adjuvant along with homogenized particulate antigens and whole cells of *L. donovani*, resistance against challenge occurred. Isolation of immunogenic components of leishmanial antigens, as was done by Nguyen et al. (1984), will increase the feasibility of developing effective vaccination programs for control of leishmaniasis.

Problems Related to Control of Leishmaniasis

Leishmaniasis has been controlled in some endemic regions by intensive insecticide spraying, extermination of infected and stray dogs and chemotherapy of infected persons (Lainson and Shaw, 1978). However, worldwide control of leishmaniasis has been retarded by a lack of information concerning the taxonomy and methods of species identification. Serodiagnosis is hampered by serological cross-reactions between different species of *Leishmania* and also with *Trypanosoma cruzi*. More information is needed on how to prevent intracellular development and multiplication of amastigotes within macrophages.

Problems With Taxonomy

The importance of proper taxonomic classification in the control of leishmaniasis withing various regions was emphasized by Chance (1979) in the following statement: "The long term prospect of controlling leishmaniasis by vaccination will only be achieved if we have exact knowledge of the indentity of the organisms from which atntigen is prepared and also what species can be protected against." Currently the taxonomy of *Leishmania* species is based on clinical pathology in human beings, and on the epidemiology and geographical distribution of the parasite. These criteria do not provide a complete list of parameters for identification of *Leishmania*. Taxonomy based upon the clinical picture might be misleading because organisms of distinctly different species might produce similar clinical disease regardless of the geographical area. Also, one species of parasite may produce different clinical conditions in various host species (Hoogstral and Heyneman, (1969).

In 1979, Zuckerman expressed a need for a revised taxonomy of the species of *Leishmania* based on intrinsic characteristics such as metabolic enzymes, deoxyribonucleic acid (DNA) buoyant density and the numbers of subpellicular microtubules.

Biochemical Characterization of Metabolic Enzymes. Electrophoretic mobility studies of malate dehydrogenase

(Gardener et al., 1974), alanine aminotransferase and aspartate aminotransferase (Kilgour et al., 1974) were used to differentiate species and subspecies of Leishmania. Also Kreutzer and Christensen (1980) reported that L. braziliensis, L. hertigi, L. mexicana, L. donovani, L. tropica and L. adleri each had distinct electrophoretic patterns for aspartate aminotransferase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and fructokinase. The electrophoretic patterns for other enzymes were closely similar for all the species of Leishmania. Kreutzer et al. (1983) differentiated five major groupings of Leishmania within 44 isolates using electrophoretic patterns of twenty five enzymes. Relationship was based on similarities of electrophoretic patterns of the enzymes.

DNA Buoyant Density. A buoyant density study of nuclear and kinetoplast DNA was done by Chance et al. (1973). They differentiated Old World and New World and also lizard strains of Leishmania. Another study by Chance et al. (1974) involving buoyant density of nuclear and kinetoplast DNA differentiated a number of Leishmania species. They demonstrated differences between L. tropica major and L. tropica minor, and also L. braziliensis guayanensis and L. braziliensis braziliensis; they also found that L.

hertigi was distinct from the other Leishmania species they tested.

Cell Size and Number of Microtubules. Differentiation of amastigotes was done by Shaw and Lainson (1976). They distinguished L. mexicana amazonensis and L. braziliensis braziliensis. Gardener et al. (1977) substantiated the result of Shaw and Lainson; the group also differentiate L. donovani, L. braziliensis and the "mexicana complex". Kocan et al. (1983), using greatest diameter measurements and microtubule counts, showed that a canine leishmanial isolate first observed in Oklahoma by Anderson et al. (1980) was most similar to a strain of L. donovani from the Sudan. Gardener et al. (1977), Lewis (1975), and Sanyal and SenGupta (1967) also studied the numbers of microtubules in amastigotes for the purpose of differentiating species of Leishmania. Gardener et al. (1977) and Sanyal and SenGupta (1967) distinguished between L. donovani and "mexicana complex", and Lewis (1975) demonstrated differences in the numbers and spacing of subpellicular microtubules in the amastigotes of Leishmania spp. isolated from reptiles and mammals.

Serologic Cross-Reactivities of *Leishmania* species

Serologic cross-reaction among diverse species of Leishmania and Trypanosoma such as T. cruzi have

been definite obstacles to identification of *Leishmania* spp. by conventional serodiagnostic technics. Bray and Lainson (1966) showed by Ouchterlony double diffusion tests that cross-immunity between *L. mexicana* and *L. tropica* was due probably to one antigen among four or five antigens shared by both organisms. On the other hand, *L. donovani* shared many antigens with all other strains tested and consequently exhibited a wider range of serological cross-reactivity. Alexander and Phillips (1978) and Perez et al. (1979) used mouse models and observed cross-immunity between *L. mexicana* and *L. tropica*; this cross-immunity was manifested as a resistance to challenge with either homologous or heterologous organisms. The immunity was present following recovery recovery from primary infections eith either of the two organisms. Other workers have described serological cross-reactions between isolates of *L. donovani*, *L. tropica* and *L. enriettii* (Rezai et al., 1969), *T. cruzi* and *L. braziliensis* (Camargo and Rebonato, 1969), *L. donovani*, *L. tropica* and *L. mexicana* (Ciskowski, 1973), *T. brucei* and *L. tropica* or *L. mexicana* (Alexander and Phillips, 1978a). In addition, Roffi et al. (1980) reported serological cross-reactions between *L. tropica* promastigote antigens and sera from patients with toxoplasmosis, malaria and leprosy. Barriga (1981) indicated that pretest adsorption of antisera with cross-

reacting parasite antigens would eliminate the cross-reacting antibodies.

Intracellular Multiplication and Killing of *Leishmania*

Amastigotes of Leishmania spp. have the ability to multiply within the phagocytic cells of the reticulo-endothelial system. Several mechanisms have been postulated by which intracellular development is accomplished by some protozoans. These include the prevention of lysosome-phagosome fusion (Jones and Hirsch, 1972), resistance to lysosomal enzymes (Chang and Dwyer, 1976) and escape from phagolysosomes into macrophage cytoplasm (Kress et al., 1977). The mechanism by which all secretory cells in metazoans release their contents has been shown by Barret (1978) to be affected by the intracellular level of cyclic adenosine monophosphate (cAMP). An increase in the intracellular level of cAMP brought about by prostaglandin E prevents the formation of phagolysosmes in leucocytes (Barret, 1978). Thus amastigotes may survive in the macrophages by directly or indirectly stimulating the production of cAMP.

Contrary to speculation that amastigotes have the ability to prevent formation of phagolysosomes, as was described for Toxoplasma gondii by Jones and Hirsch (1972), Berman et al. (1981) and Chang and Dwyer (1976) observed that amastigotes of L. donovani were able to multiply within phagolysosomes. Chang and Dwyer (1976)

used lysosomal markers and electron microscopy to confirm that there was formation of phagolysosomes. Consequently, they speculated that the intracellular survival of *L. donovani* appeared to be a result of resistance to macrophage lysosomal enzymes.

Other methods by which intracellular killing of amastigotes can be potentiated *in vitro* include pretreatment of macrophage cultures with phenazing methosulphate (Rabinovitch et al., 1982), extracts of *Mycobacterium tuberculosis* and *Corynebacterium parvum*, or by addition of various lymphokines (Haidaris and Boventre, 1981; Nacy et al., 1981) prior to inoculation with leishmanial amastigotes. Apparently, these various treatments enhance the production of intracellular hydrogen peroxide which is believed to be responsible for actually killing the organisms (Haidaris and Boventre, 1982; Pearson and Steigbigel, 1981). A pharmacological agent that can stimulate the production of hydrogen peroxide in activated macrophages was isolated from *M. tuberculosis* and identified as trehalose diester. Inraperitoneal injections with this compound produced antitumor activity in mice (Lepoivre et al., 1982). However, the possible effect of this compound on the fate of intracellular stages of *Leishmania* spp. remains to be elucidated.

CHAPTER II

MATERIALS AND METHODS

Organisms Compared in Serological Studies

Four isolates of *Leishmania* species and a *Trypanosoma* species were used to prepare antigens which were compared in this investigation.

Leishmania sp. (OKD-11) was isolated from a naturally infected dog from Oklahoma in 1979. The isolate was maintained by serial passage on MaeKelt's and Novy-MacNeal-Nicolle (NNN) media with intermittent storage by freezing at -70° C in 10% glycerin and physiological saline.

The following isolates were obtained from the Walter Reed Army Institute for Research (WRAIR):

Leishmania donovani, origin of isolate not on record.

Leishmania tropica, origin of isolate not on record.

Leishmania mexicana, strain 1156 II, originally isolated from a patient at Veterans Administration Hospital, San Antonio, Texas.

Trypanosoma cruzi, strain Md-R, isolated from juvenile female racoon, *Procyon lotor lotor*.

All the organisms listed were maintained by serial passage on MaeKelt's medium as described above. All isolates had been maintained by alternate culture and freeze storage for more than 3 years.

Antigen Preparation and Standardization

Promastigotes of each of the species of *Leishmania* and epimastigotes of *T. cruzi* were cultured in 22 cm^2 tissue culture flasks containing MaeKelt's medium with 7.5% defibrinated rabbit blood. The overlay solution was 5.0 ml of 0.5% peptone in physiological saline supplemented with 0.2 ug / ml of vitamin B12, and 0.2 ml of penicillin-streptomycin solution (10,000 units / ml and 25 mg / ml, respectively). Flasks were inoculated with organisms and incubated at 23-25°C for about 7 days.

Following incubation, overlays from three small flasks (25 cm^2) containing cultured organisms were transferred into a single 75 cm^2 tissue culture flask containing MaeKelt's medium without rabbit blood. Approximately 50 ml of supplemented peptone-saline solution was also added to the flask. After a second incubation period of 10-14 days, the overlay fluid from the large flask was transferred into sterile 50 ml centrifuge tubes and centrifuged at 4,000 x g for 10 min.

The sediments containing the organisms were pooled and washed 3 times in sterile physiological saline (0.85% NaCl) by centrifugation for 10 min at 4,000 x g. The

sediment from the final wash was resuspended in 2.0-3.0 ml of sterile physiological saline and transferred into a sterile, 16 x 125 mm test tube. This suspension was then frozen and thawed 3 times and homogenized for 3 min at 27,000 rpm with a Brinkman Polytron (Brinkman Instruments, Westbury, NY). The homogenized material was centrifuged at 12,000 x g for 20 min at 5°C using a Sorval Superspeed RC2-B (Dupont Company, Newtown, CN). The resulting sediment was then washed twice in physiological saline by alternate resuspension and centrifugation. The washed solids were designated as particulate antigens.

The particulate antigen concentrations were standardized according to the optical density in physiological saline and protein concentrations measured by Bio-Rad (Bio-Rad Lab., Richmond, CA) protein assays. The absorbance readings for each particulate antigen suspension were determine at a wavelength of 330 nm using a LKB ULTROSPEC 4050 (LKB; Bromma, Sweden). In order to achieve balanced optical density, the antigens were diluted to match the absorbance for the least concentrated antigen.

The Bio-Rad protein assay was done by making five serial dilutions of a protein standard of known concentration in phosphate buffered saline (pH 7.3) containing 0.1% sodium azide (PBS-sodium azide). A Bio-Rad dye reagent was diluted 1:5 in PBS-sodium azide;

5.0 ml of diluted reagent and 0.2 ml of the protein standards and particulate antigens were pipetted into seperate, clean test tubes. A control tube which contained 0.2 ml of PBS-sodium azide was also included. The tubes were stirred on a vortex mixer and incubated for 15-20 min. The optical density was read at a wavelength of 595 nm. Protein concentrations of the particulate antigens were determined by extrapolation from a linear regression curve of control values using a Texas Instrument TI 55 calculator. Standardization of protein concentration for particulate antigens was achieved by diluting each antigen preparation to match the calculated concentration of the least concentrated antigen.

Triton X-100 Solubilization of Particulate Antigens

Particulate antigens were solubilized using Triton X-100 detergent according to the method of Schnaitman (1971). Sedimented solids obtained after centrifugation of 3.0 ml of particulate antigens (optical density standardized) was treated with 1.0 ml of 2.0% Triton X-100 in physiological saline. The suspension was shaken for 15-20 min and centrifuged at 12,000 x g for 10 min. Both the supernatant and the precipitate were saved and designated as detergent-solubilized and detergent-treated particulate antigens, respectively. The latter was washed twice in physiological saline. Separate portions of

particulate antigens (standardized by protein concentration) were similarly treated with 2.0% Triton X-100 solution, but these suspensions were not shaken during treatment. The detergent-soluble antigen and the detergent-treated solids were collected.

Production of Leishmanial Antisera

New Zealand White rabbits were inoculated with particulate antigens produced from cultured organisms; each rabbit received antigen from a different organism. The inoculation dosage and schedule consisted of 0.25 ml of particulate antigens (obtained from approximately 2×10^7 cells) administered subcutaneously every six days. After each rabbit had received seven injections, blood was collected by ear artery puncture and allowed to clot in test tubes. Sera were harvested and stored in 2.0 ml aliquots at $-20^{\circ}C$ until needed.

Maintenance of Myeloma Cells

Mouse myeloma cells P3-X63-Ag8.653, a nonsecreting clone isolated by Kearney et al. (1979), was obtained from Dr. T. Yoshino (University of Oklahoma). The growth medium for the cells was RPMI 1640 (K. C. Biological, Lenexa, KS) supplemented with 10% fetal calf serum (K.C. Biological) and 1.0% L-glutamine (K.C. Biological). One vial of RPMI 1640 and 2.0 gm sodium bicarbonate were dissolved in 1,000 ml deionized, distilled water. Medium

was sterilized under a laminar flow hood by passing it through a sterile pressure-filtration apparatus containing a series of membrane filters including 1.2, 0.45 and 0.2 um pore sizes. The sterilized medium was collected in sterile 500 ml screw-capped bottles. Filtered medium was tested for sterility by pipetting 10 ml aliquots into 25 cm^2 tissue culture flasks; the flasks were incubated with loose caps in a humidified incubator at 37°C with 6% CO_2 in air. All subsequent incubation procedures were done in a similar manner.

Fifty ml of sterile fetal calf serum, 5.0 ml L-glutamine, 12.5 mg Gentocin (Schering Corporation, Kenilworth, N.J.) and 75 ug Fungizone (Gibco, N.Y.) was added to 450 ml of sterile RPMI 1640 just prior to use. This constituted complete, or supplemented medium. The myeloma cells were maintained in 25 cm^2 tissue culture flasks by twice weekly passages at a 1:8 ratio of culture in RPMI 1640 complete medium; incubation was as previously described. Beginning about 8 days before fusion, cells were passaged every other day.

Sensitized Spleen Cells For Fusion

Laboratory reared BALB/c mice were inoculated intraperitoneally with particulate antigens of the (OKD-11) Leishmania sp. on a weekly schedule for 2-3 weeks, or until mice became seropositive for the sensitizing antigens. A booster inoculation was given

9 days after the mice became seropositive and 4 days before harvesting spleen cells for fusion. Just prior to the fusion, the spleens from two immunized mice were removed aseptically and placed in a sterile petri dish containing 10 ml of unsupplemented RPMI 1640. The spleens were comminuted using a sterile spatula; the bulk of the fibrous debris was removed and the mixture was drawn gently in and out of a 10 ml pipet several times to disperse the cells. The suspension was transferred into a sterile 15 ml conical centrifuge tube and allowed to settle for 10 min. Approximately 9 ml of the suspension was transferred into a sterile 50 ml centrifuge tube, and the spleen cells were counted by using a haemocytometer (American Optical, Buffalo, N.Y.). The diluent used for counting cells was made up of 3 ml of 0.2% aqueous trypan blue and 1.0 ml of 4X strength physiological saline. Spleen cells were diluted in the trypan blue diluent and loaded carefully into the a haemocytometer using sterile pasteur pipet. Cells in the four large squares at the corners were counted. An average of the counts was determined and number of cells / ml was calculated using the formula: average count x 1/dilution x 10^4. The suspension was washed in unsupplemented RPMI 1640 by centrifugation for 10 min at 200 x g. The resulting pellet was resuspended in unsupplemented RPMI 1640 at a ratio to give a concentration of 10^7 cells per ml.

Macrophage Perfusion

Macrophages to function as feeder cells in post-fusion cultures were obtained aseptically from BALB/c mice. The mice were killed by cervical dislocation and soaked in 70% alcohol for 1-2 min. The peritoneal cavity was then flushed with approximately 8.0 ml of phosphate buffered saline (PBS). The macrophage suspensions were withdrawn using a sterile 6 ml syringe and pooled in a sterile 50 ml centrifuge tube. An aliquot of the suspension was diluted in diluent containing trypan blue, and the macrophages were counted using the heamocytometer. The macrophage suspension was centrifuged for 10 min at 200 x g, and the pellet was resuspended in supplemented RPMI 1640 medium at a concentration to approximate 5 x 10^5 cells / ml.

Cell Fusion Protocol

The cell fusion protocol was patterned after that of De St. Groth and Scheidegger (1980). The following things were done the day previous to fusion: PBS was prepared and filter sterilized. Hypoxanthine/aminopterine/thymidine (HAT) medium was made by mixing 1 part sterile HAT medium concentrate (K.C. Biological, Lenexa, KS) with 49 parts supplemented RPMI 1640 medium. The fusion medium was a 50% polyethylene glycol (PEG); it was prepared by adding 2 ml of

unsupplemented RPMI 1640 and 0.2 ml of sterile dimethyl sulfoxide (DMSO; Eastman Kodak, Rochester, NY) to 2.0 gm of autoclaved PEG 1450 (Midland Scientific, Omaha, NE) after the latter cooled to about 50°C. Macrophages were suspended in the diluted HAT medium (1:50) to approximeate 10^5 cells/ ml. The wells of ten 24-well tissue culture plates were filled with 1.0 ml HAT medium (1:50) and 0.05 ml macrophage-HAT suspension. The plates were incubated at 37°C in a 6.0% CO_2 atmosphere until the following day.

On the following day, spleen cells were harvested from the immunized mice immediately prior to fusion; the cells were counted, washed and suspended in unsupplemented RPMI 1640 at a concentration of approximately 10^7 cells / ml. Also, the contents of 5-6 tissue culture flasks (25 cm^2) containing actively growing myeloma cells were pooled in sterile 50 ml conical centrifuge tubes and centrifuged for 10 min at 200 x g. The resulting pellets were pooled and resuspended in 6.0 ml of unsupplemented RPMI 1640. An aliquot of this suspension was counted in a haemocytometer as described earlier. Once the count was determined, the cells were centrifuged at 200 x g for 10 min and resuspended in unsupplemented RPMI 1640 at a concentration that would approximate 1 x 10^7 cells /ml.

Approximately 5.0 x 10^7 myeloma cells were mixed with 2.0 x 10^7 spleen cells in a 50 ml centrifuge tube. The mixture was centrifuged at 200 x g and the supernatant

was removed completely. The pellet was loosened by flicking the tip of the centrifuge tube several times. Once the pellet was loosened, 1.0 ml of fusion solution was added dropwise over a 1 min period while gently swirling the tube. The fusion mixture was then incubated at 37°C in a waterbath for 1.5 min. To stop the fusion process, 20 ml unsupplemented RPMI 1640 was added (1.0 ml during the first 30 sec, 3.0 ml over the next 30 sec and the remainder in the next minute). The cell suspension was allowed to stand for 5 min after which it was centrifuged for 10 min at 200 x g. Then the pellet was washed once by centrifugation in supplemented RPMI 1640 medium. The washed pellet was loosened and resuspended in 12 ml of HAT medium; 0.05 ml of this suspension was distributed into each well of the culture plates containing HAT medium and macrophages. The fused cells were incubated at 37°C in 6.0% CO_2. The plates were retrieved from the incubator only to feed the cells.

The cells were fed according to the protocol of de St.Groth and Scheidegger (1980). The cells in each of the wells were fed 1.0 ml of HAT medium (1:50) on day 7 post-fusion. This gave a total volume of approximately 2 ml of medium / well. The initial feeding was followed by removal of approximately 1.0 ml of spent medium and addition of 1.0 ml of HAT medium (1:50) on days 10, 15, 17, 19, 21, 23, 25 and 27 post-fusion. Cells in wells showing hybridoma growth, represented as an 8+ cluster

of cells, were fed with hypoxanthine/thymidine (HT) medium. The HT medium was a 1:50 dilution of sterile HT medium concentrate (K.C. Biological, Lenexa, KS) mixed with supplemented RPMI 1640. After about 4 days in HT medium, the hybridoma cells were fed supplemented RPMI 1640 medium twice each week.

Screening Assay

Supernatants above actively growing hybridomas were screened for the presence of antibody against the particulate antigens of the OKD-11 leishmanial isolate using FIAX and ELISA serological methods.

Screening by FIAX serology was done by spotting StiQ samplers (I.D.T., Santa Clara, CA) with 25 μl of titrated particulate antigen. The StiQs were allowed to dry overnight. Fluorescein isothiocyanate-tagged goat anti-mouse IgG (Cappel Labørataories, Westchester, PA) was rehydrated with 2.0 ml of double-distilled water and then diluted 1:25 in PBS (pH 7.4) and frozen in 1.0 ml aliquots. For tests, an aliquot was diluted to a 1:200 final concentration in PBS (pH 7.4) containing 0.15% v/v Tween 20. Four rows of 12 x 75 mm test tubes were set up in a rack. The first row contained approximately 500 ul of supernatants obtained from growing hybridoma. Each supernatant was tested undiluted and in duplicate. Second and fourth rows of tubes contained 0.6 ml of PBS-Tween 20 and served as washing solutions. The third row of

tubes contained 0.5 ml of 1:200 dilution of the fluorescein-conjugated antiserum. The antigen-spotted StiQs were put in the first row of tubes containing undiluted supernatant and incubated for 30 min, washed for 10 min in the second row, transferred to the third row of conjugate for 20 min, and washed again for 10 min in the final row of tubes. All incubation and washing procedures were done on a shaker at room temperature. After the final wash, the amount of fluorescence on both faces of each StiQ was measured using a FIAX 100 Fluorometer (I.D.T., Santa Clara, CA), and the fluorescent signal units were recorded. Mouse antiserum to *Leishmania* sp. (OKD-11) was diluted 1:40 and served as a positive control. Serum from an uninfected mouse and supernatant from wells without hybridoma growth were used as negative controls.

The ELISA was patterned after Luzzio et al. (1979) and Araujo et al. (1982). Particulate antigen prepared from *Leishmania* sp. (OKD-11) was diluted in sodium carbonate buffer (pH 9.6) made by dissolving 1.59 gm sodium carbonate and 2.93 gm sodium bicarbonate in 1.0 liter distilled water. The antigen was diluted to a concentration of approximately 9.3 μg protein /ml as determined by Bio-Rad protein assay. The wells of ELISA-treated microtiter plates (NUNC, Santa Anna, CA) were coated with antigens by adding 0.1 ml of the diluted antigen and incubating them overnight in a humidified

chamber while rocking at room temperature (20-25°C). The coated wells were then washed three times in phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (Appendix I). Washings were done using a Nunc Immunowash 12 (Nunc, Santa Anna, CA). The last wash solution was allowed to remain for approximately 2 min after which it was removed by flicking the plate, and the top of the plate was blotted dry. All subsequent washings of ELISA plates were done in similar manner. Supernatants (0.1 ml) from growing hybridomas were placed into each of 2 wells. A 1:75 dilution of mouse anti-*Leishmania* sp. (OKD-11) in PBS-Tween 20 was used as a positive control. Supernatant from hybridoma-negative wells and PBS-Tween 20 were used as negative controls. Plates were incubated for 2 hr in a humidified chamber while rocking at 20-25° C and washed as described earlier. This was followed by addition of 0.1 ml of 1:1,000 dilution of anti-mouse IgG conjugated with horseradish peroxidase (Sigma Chemical, St. Louis, MO) to each well. Another 2 hr incubation and another washing were followed by addition of 0.1 ml of freshly prepared substrate (Ortho phenylinediamine, Sigma Chemical, St. Louis, MO) solution (APPENDIX II) to each well. Plates were incubated at room temperature for 1 hr, in the dark under an opaque box. The reaction was stopped by adding 20 μl of 2.5 M sulfuric acid and the absorbance was read at a wavelength of 490 nm using an EIA Reader, Model EL 307 (Biotek Instruments, Inc.,

Burlington, VT).

Hybridomas secreting detectable antibodies were expanded by subculturing in 2 additional wells. Supernatants over these hybridomas were screened against *T. cruzi* particulate antigens. Those that were positive were also cloned and saved.

Hybridoma Cloning

Cloning by limiting dilution was done in sterile 96-well flat-bottomed plates according to Oi and Herzenberg (1980). Hybridomas in antibody-positive wells were dispersed by mixing with sterile pasteur pipets. Cell concentrations were determined by haemocytometer counts as described previously. The hybridomas were diluted in cloning medium containing 10^5 macrophages / ml of supplemented RPMI 1640 medium, and the concentration was adjusted to approximately 240 hybridoma cells / ml of cloning medium. A 1.0 ml aliquot of this dilution was further diluted to 4.6 ml and each of 36 wells was inoculated with 0.1 ml of this mixture; the remaining 1.0 ml of cell suspension was mixed with 4 ml of cloning medium and plated in 0.1 ml volumes into another 36 wells. A third dilution was done by adding 1.4 ml of cloning medium to the remaining suspension and the last 24 wells were inoculated with 0.1 ml portions of this mixture. These plates were incubated at 37° C in 6.0% CO_2 and retrieved from the incubator on days 5

and 12 post-cloning in order to feed the cells. The cells were fed by removal of approximately 2 drops of medium from each well and replacing it with 2 drops of fresh supplemented RPMI 1640 medium. Supernatants in wells with single cell clones were tested for *Leishmania*-specific antibody. The cells in antibody-positive wells were expanded and used for mass production of antibody by the ascites fluid method in mice, or by growth to extinction in tissue culture flasks.

Ascites antibodies were produced in BALB/c mice according to the method of DeIbarra et al. (1982). The mice were inoculated intraperitoneally with 0.5 ml of pristane (Aldrich Chemicals Co., Milwaukee. WI). Five days later, the mice were inoculated with approximately 10 antibody-producing hybridoma cells. Ascites fluid was collected at different intervals following distension of the peritoneal cavity. The ascites fluid was centrifuged at 200 x g for 10 min and the supernatant was collected and frozen.

Indirect Fluorescent Antibody Test.

This test was used to determine the specificity of monoclonal antibodies against both promastigotes of *Leishmania* sp. (OKD-11) and epimastigotes of *T. cruzi*. Mouse antiserum against *Leishmania* sp. (OKD-11) and negative mouse serum were used as controls. The tests were performed as described by DeIbarra et al.

(1982). Actively growing cultures were washed twice in PBS by centrifugation at 4,000 x g for 10 min. The cells were resuspended in PBS to produce approximately 20-40 organisms/ microscopic field at 400 x magnification. Smears of the organisms were made on microscope slides, air-dried, fixed in acetone for 10 min and reacted with monoclonal antibodies, or control mouse serum for 1-2 hr. Slides were washed in PBS for 10 min, reacted with fluorescein-conjugated antimouse IgG for 1-2 hours, washed again and air-dried. The slides were mounted with cover slips using 90.0% glycerol in PBS and examined under a Zeiss Microscope with Epifluorescence. A distinct yellow glow was evidence of fluorescence.

Fluorometric Analysis of Antibody Binding

Each of the particulate antigens and its homologous rabbit antiserum were titrated for optimum concentrations prior to testing. During titrations, the antiserum and conjugate dilutions were kept constant while varying the volume of antigen suspension applied to the StiQ Samplers. Each volume of antigen was run in duplicate. The pregain signals were read in the fluorometer and the amount of antiserum, conjugate and antigen were adjusted based on the highest pregain signal values. StiQ samplers were each spotted with 30.0 μl portions of titrated antigen on the front surface; the face without the antigen acted as a control to measure nonspecific fluorescein

conjugate binding. The spotted StiQs were dried overnight at room temperature. Each vial of fluorescein isothiocyanate-tagged goat anti-rabbit IgG (Cappel Laboratories, Westchester, PA) was rehydrated with 2.0 ml of double-distilled water and then diluted 1:25 in PBS (pH 7.4). For tests, an aliquot was diluted again to a 1:300 final concentration in PBS (pH 7.4) containing 0.15% v/v Tween 20. The procedure was as described previously except that each antigen was run in duplicate against each antiserum. After the final wash, the amount of fluorescence on both faces of each StiQ was measured using the FIAX 100 fluorometer, and the pregain signal was recorded. Using the StiQ which registered the highest pregain signal, the fluorometer was adjusted to read 190 and all StiQs were read again. The fluorescence values from the control surface was subtracted from that on the antigen-coated side and recorded as fluorescent signal units (FSU). Control sera with predetermined FIAX values from previous FSU measurements were used to relate serum antibody levels each time a test was performed.

ELISA Measurement of Antibody Levels

Antibody levels of each polyclonal antiserum against the particulate antigen were measured by ELISA as described previously. Particulate antigens were coated

on NUNC ELISA-treated microtiter plates. Antisera prepared in rabbits and antisera obtained from naturally infected dogs with titers to either *Leishmania* sp., or *T. cruzi* were reacted with particulate antigens prepared from each of the five organisms under study. Antigen-antibody reactions were recorded as ELISA scan values obtained at a wavelength of 490 nm using the EIA Reader. Negative control sera were obtained from an uninfected dog and rabbit; PBS-Tween 20 was also used as a negative control for detecting nonspecific conjugate binding.

Preabsorptiion of Antisera with *L. mexicana* and *T. cruzi* Particulate Antigens

The absorption procedure was patterned after that of Camargo and Rebonato (1969). Polyclonal rabbit antisera and sera from four dogs were used in these tests. Two of the dogs (Arrow and Jeremy) had natural and experimental leishmanial infections, respectively. A third dog (Carpenter) was infected naturally with *T. cruzi*. The fourth dog (Miller) was negative for either of the organisms. A portion of each antiserum was diluted 1:100 in PBS-Tween (pH 7.4) and individual 2.0 ml portions were absorbed with precipitate obtained after centrifugation of particulate antigen suspensions of *L. mexicana* or *T. cruzi*. Equal volumes of the absorbing antigens were used in the absorption of each

of the antisera. Absorption was done on a shaker for approximately 1.0 hr at room temperature after which it was centrifuged at 12,000 x g for 10 min. The absorbed sera were removed as supernatant and then tested by ELISA for specific antibody responses to each of the 5 particulate antigens under study.

Ouchterlony Gel Diffusion

Double gel-diffusion was performed by the Ouchterlony technic in 60 x 15 mm plastic petri dishes using a modification of the methods of Halbert et al. (1955). Agarose (0.9%, w/v) was prepared by dissolving 0.9 gm agarose (Sigma Chemical, St. Louis, MO) in 100.0 ml barbital buffer, pH 8.6 (Sigma Chemical). Merthiolate (0.01%, w/v) was added as a preservative. The 0.9% agarose was distributed in 5.0 ml aliquots into the plastic petri dishes. Seven antigen wells 3.0 mm in diameter surrounded an antibody well 4.0 mm in diameter. The antigen wells were 7.0 mm away from the antibody well. The rabbit antisera, described previously, were tested against each of the detergent-solubilized antigens. Ouchterlony plates were kept for approximately one week in an air-tight, humidor at room temperature. Antibody wells were refilled once or twice as needed. Gels were examined for precipitin bands every day beginning the next day after incubation.

Immunoelectrophoresis

Immunoelectrophoresis was done as specified by Garvey et al. (1979). Barbital buffer was made by dissolving one vial of barbital powder (Sigma Chemical) in 1,000 ml of distilled water. The pH was adjusted to 8.6. Agarose gel solutions (1.0% and 2.0%, w/v) were prepared by dissolving 1.0 and 2.0 gm, respectively, of agarose (Sigma Chemical) in 100 ml of barbital buffer containing 1.0 ml of Triton X-100. Microscope slides (75 x 25 mm) were cleaned with 90% ethanol and coated with warm 2.0% agarose. After the agarose hardened, the slides were set on a horizontal surface. The agarose gel solution (1.0%) was distributed gently over the surface of each slide (3.0 ml/ slide) using a serological pipet. The agarose was allowed to harden and the slides were stored overnight in humidified chambers kept at 4°C.

Two antigen wells were cut approximately 10 mm apart and the agar plugs were removed. The wells were filled with 2-3 μl of Triton X-100-solubilized antigen. After the antigen had diffused from the wells, the wells were filled with melted 1.0% agarose. Electrophoresis was done at 10 V/cm for 1.0 hr using a LKB 2117 Multiphor and LKB 2197 Power Supply (LKB, Houston, TX). After electrophoresis a trough was cut equidistance between the two antigen well locations; the agar was removed and the trough was filled with antiserum. The slides were

placed in a humidified chamber at 4°C for up to 48 hr. After incubation, the slides were washed thrice in 0.3 M NaCl solution for 24 hr and dried at room temperature. The dried agar film was stained for 10 min with acid fuchsin (Sigma Chemical). Destaining was done for 15-20 min with a solution made up of methyl alcohol, distilled water and glacial acetaic acid in 5:4:1 ratio, respectively. The slides were air-dried in a vertical position and observed for precipitin bands.

Data Analysis

The FIAX and ELISA measurements of serological reactions involving polyclonal rabbit antisera and antigens were compared by determining the correlation coefficients for the two measurements. The effects of antisera absorption with particulate antigens of _L. mexicana_ and _T. cruzi_ on antigen-antibody reactions as determined by ELISA measurements were evaluated by the Duncan's Multiple Range Test and Tukey's Analysis of Variance.

CHAPTER III

RESULTS

Monoclonal Antibodies

The fusion efficiency obtained in the spleen cell and myeloma fusions is provided in Table I. Approximately 55% of 240 wells contained hybridomas; wells were considered positive for hybridoma when clusters of 8 or more cells were present.

Supernatants over hybridomas were initially screened for anti-leishmanial antibody by FIAX serology, but the system proved not to be useful for screening purposes; thus subsequent screening was done by the ELISA method. A comparison of the FIAX and ELISA screening tests is given in Table II which contains data for only selected supernatants. The FIAX method did not detect antibody in supernatants from any of the hybridomas. On the other hand, the ELISA method was sensitive enough to detect antibodies against OKD-11 antigen in the supernatant media. Initially, 7 hybridomas were found to be secreting antibodies to OKD-11 antigen: approximately 5.3% of the total hybridoma-positive wells. After cloning the antibody-positive hybridomas, only four continued to secrete antibodies; the clones were designated 3A2, 5B2,

TABLE I

THE CLONING EFFICIENCY OF THE FUSION PROTOCOL[a] USED FOR PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST AN ISOLATE OF LEISHMANIA SP. FROM OKLAHOMA (OKD-11)

Tissue Culture Plates (24 Wells / Plate)	Numbers of Wells / Plate With Hybridomas[b]
I	15
II	11
III	13
IV	15
V	8
VI	10
VII	12
VIII	19
IX	17
X	11

[a] Protocol as described by De St. Groth and Scheidegger, 1980, J. Immunol Methods, 35: 1-21.

[b] Myeloma-Spleen cells hybridomas, not necessarily producing antibodies.

TABLE II

COMPARISON OF FIAX AND ELISA SEROLOGICAL METHODS FOR SCREENING HYBRIDOMA CULTURES FOR ANTIBODIES TO PARTICULATE ANTIGENS OF THE OKD-11 LEISHMANIAL ISOLATE FROM DOGS FROM OKLAHOMA

Plate Number	Well Number	FIAX Pregain Values[a]	ELISA Values[b]
I	D4	-1	0.37
"	"	-1	0.42
II	A2	0	0.37
"	"	0	0.33
V	B2	0	0.45
"	"	0	0.41
VI	B2	-1	0.39
"	"	0	0.39
Positive control (Mouse antiserum)		3	0.67
"		4	0.65
Negative control (supernatant)		0	0.12
"		0	0.10

[a] Fluorescent signal measured by a FIAX 100™ Fluorometer.

[b] Optical density measured by an EIA reader at a wavelength of 490 nm.

8C3 and 12E8.

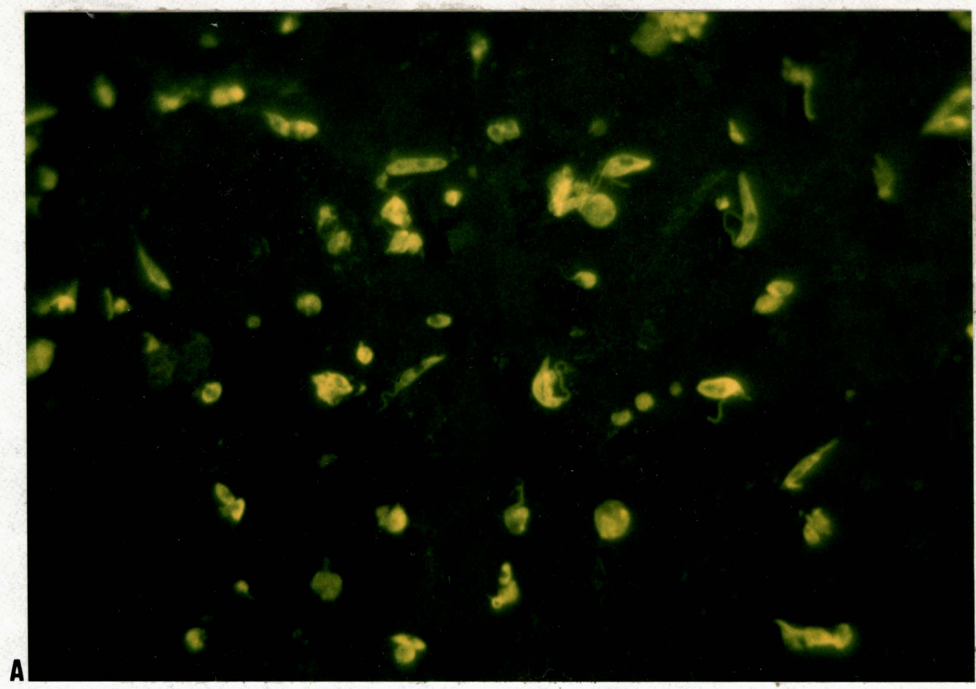
Indirect Fluorescent Antibody Tests

The binding patterns of monoclonal antibody 3A2 to promastigotes of *Leishmania* sp. (OKD-11) and epimastigotes of *T. cruzi* are shown in Figure 1. The fluorescence patterns indicated that this antibody bound specifically to surface membranes, kinetoplasts, and flagellar pockets of both species. Binding specificities for antibody from clone 5B2 are shown in Figure 2. Here the predominant binding was to intracellular kinetoplasts. There was no antibody binding observed by IFA for clones 8C3 and 12E8 (Figs. 3 and 4, respectively). The IFA results for clones 8C3 and 12E8 appeared similar to those obtained from a negative control mouse (Fig. 6).

Monoclonal Antibodies and Particulate Antigens

ELISA results indicating the binding efficiencies for the 4 monoclonal antibodies obtained after hybridoma cloning are contained in Table III. None of these antibodies showed absolute species specificity for any of the particulate antigens. Three of the four monoclonal antibodies cross-reacted with all antigens. Although antibodies from clones 8C3 and 12E8 could not be detected by IFA tests (Figs 3 and 4), clone 12E8 antibody was detected by ELISA. Supernatants from clone 8C3 were

Figure 1. Immunofluorescence Showing Monoclonal Antibody (Clone 3A2) Binding to Pellicular Membranes and Kinetoplasts of (A) Promastigotes of *Leishmania* sp. (OKD-11) and (B) Epimastigotes of *Trypanosoma cruzi*; X400.


A

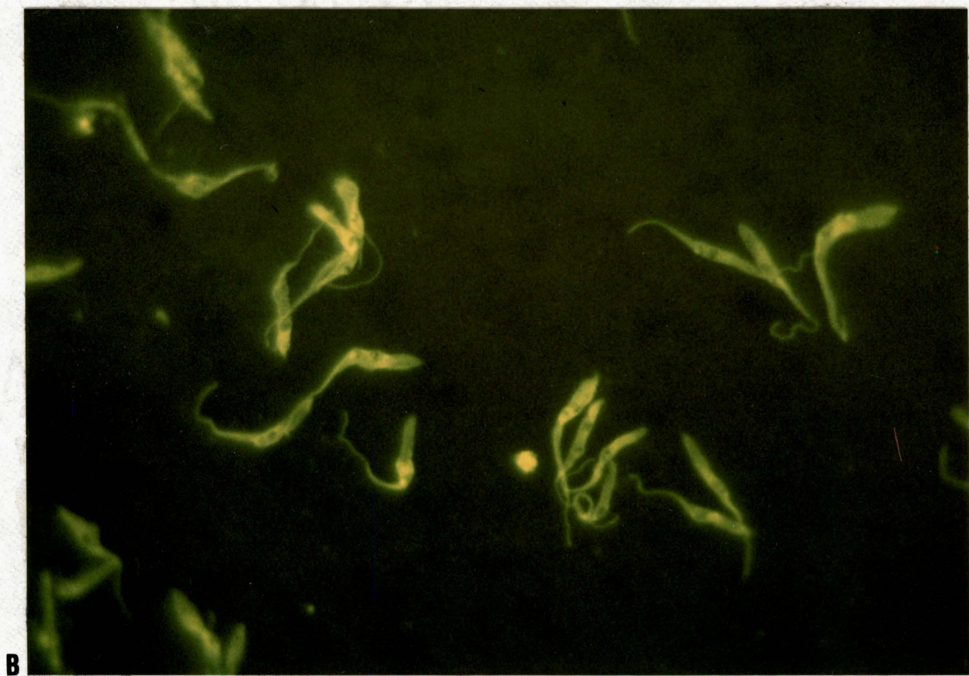
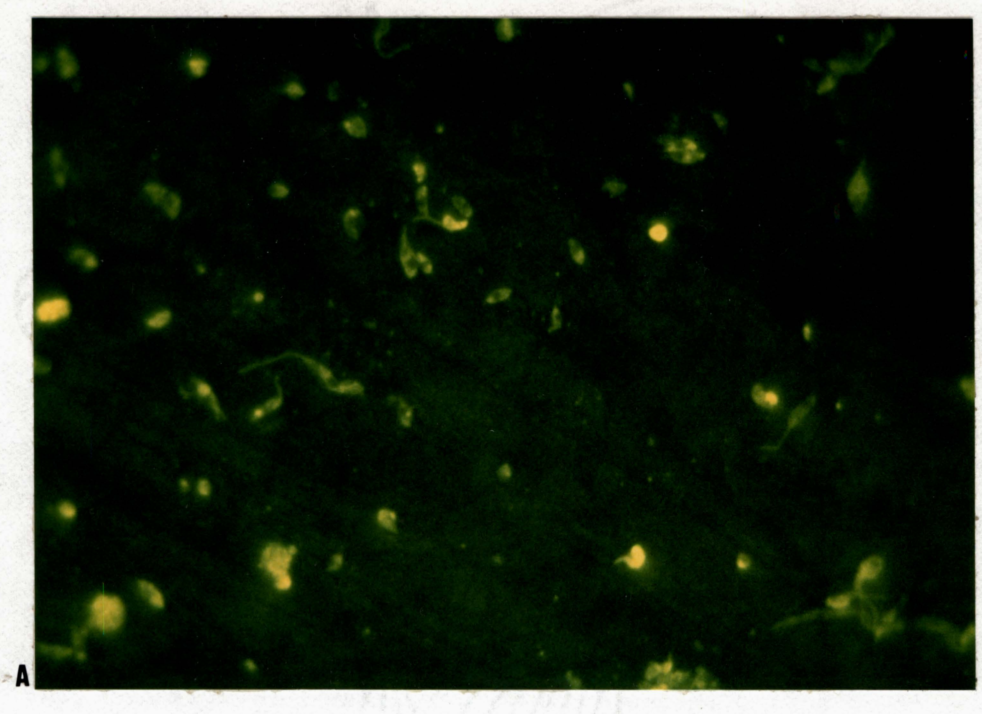

B

Figure 2. Immunofluorescence Showing Monoclonal Antibody (Clone 5B2) Binding to Kinetoplasts of (A) Promastigotes of *Leishmania* sp. (OKD-11) and (B) Epimastigotes of *Trypanosoma cruzi*; X400.


A

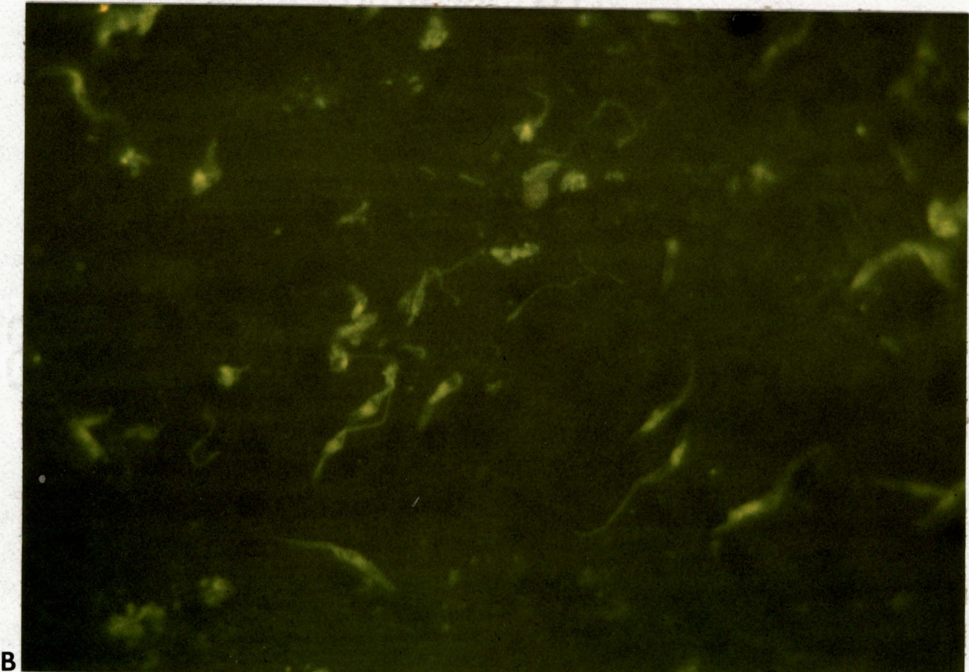

B

Figure 3. Immunofluorescence Showing Lack of Binding of Monoclonal Antibody (Clone 8C3) to (A) Promastigotes of Leishmania sp. (OKD-11) and (B) Epimastigotes of Trypanosoma cruzi; X400.

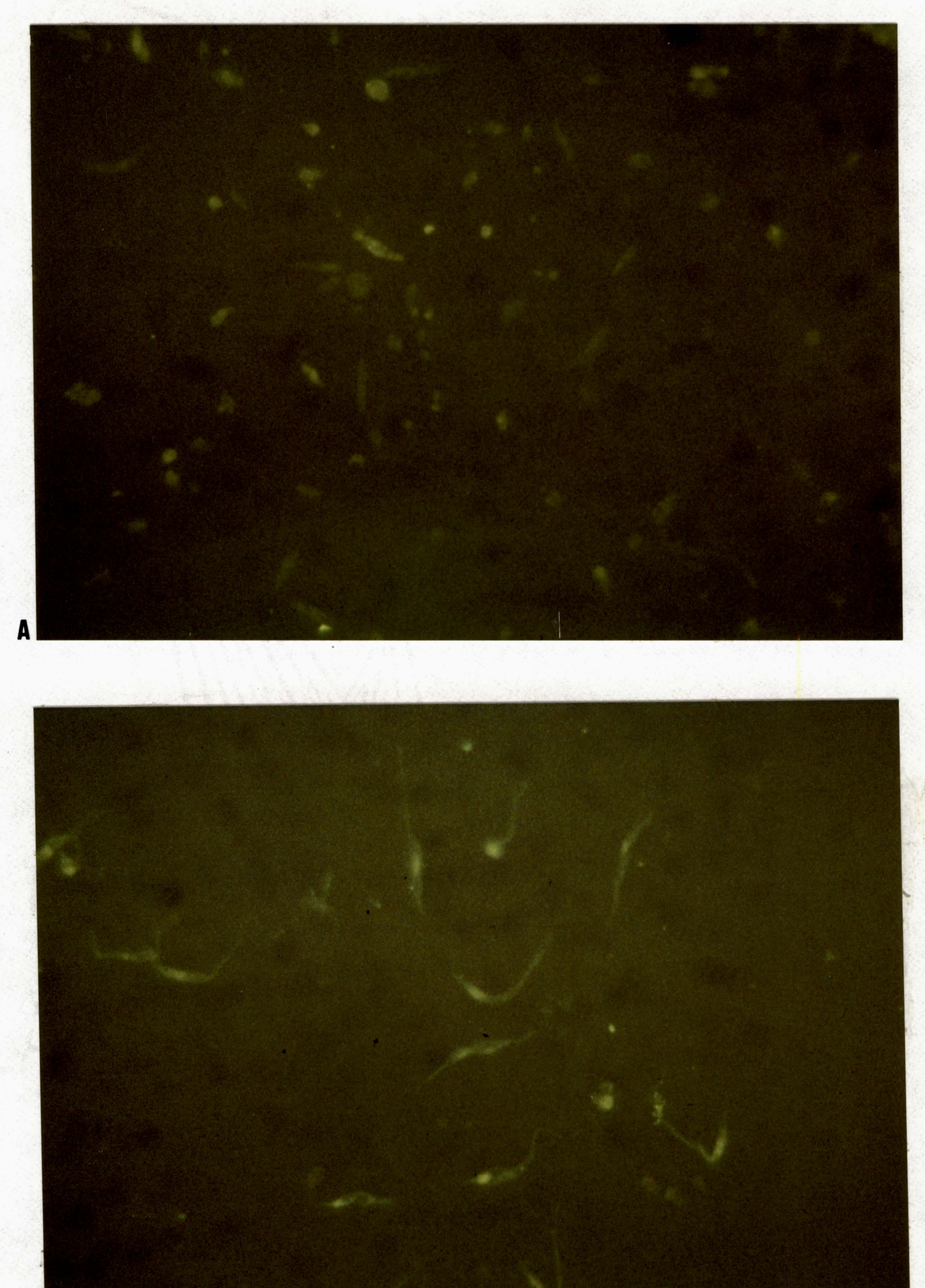
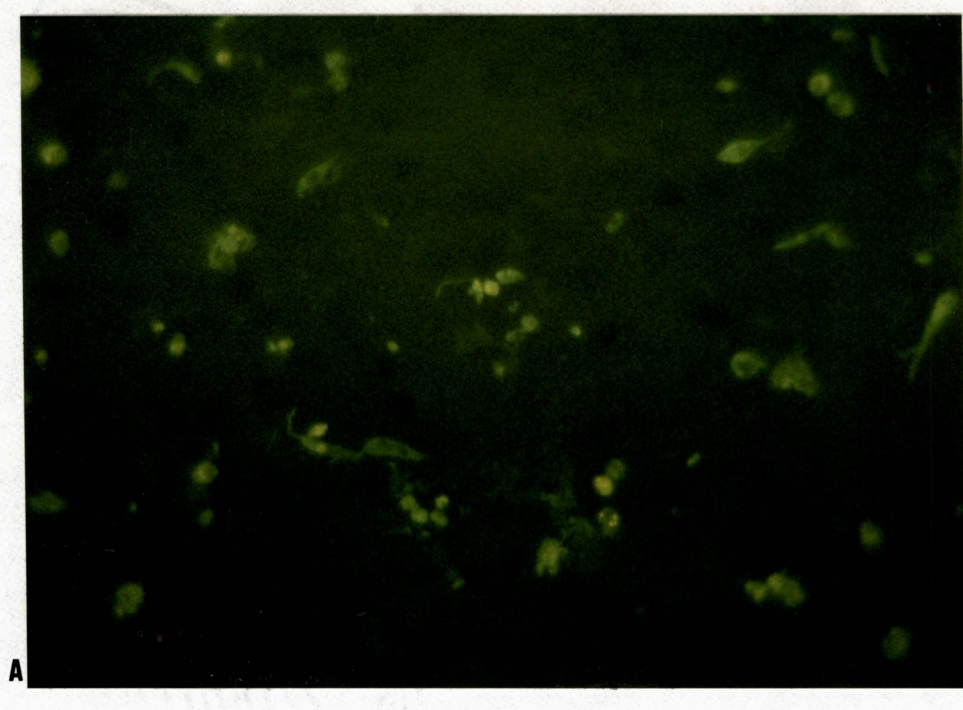

A
B

Figure 4. Immunofluorescence Showing the Lack of Binding of Monoclonal Antibody (Clone 12E8) to (A) Promastigotes of *Leishmania* sp. (OKD-11) and Epimastigotes of *Trypanosoma cruzi*; X400.


A

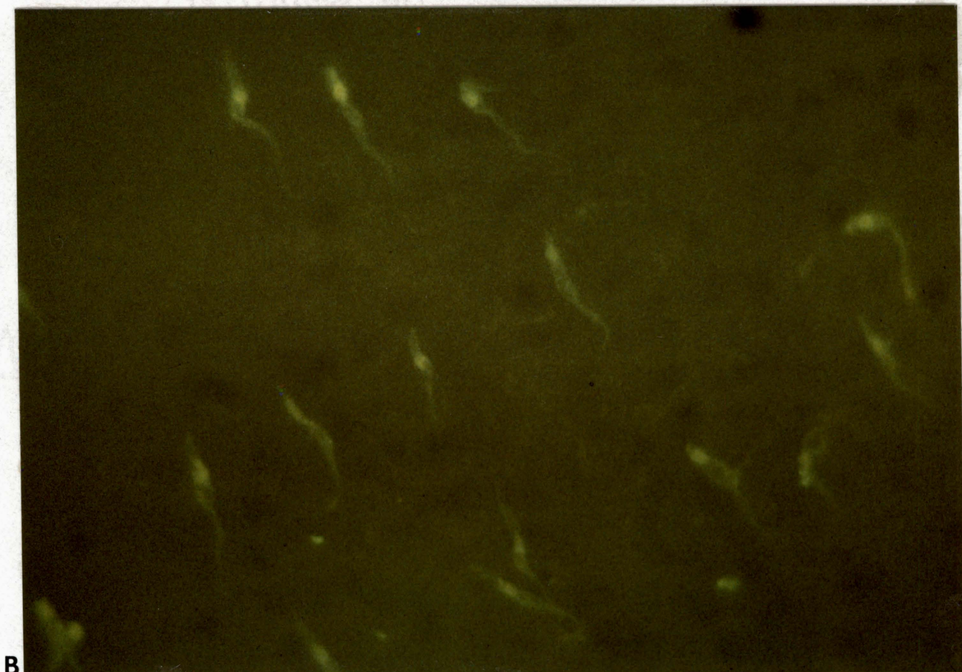
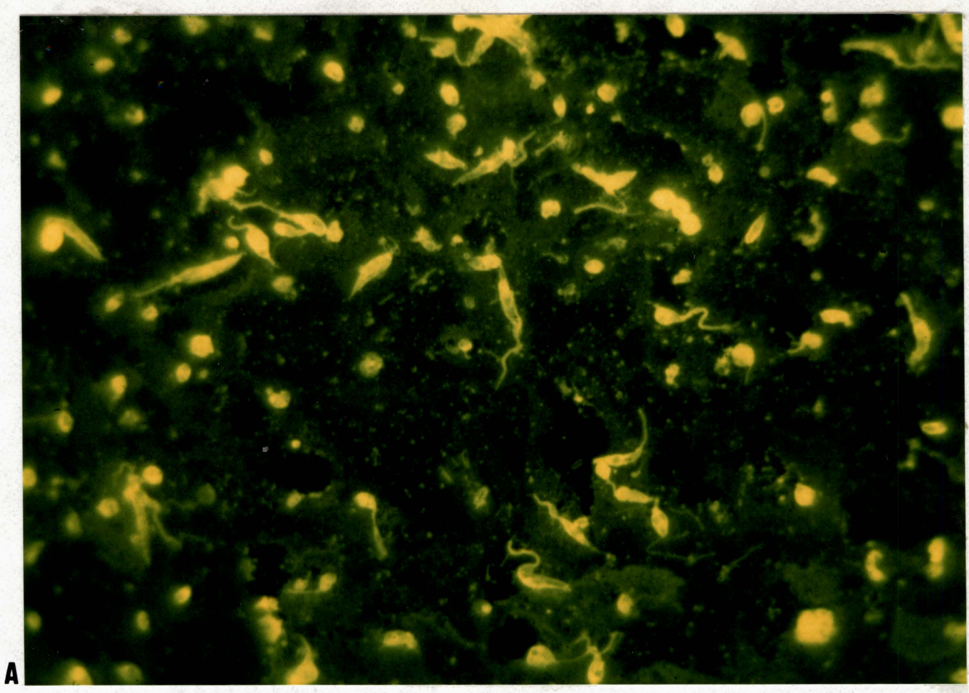
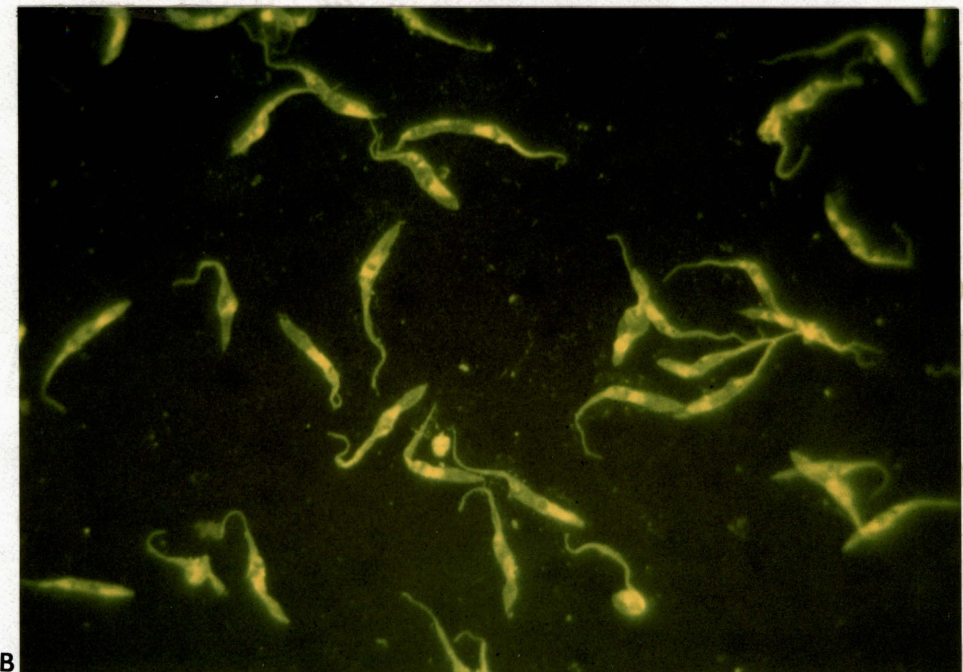

B

Figure 5. Immunofluorescence Showing Binding of Polyclonal Mouse anti-*Leishmania* sp. (OKD-11) to Pellicular Membranes, Kinetoplasts and Flagella of (A) Promastigotes of *Leishmania* sp. (OKD-11) and (B) Epimastigotes of *Trypanosoma cruzi*; X400.

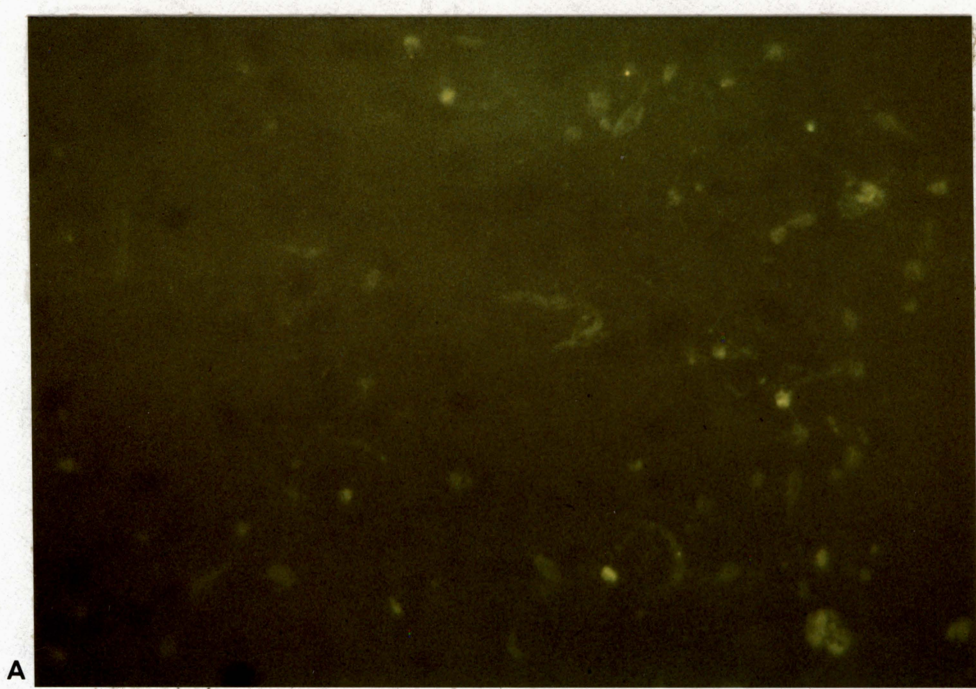


A



B

Figure 6. Immunofluorescence Showing the Lack of Binding of Mouse Serum to (A) Promastigotes of *Leishmania* sp. (OKD-11) and (B) Epimastigotes of *Trypanosoma cruzi*; X400.


A

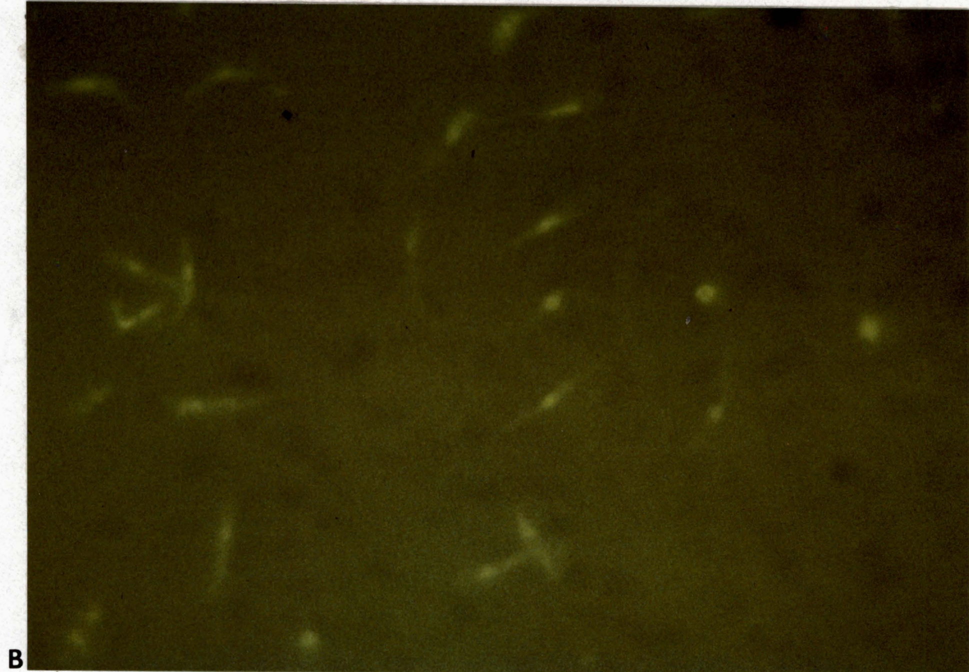

B

TABLE III

ELISA VALUES INDICATING BINDING CAPACITY OF FOUR MONOCLONAL ANTIBODIES PRODUCED AGAINST PARTICULATE ANTIGENS OF *LEISHMANIA* SP. (OKD-11) TO PARTICULATE ANTIGENS OF *LEISHMANIA* SPECIES AND *TRYPANOSOMA CRUZI*

Antigens	Control[a]	Monoclonal Antibodies			
		8C3[b]	3A2[b]	5B2[c]	12E8[b]
Leishmania sp. (OKD-11)	0.10	0.08	0.15	0.23	0.14
L. donovani	0.08	0.07	0.09	0.26	0.11
L. tropica	0.09	0.10	0.16	0.27	0.15
L. mexicana	0.08	0.07	0.17	0.32	0.13
T. cruzi	0.11	0.09	0.24	0.25	0.14

[a] Supernatant in well without hybridoma.

[b] Antibodies produced *in vitro*.

[c] Antibodies produced by ascites method.

negative by all the described methods.

Antibody from clone 5B2 had its highest affinity for L. mexicana antigen. Although ascites-produced monoclonal antibody from clone 5B2 showed a higher antibody concentration, antibodies from clones 3A2 and 5B2 produced similar ELISA values when reacted with T. cruzi antigen (Table III). Also, the reactions of 3A2 antibody were similar to those of 12E8 except for the reactions with T. cruzi antigen to which 3A2 had higher ELISA values than clone 12E8.

Polyclonal Antibodies

Comparison of Reactions to Particulate Antigens

A comparison of FIAX and ELISA reactons for rabbit antisera produced against OKD-11, L. donovani, L. tropica, L. mexicana and T. cruzi particulate antigens is given in Table IV. The range in the correlation coefficients between FIAX fluorescent signals and ELISA values was 0.8245 to 0.9579. The anti-L. mexicana values for the two tests had the highest correlation and anti-L. donovani the lowest.

Table IV also shows that interspecific cross-reactions occurred. Particulate L. mexicana had the highest affinity for all the antisera. Antiserum to Leishmania sp. (OKD-11) exhibited strong affinity to L. mexicana antigen, whereas reactions of anti-L.

TABLE IV

QUANTITATIVE COMPARISON OF ELISA (EL) AND FIAX (FX) VALUE FOR FIVE RABBIT POLYCLONAL ANTISERA AGAINST EACH OF THE PARTICULATE ANTIGEN PREPARATIONS

Antigens[b]	Antisera[a]											
	Negative control		*Leishmania* sp. (OKD-11)		*L. donovani*		*L. tropica*		*L. mexicana*		*T. cruzi*	
	EL	FX	EL	FX	EL	FX	EL	FX	EL	FX	EL	FX
Leishmania sp. (OKD-11)	0.14	7	0.71	49	0.28	24	0.18	40	0.22	20	0.24	10
L. donovani	0.10	10	0.33	19	0.20	30	0.20	30	0.20	21	0.13	25
L. tropica	0.09	5	0.30	16	0.15	18	0.15	20	0.17	19	0.17	9
L. mexicana	0.19	35	0.84	144	0.35	54	0.27	90	0.64	109	0.48	122
T. cruzi	0.10	11	0.28	15	0.24	28	0.19	13	0.24	53	0.35	49
Correlation Coefficient	0.8613		0.8869		0.8245		0.8785		0.9579		0.8963	

[a] Antisera made by subcutaneous inoculation of antigens into New Zealand white rabbits.

[b] Antigen preparations standardized by protein assay.

mexicana serum to OKD-11 antigen were low.

Reactions to Detergent-Soluble and Detergent-Insoluble Antigens

The quantitative antibody binding characteristics of each antigen when solubilized in Triton X-100 were compared with those of untreated particulate antigens (Table V). Generally, the detergent-solubilized antigens appeared to bind more antibody than did untreated antigens except for those of *L. donovani*. The corresponding detergent-insoluble fractions remaining after centrifugation did not exhibit significant amounts of antibody binding; however, the residual *L. donovani* antigen showed a marginal amount of antibody binding.

Comparison of antibody binding of untreated and detergent-treated particulate antigens prepared without continuous mixing is shown in Table VI. All of the detergent-insoluble antigens reacted strongly with each of the five polyclonal antisera; these measurements were greater than those obtained with the corresponding, untreated particulate antigens. The soluble portions of the detergent-treated antigens did not appear to bind antibody.

Absorption of Antisera With Particulate Antigens

Responses for rabbit antisera preabsorbed with either *L. mexicana* or *T. cruzi* antigens were measured

TABLE V

MEAN FIAX VALUES[a] OF SEROLOGICAL REACTIONS INVOLVING POLYCLONAL RABBIT ANATISERA AGAINST PARTICULATE ANTIGEN (PA) AND TRITON X-100-SOLUBILIZED ANTIGEN (SA) WITH AGITATION

Antisera	Antigens[b]									
	Leishmania sp. (OKD-11)		*L. donovani*		*L. tropica*		*L. mexicana*		*T. cruzi*	
	PA	SA	PA	SA	PA	SA	PA	SA	PA	SA
Negative control	21	24	18	22	9	14	12	19	13	31
Leishmania sp. (OKD-11)	137	121	12	40	16	53	9	35	9	30
L. donovani	40	109	53	44	40	59	27	41	21	63
L. tropica	97	122	103	74	36	80	24	58	30	69
L. mexicana	65	110	74	46	32	56	36	40	47	69
T. cruzi	62	69	95	46	6	50	45	57	47	144

[a] FIAX values measured as fluorescent signal units for 3 replicate samples.

[b] Particulate antigens standardized by optical density measurement at 330 nm wavelength.

TABLE VI

MEAN FIAX VALUES[a] OF SEROLOGICAL REACTIONS INVOLVING POLYCLONAL RABBIT ANTISERA AGAINST PARTICULATE ANTIGENS (PA) AND DETERGENT-TREATED PARTICULATE ANTIGENS (DT) WITHOUT AGITATION

Antisera	Antigens[b]									
	Leishmania sp. (OKD-11)		*L. donovani*		*L. tropica*		*L. mexicana*		*T. cruzi*	
	PA	DT	PA	DT	PA	DT	PA	DT	PA	DT
Negative control	6	16	9	33	11	38	9	45	11	36
Leishmania sp. (OKD-11)	49	61	19	62	16	74	144	73	15	65
L. donovani	24	74	30	71	18	72	54	68	28	66
L. tropica	40	39	30	47	20	45	90	47	13	56
L. mexicana	20	79	21	89	19	84	109	108	53	97
T. cruzi	10	65	25	66	9	68	122	84	49	74

[a] FIAX values measured as fluorescent signal units for 3 replicate samples.

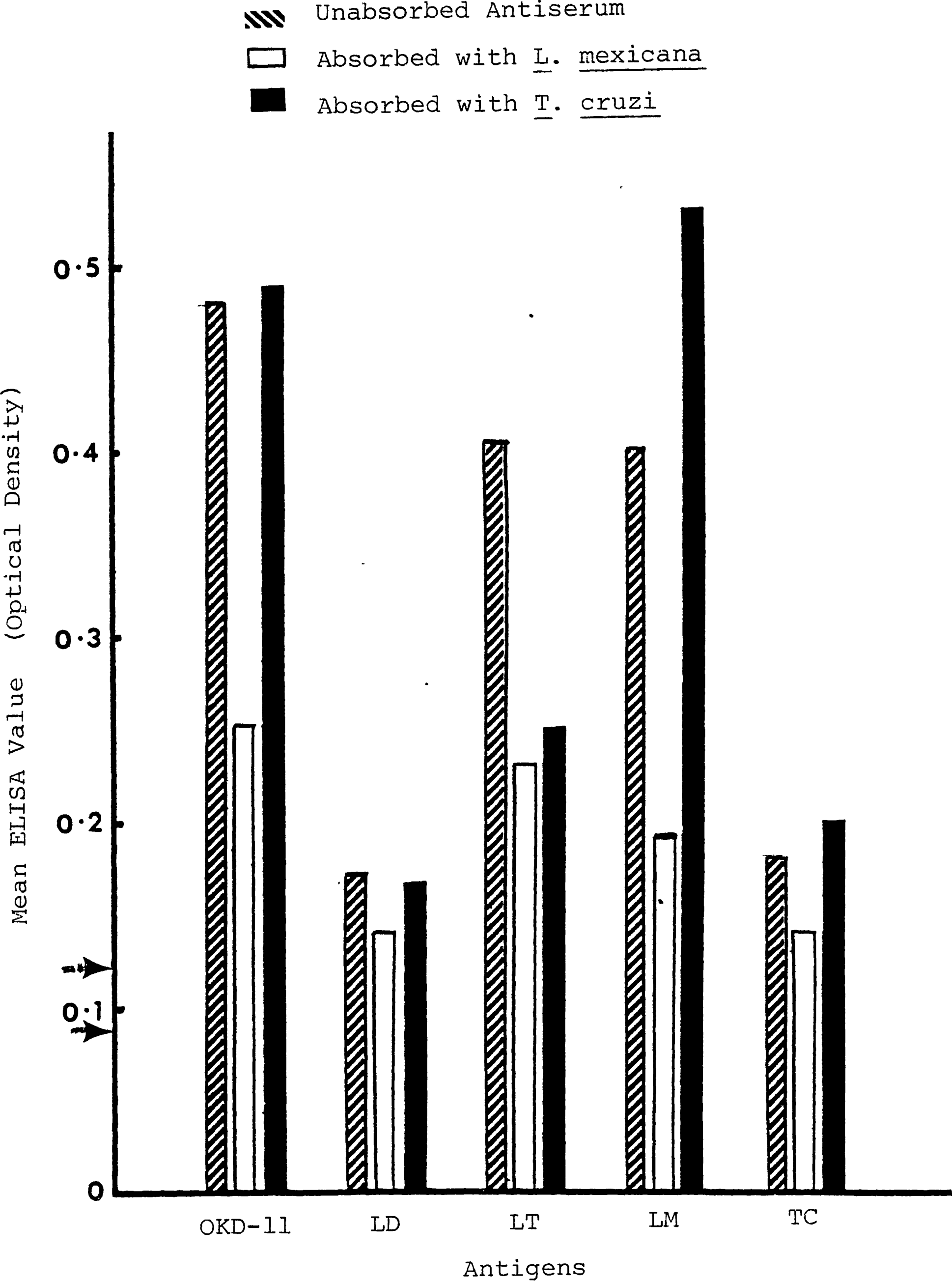
[b] Antigen preparations standardized by protein assay.

by ELISA and compared to responses of unabsorbed antisera (Figs. 7-11). ELISA values for anti-*Leishmania* sp. (OKD-11) serum are shown in Figure 7. The unabsorbed anti-OKD-11 reacted strongly with antigens of OKD-11, *L. tropica* and *L. mexicana*. After absorption with *L. mexicana*, the amount of antibody binding was significantly reduced for all three antigens. Binding to OKD-11 was the most affected. Absorption with *T. cruzi* produced significantly decreased values for *L. tropica*. In general, anti-OKD-11 responses to *L. donovani* and *T. cruzi* were weak in the unabsorbed serum and were not significantly affected by the absorption. Overall, the antibody binding characteristics of OKD-11 were most similar to *L. mexicana*.

ELISA values for absorbed anti-*L. donovani* serum are shown in Figure 8. Unabsorbed anti-*L. donovani* exhibited cross-reactions with each of the antigens. After absorption with either *L. mexicana* or *T. cruzi*, the ELISA values for all antigens were significantly reduced.

Figure 9 shows ELISA results for absorptions of anti-*L. tropica* serum. Whereas absorption with *L. mexicana* resulted in slightly decreased responses to OKD-11, *L. tropica* and *L. mexicana*, absorption with *T. cruzi* resulted in an appreciable reduction in the amount of antibody available for binding to OKD-11 and *L. donovani* antigens. In general, the reactions

Figure 7. Mean optical density for ELISA reactions of rabbit anti-*Leishmania* sp. (OKD-11) serum with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrows (➡, ➡) indicate baseline responses for absorbed anti-*L. mexicana* and anti-*T. cruzi*, respectively, against homologous absorbing antigens.



Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
Mean ELISA Value (Optical Density)
0·5
0·4
0·3
0·2
0·1
0
OKD-11
LD
LT
LM
TC
Antigens

Figure 8. Mean optical density for ELISA reactions of rabbit anti-*L. donovani* serum with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrows (➔, ➔) indicate baseline responses for absorbed anti-*L. mexicana* and anti-*T. cruzi*, respectively, against homologous absorbing antigens.

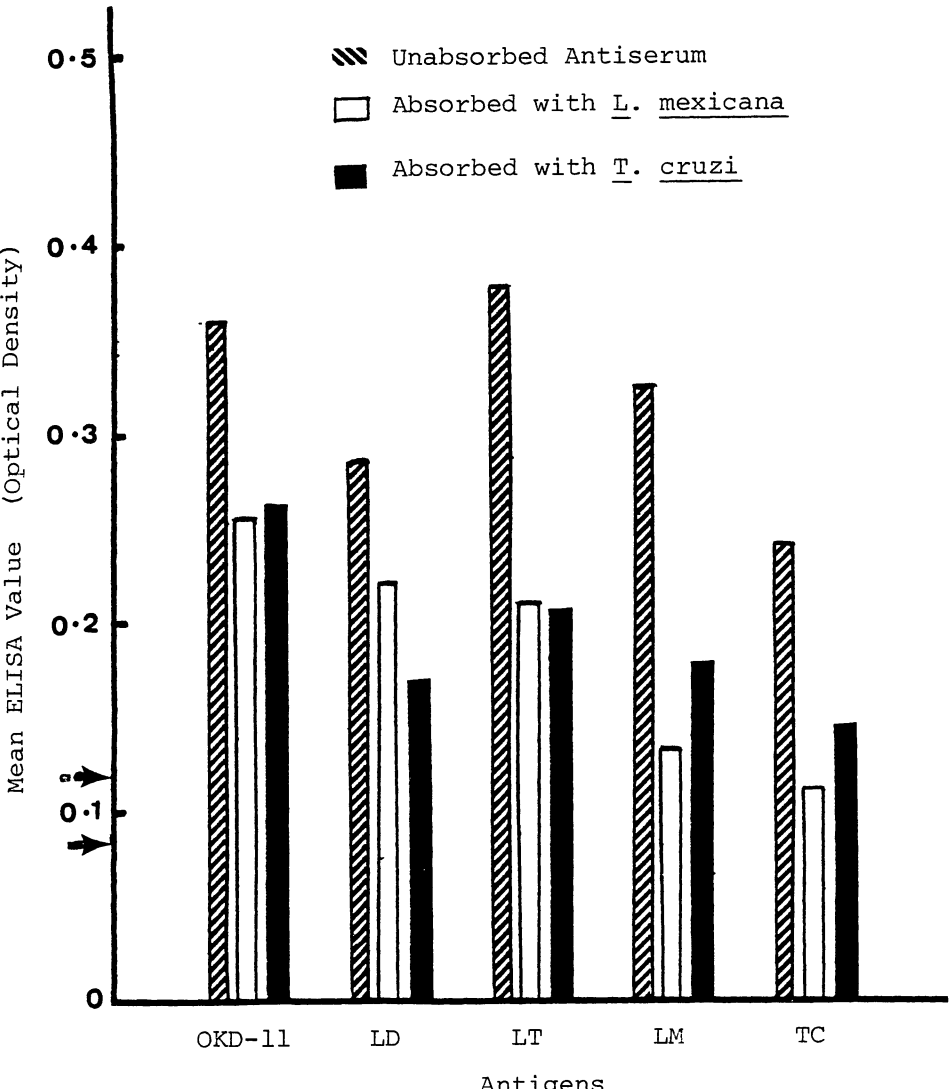
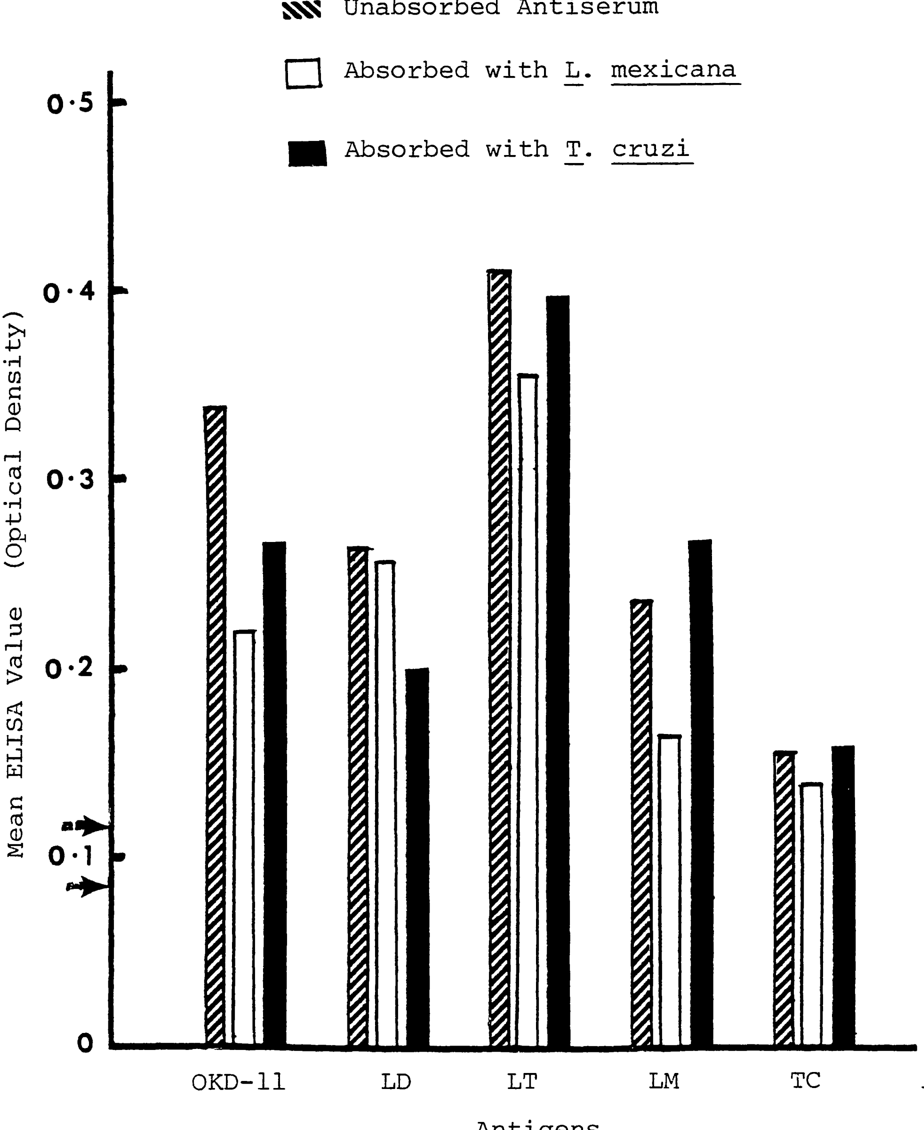

Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
0·5
0·4
0·3
0·2
0·1
0
Mean ELISA Value (Optical Density)
OKD-11
LD
LT
LM
TC
Antigens

Figure 9. Mean optical density for ELISA reactions of rabbit anti-*L. tropica* serum with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrows (➔, ➔) indicate baseline responses for absorbed anti-*L. mexicana* and anti-*T. cruzi*, respectively, against homologous absorbing antigens.



Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
0·5
0·4
0·3
0·2
0·1
0
Mean ELISA Value (Optical Density)
OKD-11
LD
LT
LM
TC
Antigens

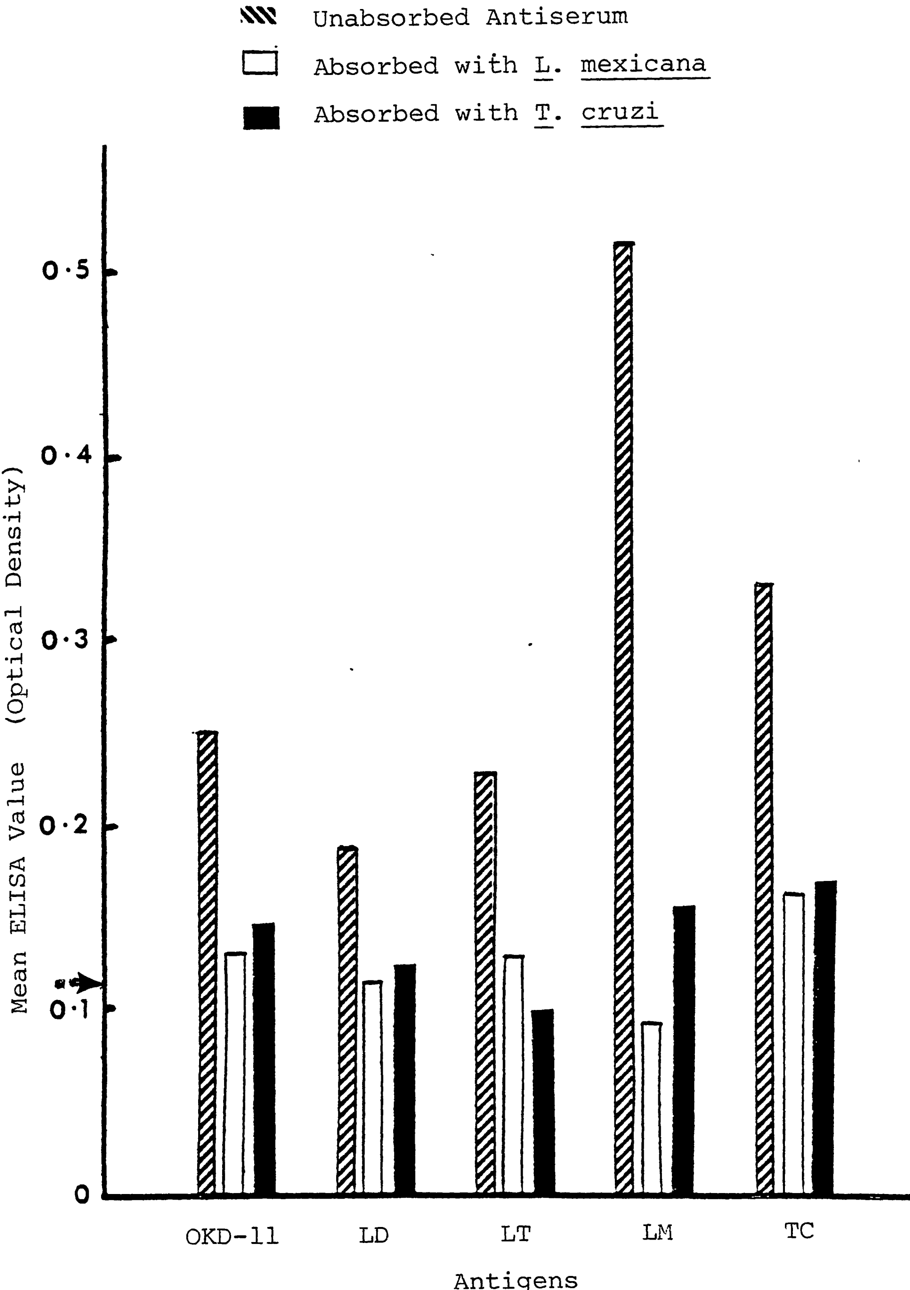
for OKD-11, *L. tropica* and *L. mexicana* showed similar binding patterns.

ELISA reactions for absorbed anti-*L. mexicana* serum are shown in Figure 10. The unabsorbed antiserum cross-reacted with all the antigens. Absorption with either *L. mexicana* or *T. cruzi* significantly reduced the ELISA values for each of the five antigens. Reactions against the *L. mexicana* antigen were most affected by absorption indicating similarity between *L. mexicana* and *T. cruzi*.

Figure 11 shows the ELISA measurement of reactions of anti-*T. cruzi* serum against the various particulate antigens. Absorptions with either of the absorbing antigens resulted in a reduction of ELISA values for antibody reactions against each of the antigens except *L. donovani*. Antibody binding to *L. mexicana* and *T. cruzi* antigens was reduced dramatically.

Tables VII-X show statistical analyses of absorption data using Duncan's Multiple Range test and Tukey's Analysis of Variance. The antigen groupings indicate binding specificity for all of the polyclonal rabbit antisera following absorption with *L. mexicana* antigen, and the mean ELISA values for each of the antigens are shown in Table VII. Although Tukey's method indicated that there were no statistically significant differences between means, the Duncan's Multiple Range test indicated two statistical groupings for the means.

Figure 10. Mean optical density for ELISA reactions of rabbit anti-*L. mexicana* serum with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrow indicates baseline responses for absorbed anti-*T. cruzi* against homologous absorbing antigens.



Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
Mean ELISA Value (Optical Density)
0·5
0·4
0·3
0·2
0·1
0
OKD-11
LD
LT
LM
TC
Antigens

Figure 11. Mean optical density for ELISA reactions of rabbit anti-*T. cruzi* serum with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrow indicates baseline responses for absorbed anti-*L. mexicana* against homologous absorbing antigens.

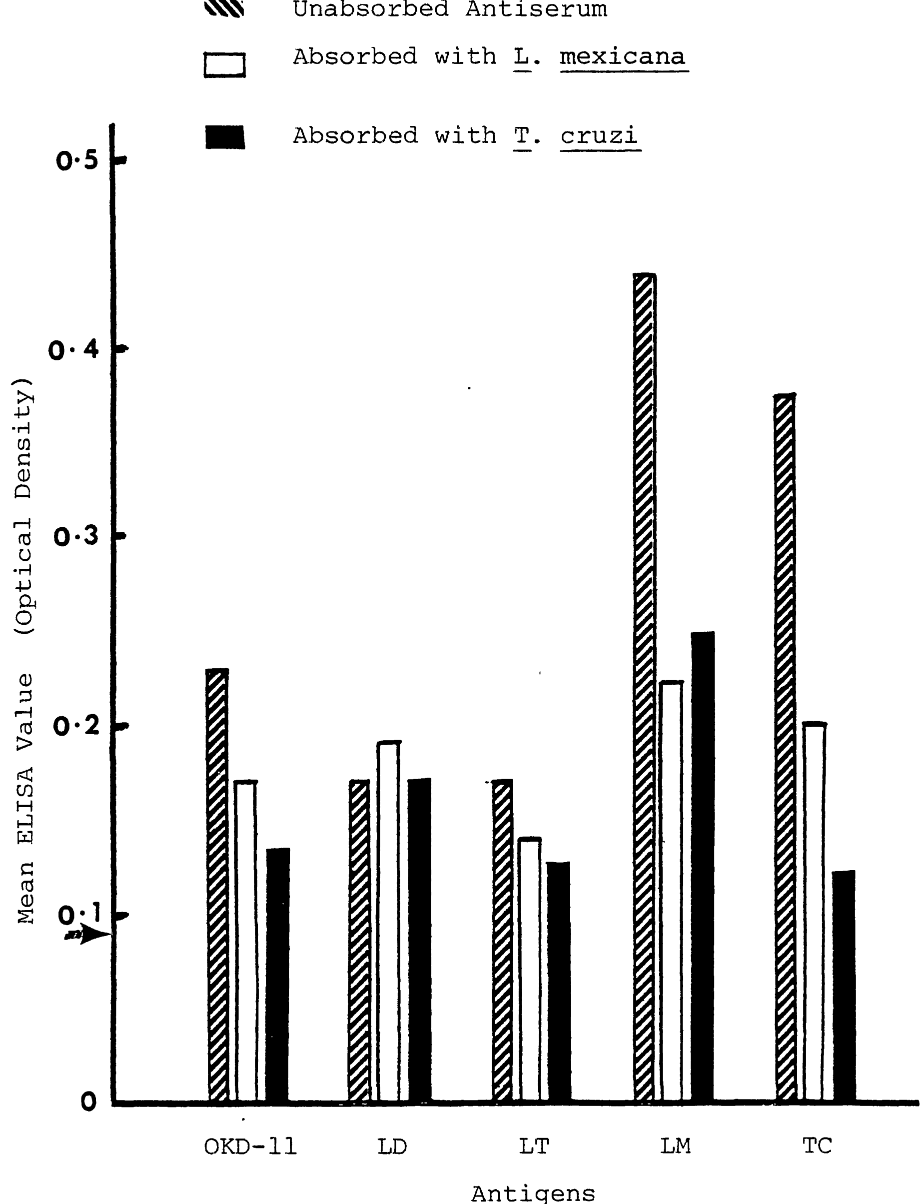

Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
0·5
0·4
0·3
0·2
0·1
0
Mean ELISA Value (Optical Density)
OKD-11
LD
LT
LM
TC
Antigens

TABLE VII

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTIGEN-ANTIBODY RESPONSES WHEN POLYCLONAL ANTISERA FROM RABBITS WERE ABSORBED WITH *L. MEXICANA* PARTICULATE ANTIGEN AND REACTED TO EACH OF THE ANTIGENS

Antigens	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey[c]
L. mexicana	0.271	A	A
		A	A
Leishmania sp.	0.269	A	A
(OKD-11)		A	A
L. tropica	0.263	A	A
			A
T. cruzi	0.204	B	A
		B	A
L. donovani	0.200	B	A

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

Leishmania mexicana, OKD-11 and L. tropica were not significantly different and formed one group; T. cruzi and L. donovani comprised the second group. Antigen groupings for tests where the antisera were absorbed with T. cruzi are shown in Table VIII. The antigens were arranged into four groups as a result of data analysis by Duncan's Multiple Range Analysis. These groups included L. mexicana and OKD-11, OKD-11 and L. tropica, L. tropica and T. cruzi, T. cruzi and L. donovani. With Tukey's analysis, there were two groups of antigens. The first group was L. mexicana, OKD-11 and L. tropica; the second group was L. tropica, T. cruzi and L. donovani. Difference in the groupings of antigens (Tables VII and VIII) show that there was more cross-reaction with L. mexicana than with T. cruzi. Absorption of antisera with L. mexicana resulted in little difference in the amount of antibody binding to antigens, whereas absorption with T. cruzi yielded a greater difference.

Table IX shows mean response groupings for each of the antisera following absorption with L. mexicana antigen and reactions to all of the antigens. Both the Duncan's and Tukey's analyses showed that mean responses of the antisera to all of the antigens did not differ significantly. Antisera groupings resulting from preabsorption with T. cruzi are shown in Table X. Here, analysis by each of the methods resulted in two

TABLE VIII

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTIGEN-ANTIBODY RESPONSES WHEN POLYCLONAL ANTISERA FROM RABBITS WERE ABSORBED WITH *T. CRUZI* AND REACTED TO EACH OF THE ANTIGENS

Antigens	Mean ELISA Value	Statistical Method [a]	
		Duncan [b]	Tukey [c]
L. mexicana	0.329	A	A
		A	A
Leishmania sp. (OKD-11)	0.297	A B	A
		B	A
L. tropica	0.265	C B	A B
		C	B
T. cruzi	0.208	C D	B
		D	B
L. donovani	0.190	D	B

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

TABLE IX

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTISERA RESPONSES WHEN EACH POLYCLONAL ANTISERUM FROM RABBIT WAS ABSORBED WITH *L. MEXICANA* ANTIGEN AND REACTED TO ALL ANTIGENS

Antisera	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey[c]
Leishmania sp.	0.256	A	A
(OKD-11)		A	A
L. tropica	0.254	A	A
		A	A
L. donovani	0.252	A	A
		A	A
T. cruzi	0.232	A	A
		A	A
L. mexicana	0.214	A	A

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

TABLE X

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTISERA RESPONSES WHEN EACH POLYCLONAL ANTISERUM FROM RABBIT WAS ABSORBED WITH *T. CRUZI* PARTICULATE ANTIGEN AND REACTED TO ALL ANTIGENS

Antisera	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey
Leishmania sp.	0.326	A	A
(OKD-11)		A	A
L. tropica	0.259	A B	A B
		B	A B
L. donovani	0.254	B	A B
		B	A B
T. cruzi	0.221	B	B
		B	B
L. mexicana	0.220	B	B

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

antisera groupings. The two groups obtained with Duncan's Multiple Range test were OKD-11 and *L. tropica*, and *L. tropica*, *L. donovani*, *T. cruzi* and *L. mexicana*. According to Tukey's Analysis, the first group included OKD-11, *L. tropica* and *L. donovani*; the second group was made up of *L. tropica*, *L. donovani*, *T. cruzi* and *L. mexicana*. The variations in the groupings caused by absorptions with either of the absorbing antigens showed that each antiserum reacted more strongly with *L. mexicana* than with *T. cruzi*.

Responses of Polyclonal Dog Sera

Figures 12-15 show comparative ELISA serology for sera from two *Leishmania*-infected dogs (Arrow and Jeremy), a *Trypanosoma cruzi*-positive dog (Carpenter) and an uninfected control dog (Miller) that were preabsorbed with particulate antigens of *L. mexicana* and *T. cruzi* and reacted to each of the 5 particulate antigens.

The ELISA values for serum from the uninfected dog are shown in Figure 12. The mean ELISA value for the negative control serum was approximately 0.265 (Tables XIII and XIV) and these baseline values were not significantly changed by preabsorption of the serum.

Figure 13 shows the reactions of anti-OKD-11 serum from a naturally infected dog (Arrow). There was

Figure 12. Mean optical density for ELISA reactions of serum from unifected dog (Miller) with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC).

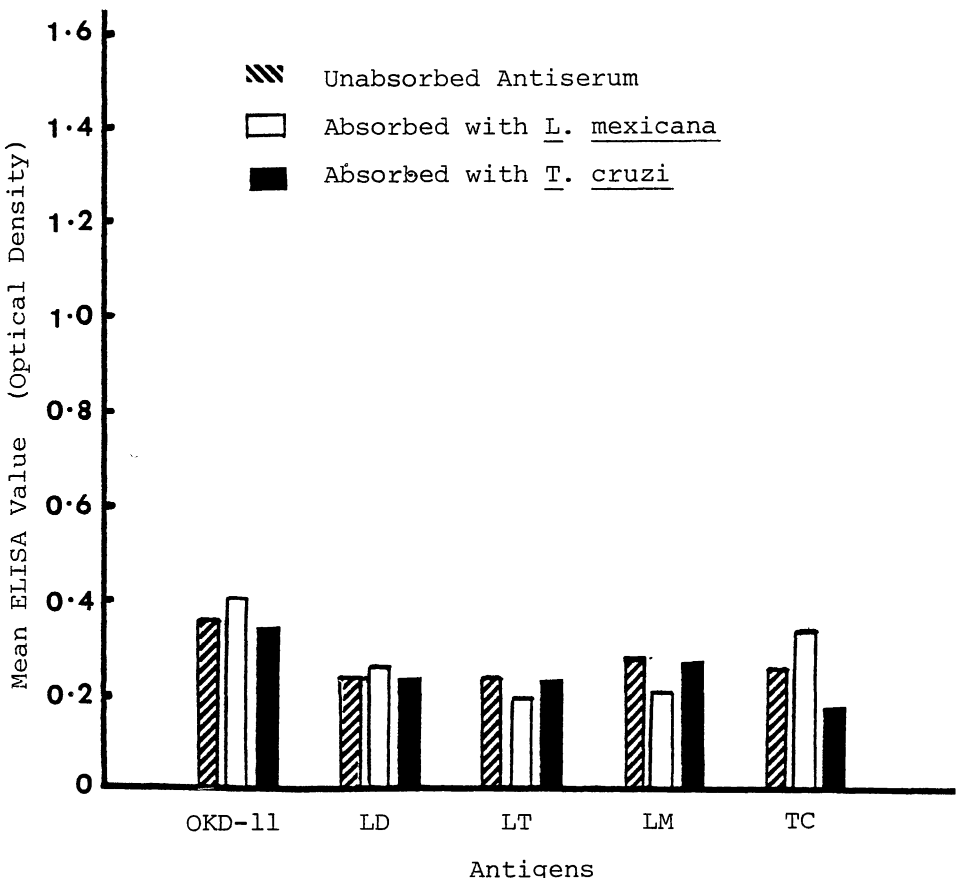
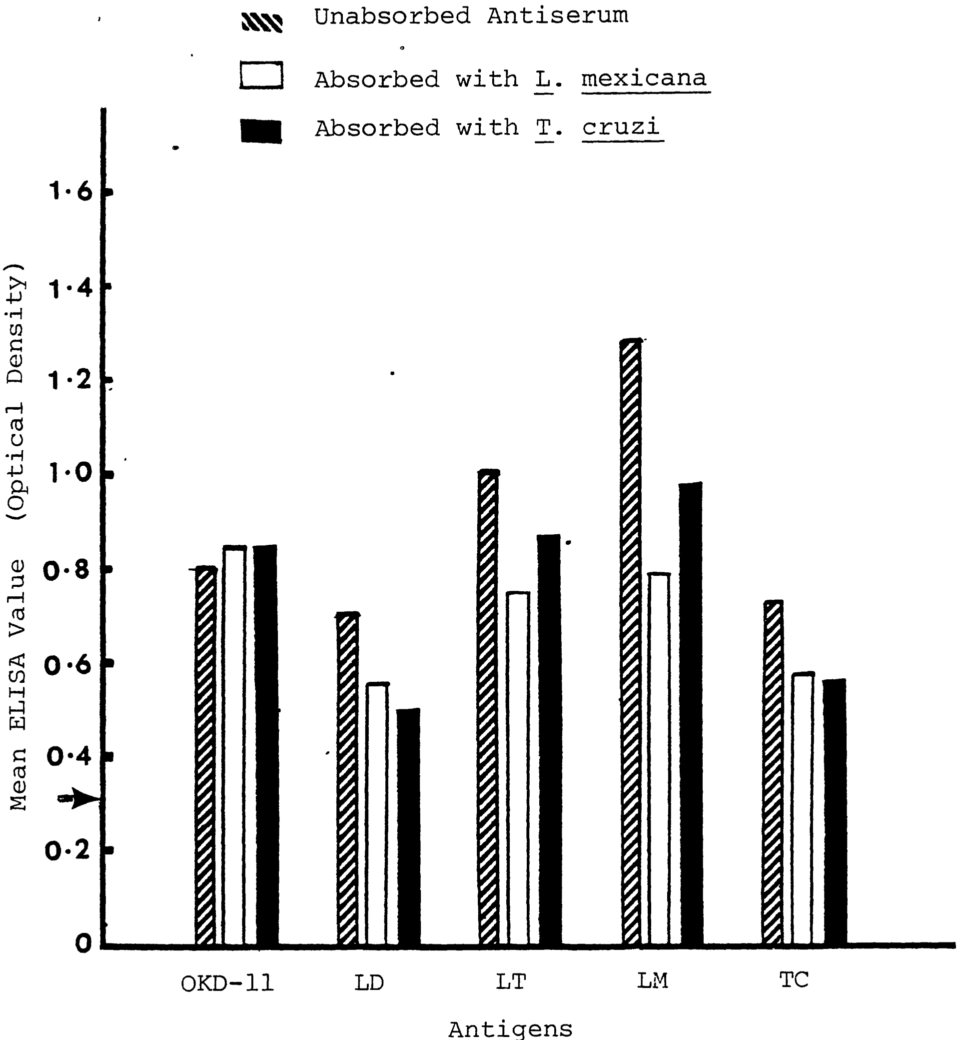

1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Mean ELISA Value (Optical Density)
Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
OKD-11
LD
LT
LM
TC
Antigens

Figure 13. Mean optical density for ELISA reactions of serum from a naturally infected dog (Arrow) with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrow indicates mean ELISA value of negative serum.



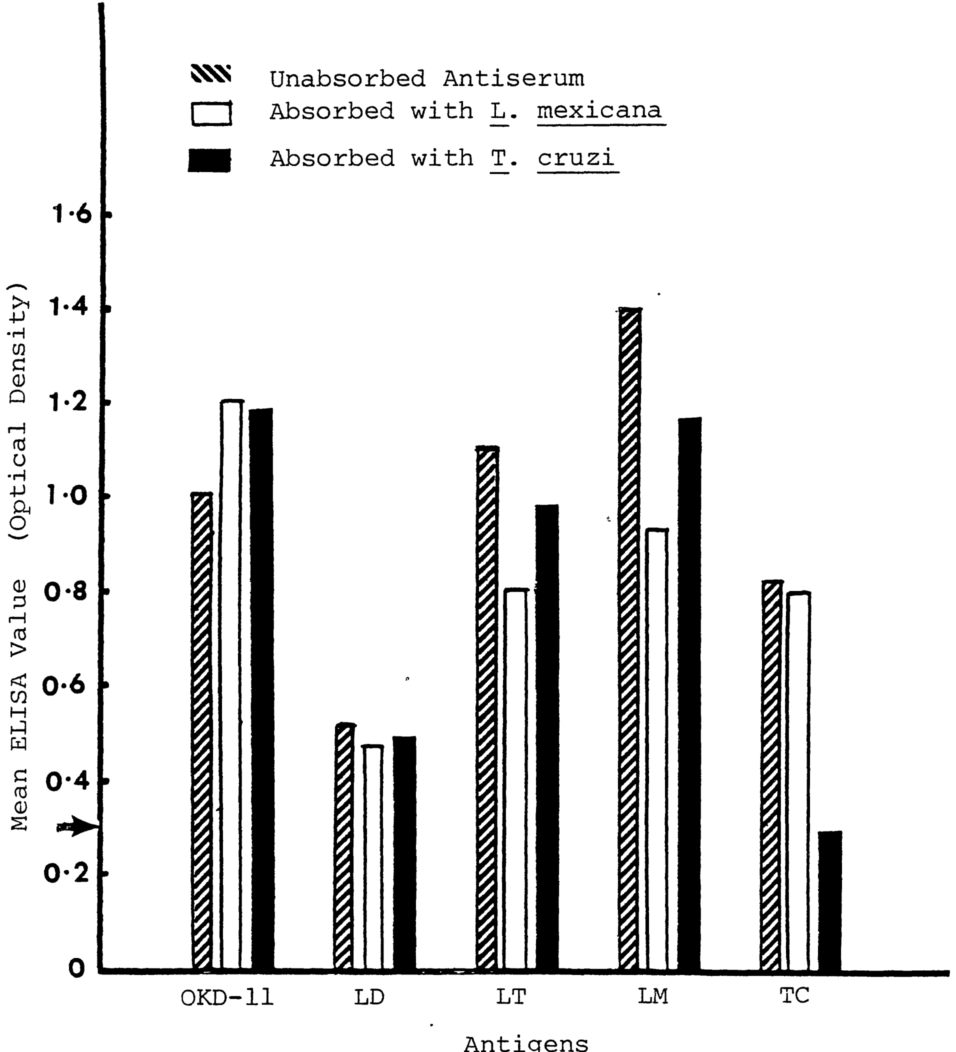
Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Mean ELISA Value (Optical Density)
OKD-11
LD
LT
LM
TC
Antigens

significant antibody binding with all 5 antigens: the highest amount of binding was with L. mexicana. Preabsorption with either L. mexicana or T. cruzi did not significantly affect the amount of binding to the homologous OKD-11 antigen. On the other hand, ELISA values obtained with the other antigens were decreased by the absorption.

Serological reactions of unabsorbed and preabsorbed antiserum from a dog experimentally infected with the canine Leishmania sp. (Jeremy) are compared in Figure 14. This antiserum also cross- reacted strongly with all the antigens; the highest amount of binding was against L. mexicana. Preabsorption significantly decreased the amount of antibody that bound to L. tropica, L. mexicana and T. cruzi. ELISA values for reactions with OKD-11 and L. donovani antigens were not significantly affected by preabsorption with either of the absorbing antigens.

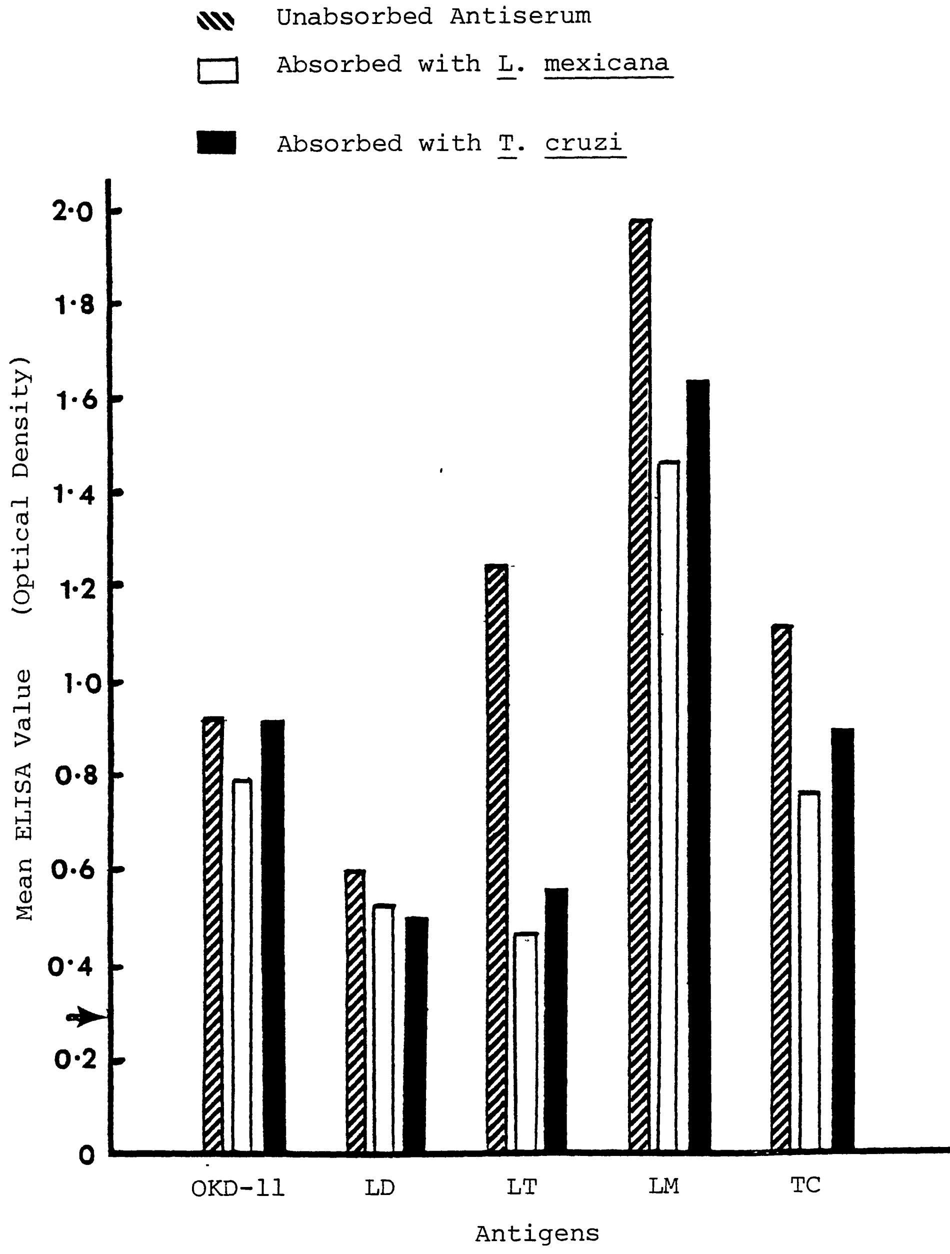
Unabsorbed antiserum from a dog with a natural T. cruzi infection (Carpenter) also reacted strongly with all the antigens as can be seen in Figure 15. Reactions with L. mexicana produced the highest ELISA values. Preabsorptions of the antiserum with each of the absorbing antigens reduced the ELISA values for reactions with L. tropica, L. mexicana and T. cruzi antigens. The amount of reaction to L. tropica was depressed most, and reactions with OKD-11 and L. donovani were

Figure 14. Mean optical density for ELISA reactions of serum from an experimentally infected dog (Jeremy) with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrow indicates mean ELISA value of negative serum.



Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
Mean ELISA Value (Optical Density)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
OKD-11
LD
LT
LM
TC
Antigens

Figure 15. Mean optical density for ELISA reactions of serum from a naturally infected dog (Carpenter) with particulate antigens of *Leishmania* sp. (OKD-11), *L. donovani* (LD), *L. tropica* (LT), *L. mexicana* (LM) and *Trypanosoma cruzi* (TC). Arrow indicates mean ELISA value of negative serum.



Unabsorbed Antiserum
Absorbed with L. mexicana
Absorbed with T. cruzi
Mean ELISA Value (Optical Density)
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
OKD-11
LD
LT
LM
TC
Antigens

not significantly affected by preabsorption.

Statistical analyses of tha absorption data for the canine antisera are shown in Tables XI through XIV. Groupings of the antigens based on the reactions of antisera to each antigen are shown in Tables XI and XII; Tables XIII and XIV show the groupings of individual antiserum when reacted to all antigens.

Data in Table XI show the antigen groupings when all the dog sera were preabsorbed with _L. mexicana_ antigen and reacted to each of the 5 antigens. Both statistical methods indicate that _L. mexicana_ antigen differed significantly from the other antigens. Antigens OKD-11, _L. tropica_ and _T. cruzi_ formed a second group with similar mean ELISA values. Whereas the Duncan's method indicated that _L. donovani_ differed significantly from the other antigens, analysis by Tukey's method showed that it was similar to _T. cruzi_. Table XII contains the antigen groupings that resulted from reactions of dog sera absorbed with _T. cruzi_ antigens. Reactions to _Leishmania mexicana_ differed significantly from those of other antigens. Two other groups of antigens were differentiated by the Duncan's analysis: OKD-11 and _L. tropica_ formed one group, and _T. cruzi_ and _L. donovani_ formed the other. Antigens in each grouping exhibited a similar amount of antibody binding. These analyses showed a relationship among the following species: OKD-11, _L. mexicana_ and

TABLE XI

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTIGEN-ANTIBODY RESPONSES WHEN POLYCLONAL ANTISERA FROM DOGS WERE ABSORBED WITH *L. MEXICANA* AND REACTED TO EACH OF THE ANTIGENS

Antigens	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey[c]
L. mexicana	1.041	A	A
Leishmania sp.	0.797	B	B
(OKD-11)		B	B
L. tropica	0.724	B	B
		B	B
T. cruzi	0.675	B	B C
			C
L. donovani	0.482	C	C

[a] Means with the same letters are not significantly different (alpha = 0.05)

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

TABLE XII

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTIGEN-ANTIBODY RESPONSES WHEN POLYCLONAL ANTISERA FROM DOGS WERE ABSORBED WITH *T. CRUZI* AND REACTED TO EACH OF THE ANTIGENS

Antigens	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey[c]
L. mexicana	1.128	A	A
Leishmania sp.	0.804	B	B
(OKD-11)		B	B
L. tropica	0.780	B	B
			B
T. cruzi	0.608	C	B C
		C	C
L. donovani	0.471	C	C

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

L. tropica.

Table XIII shows statistical groupings obtained when each antiserum was preabsorbed with L. mexicana antigen and reacted to all antigens. Serum from the negative control dog (Miller) had the lowest mean ELISA values and differed significantly from the other 3 antisera. Mean ELISA values for the T. cruzi-infected dog (Carpenter) and the Leishmania-infected dog (Jeremy) did not differ significantly. Nor did means for the Leishmania-infected dogs (Jeremy and Arrow) differ significantly. Statistical analysis of ELISA values for the dog sera after preabsorption with T. cruzi are shown in Table XIV. The Duncan's Multiple Range test showed that the mean ELISA values for the T. cruzi-positive dog (Carpenter) were significantly different from those of the Leishmania-infected dogs (Arrow and Jeremy); also, the latter two sera did not differ significantly from each other. Antiserum from the negative control dog (Miller) had the lowest mean ELISA values and differred significantly from the other three sera. The groupings for L. mexicana-absorbed sera by Tukey's Analysis of Variance (Table XIV) were similar to groupings obtained from sera absorbed with L. mexicana in Table XIII.

Double Gel Diffusion

Figures 16-20 illustrate the precipitin lines which

TABLE XIII

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTISERA RESPONSES WHEN EACH POLYCLONAL ANTISERUM FROM DOG WAS ABSORBED WITH *L. MEXICANA* AND REACTED TO ALL ANTIGENS

Antiserum	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey[c]
Carpenter	0.993	A	A
		A	A
Jeremy	0.910	A B	A B
		B	B
Arrow	0.801	B	B
Miller	0.271	C	C

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

TABLE XIV

MULTIPLE RANGE ANALYSIS OF MEAN ELISA VALUES FOR ANTISERA RESPONSES WHEN EACH POLYCLONAL ANTISEUM FROM DOG WAS ABSORBED WITH *T. CRUZI* AND REACTED TO ALL THE ANTIGENS

Antiserum	Mean ELISA Value	Statistical Method[a]	
		Duncan[b]	Tukey[c]
Carpenter	1.045	A	A
			A
Jeremy	0.901	B	A B
		B	B
Arrow	0.829	B	B
Miller	0.259	C	C

[a] Means with the same letters are not significantly different (alpha = 0.05).

[b] Duncan's Multiple Range Analysis.

[c] Tukey's Analysis of Variance.

formed between the antigens and antibodies in the gel diffusion tests. In all cases, only single precipitin lines formed. When anti OKD-11 serum was diffused against each of the detergent-solubilized antigens (Fig. 16), lines of identity formed only with those of L. mexicana and T. cruzi; precipitin bands were not visible for any of the other antigens which indicated antigenic similarity between OKD-11, L. mexicana and T. cruzi organisms.

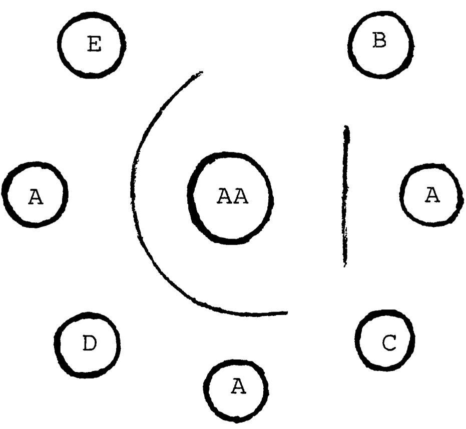
Rabbit anti-L. donovani serum produced a precipitin line against L. donovani antigen but not against the others (Fig. 17); this signified a strong specificity for its homologous antigen. The rabbit anti-L. tropica serum formed precipitin lines with OKD-11 and L. tropica antigens (Fig. 18).

Figure 19 shows that Leishmania mexicana, OKD-11, L. tropica and T. cruzi antigens all cross-reacted with the anti-L. mexicana serum; a precipitin line did not form with the L. donovani antigen. Each antigen, except that of L. donovani, may have had some common antigenic component that was responsible for the precipitin band. Rabbit anti T. cruzi serum showed cross-reactivity with all the antigens studied (Fig. 20).

Immunoelectrophoresis

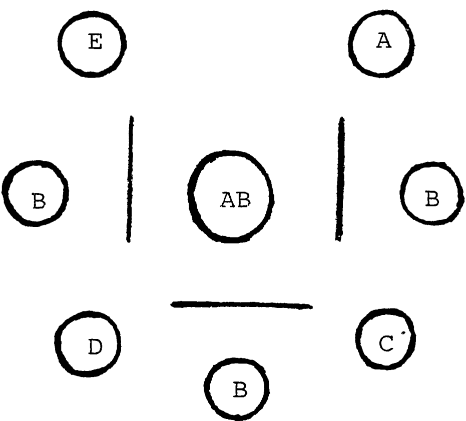
Only one precipitin arc was observed with each

Figure 16. Double gel Diffusion Diagram Showing Precipitin Reactions Between anti-*Leishmania* sp. (OKD-11) Serum and Detergent-Solubilized Antigens of 4 *Leishmania* species and *Trypanosoma cruzi*.



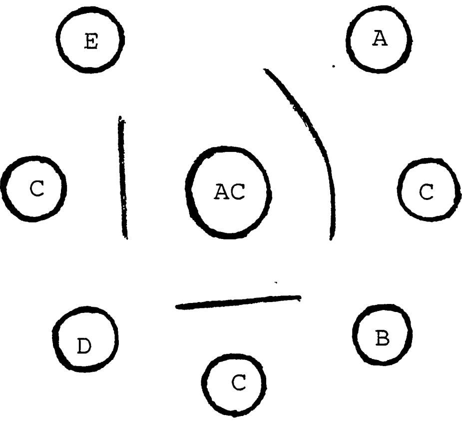
AA - Rabbit anti-Leishmania sp. (OKD-11)
A - Leishmania sp. (OKD-11) antigen
B - L. donovani antigen
C - L. tropica antigen
D - L. mexicana antigen
E - T. cruzi antigen

Figure 17. Double Gel Diffusion Diagram Showing Precipitin Reactions Between anti-*L. donovani* Serum and Detergent-Solubilized Antigens of 4 *Leishmania* species and *Trypanosoma cruzi*.



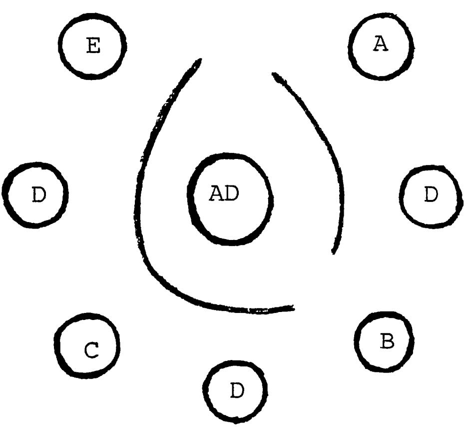
AB - Rabbit anti-*L. donovani*
A - *Leishmania* sp. (OKD-11) antigen
B - *L. donovani* antigen
C - *L. tropica* antigen
D - *L. mexicana* antigen
E - *T. cruzi* antigen

Figure 18. Double Gel Diffusion Diagram Showing Precipitin Reactions Between anti-*L. tropica* Serum and Detergent-Solubilized Antigens of 4 *Leishmania* species and *Trypanosoma cruzi*.



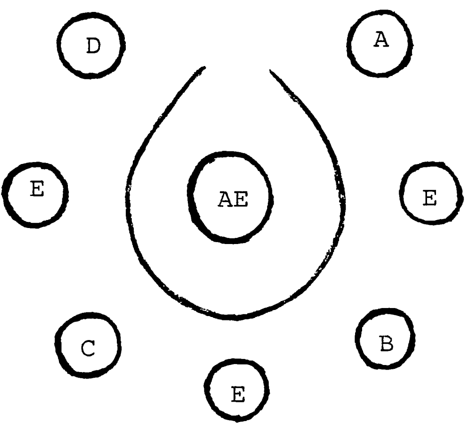
AC - Rabbit anti-*L. tropica*
A - *Leishmania* sp. (OKD-11) antigen
B - *L. donovani* antigen
C - *L. tropica* antigen
D - *L. mexicana* antigen
E - *T. cruzi* antigen

Figure 19. Double Gel Diffusion Diagram Showing Precipitin Reactions Between anti-*L. mexicana* Serum and Detergent-Solubilized Antigens of 4 *Leishmania* species and *Trypanosoma cruzi*.



AD - Rabbit anti-*L. mexicana*
A - *Leishmania* sp. (OKD-11) antigen
B - *L. donovani* antigen
C - *L. tropica* antigen
D - *L. mexicana* antigen
E - *T. cruzi* antigen

Figure 20. Double Gel Diffusion Diagram Showing Precipitin Reactions Between anti-*T. cruzi* Serum and Detergent-Solubilized Antigens of 4 *Leishmania* species and *Trypanosoma cruzi*.



AE - Rabbit anti- T. cruzi
A - Leishmania sp. (OKD-11) antigen
B - L. donovani antigen
C - L. tropica antigen
D - L. mexicana antigen
E - T. cruzi antigen

antigen in the immunoelectrophoresis tests. The arc was formed at the points where antigens were applied on the gel. Variation of voltage and time of electrophoresis did not change the electrophoresis patterns. Anti *Leishmania donovani* serum apparently did not bind to its homologous antigen in a visible way because no precipitin arc was observed. Formation of precipitin arcs at the points of antigen application suggested a lack of movement of the proteins during electrophoresis.

CHAPTER IV

DISCUSSION

A potential use of particulate antigens for diagnosis of leishmaniasis was demonstrated in this study. Washed particulate antigens elicited measurable antibody levels in rabbits without the use of an adjuvant. Although serologic cross-reactivities among the organisms were evident in both the monoclonal and polyclonal antibody studies, quantitative differences in affinity were demonstrated through absorption of antisera and subsequent statistical analysis of ELISA measurements. The results of absorption tests and statistical analyses indicated that the canine isolate of *Leishmania* sp. (OKD-11) from Oklahoma was more similar to *L. mexicana* than to any of the other isolates studied.

Monoclonal Antibody Studies

The fusion efficiency of 55% during hybridoma production was considered low. This low fusion percentage may have been caused by any of several factors including, for example, the viability of myeloma cells or unsatisfactory fusion technic. The viability of myeloma cells is important in their fusion potential. Because

the myeloma cells were passaged every other day for approximately 8 days before fusion, adequate viability of the myeloma cells was presumably maintained. Therefore, fusion technic was more likely responsible for the low fusion efficiency.

Screening of hybridoma supernatants for antibodies was more efficiently accomplished using ELISA than FIAX serology. The greater sensitivity of the ELISA method made it more useful for supernatant screening. Seemingly, antibody concentrations in the supernatants were below levels detectable by FIAX methodology.

Indirect Fluorescent Antibody Tests

The indirect fluorescent antibody tests indicated that some of the monoclonal antibodies produced in this study had binding specificities for kinetoplasts of both *Leishmania* sp. (OKD-11) and *T. cruzi*. Thus, the kinetoplast proteins were probably responsible for some of the serological cross-reactions observed between these organisms. The kinetoplasts were no doubt present in the particulate antigen preparation because special measures were not taken to remove them. According to Dwyer (1981), centrifugation of fractionated organisms resulted in sedimentation of kinetoplasts along with the pellicular membranes. However, the separation of the two components could be and was achieved by discontinuous sucrose gradient ultracentrifugation. No attempt was

made to remove kinetoplasts from the particulate antigens in the present study. Nevertheless, the resulting kinetoplast-specific monoclonal antibody that was produced might be useful to trace kinetoplast development during morphogenesis of these and other hemoflagellates. Also, such monoclonal antibody might be useful in comparative studies of kinetoplasts and to remove cross-reacting kinetoplastic antigenic determinants from antigen preparations.

The binding of another monoclonal antibody (3A2) to the pellicular membrane and flagella (Fig. 1) indicated the presence of similar antigenic components in these two structures. The most likely component common to both structures is tubulin protein (De Souza et al., 1983). This protein was probably the one most likely labelled by the 3A2 monoclonal antibody.

Enzyme-Linked Immunosorbent Assay

Each of the monoclonal antibodies that was isolated, except that produced by clone 8C3, reacted with particulate antigens from all five species tested demonstrating a lack of species specificity. DeIbarra et al. (1982) observed that one monoclonal antibody that they produced against *L. tropica* antigen showed definite affinity for *L. donovani*, *L. braziliensis*, *L. mexicana*, *T. cruzi* and *T. brucei* antigens. The wide range of cross-reactivity

was attributed to monoclonal antibody recognition of certain antigenic determinants that were apparently common among all of those organisms studied. In the present study, some of the antigenic determinants held in common were found to reside in the pellicular membranes, flagella and kinetoplasts.

Antibody produced by one clone (12E8) could not be concentrated sufficiently to detect it by indirect fluorescent antibody tests (Fig. 4). However, antibody was detected by ELISA (Table III) demonstrating a need to use ELISA instead of less sensitive IFA tests. The low antibody concentration in 12E8 supernatant fluid was probably a result of inefficient in vitro production of antibody by the clone. Anthony et al. (1981) made similar observations with some of their clones and concluded that the indirect fluorescent antibody test was prone to giving false negative results. This group also speculated that sequestration or loss of antigen through fixation and drying might have contributed to the lack of antibody binding. Thus, the IFA test is not an effective screening method.

The data in Table III show that the ELISA values signifying the amount of monoclonal antibodies which bound to T. cruzi antigens was higher than that for Leishmania sp. (OKD-11), the sensitizing antigen. A possible explanation for this might be derived from the immunofluorescent photographs (Figs. 1-6) which revealed

that *T. cruzi* epimastigotes were markedly larger and considerably more uniform in size and shape and had larger kinetoplasts than did the promastigotes of the OKD-11 isolate. Because the *T. cruzi* kinetoplasts were larger and would have a higher proportion of antigenic determinants per kinetoplast, they would bind proportionately more of the monoclonal antibody. It could be that the higher concentration of kinetoplast antigen accounts for the higher ELISA values recorded with the *T. cruzi* antigens.

Antibody from clone 8C3 showed little or no evidence of binding in either the IFA (Fig. 3) or ELISA tests (Table III) in spite of the presence of ELISA-detectable antibody previous to hybridoma cloning. This failure to bind could be attributed to a loss of antibody-producing ability by the 8C3 clone. A similar phenomenon was observed by Goding (1980) who reported that approximately 50-70% of all hybridomas that initially secrete antibodies will lose the ability to produce antibodies. He indicated that stress to cells from overgrowth in media could be responsible for loss of antibody production.

Changes in bovine serum lots used to supplement media during *in vitro* cultivation may have affected the 8C3 clone because not all lots of commercial serum will enhance growth of cells. Also, a loss of spleen cell chromosomes by the hybridomas could have been responsible

for loss of antibody production as has been demonstrated by Taggart and Samloff (1983) and by Oi and Herzenberg (1980).

Polyclonal Antisera

The use of particulate antigens in the ELISA and FIAX solid-phase assays indicated considerable variation in the two tests. Of primary concern was getting particulate antigens to adsorb to the wells of the ELISA-treated microtiter plates and the cellulose-nitrate surfaces of the StiQ samplers. Obviously, some antigen could have dislodged from StiQ surfaces because of vigorous shaking during the incubation and washing processes. Poor binding of the particulate antigens to the microtiter plates could have led to low or erratic ELISA values. Better methods of binding particulate antigens to surfaces or increase in numbers of test replicates could have improved the consistency and accuracy of the analyses. In spite of these and other possible sources of variation, the correlation coefficients obtained by comparing data from FIAX and ELISA (Table IV) showed that the two technics correlated satisfactorily in their quantitation of the serological reactions.

Antigens and Serological Cross-Reactions

Interspecific cross-reactions were confirmed for

all the antisera tested. Evidently, similar antigenic components were present in the antigens prepared from each of the parasites. Indirect immunofluorescent tests with monoclonal antibodies (Figs. 1 and 2) indicated that pellicular membrane, flagella and kinetoplasts were involved in the serological cross-reactions. This finding might substantiate the observations of Anthony et al. (1981) and Constantine and Anthony (1983) who hypothesized that antigens associated with flagella (and another structure closely associated with the flagella) of *L. braziliensis* and certain *Trypanosoma* species, accounted for some of the cross-reactivity they observed. Constantine and Anthony also reported that the antigens associated with the kinetoplasts were species-specific as demonstrated with their monoclonal antibodies. Such species specificity was not observed in the present study.

Other observations of the presence of cross-reactive surface antigens were made by Ramasamy et al. (1983). This group observed that four strains of *L. donovani* and one strain of *L. mexicana* all possessed surface proteins of similar molecular weights; these proteins were thought to be responsible for serological cross-reactions observed among the organisms they studied.

The reactivity between an antigen of one species and antiserum produced against antigens from a different species may not be quantitatively reciprocal. This phenomenon can be seen in the strong reactions of

anti-*Leishmania* sp. (OKD-11) antibodies with *L. mexicana* antigens (Fig. 7) and the low antibody binding in the reciprocal situation (Fig. 11). An explanation for this finding might be that the antigenic determinants that accounted for the cross-reactivity were expressed in significantly different proportions among the leishmanial species. Also, there could be differences in spatial arrangements of the target antigenic determinants or even in certain surface proteins; spatial arrangements could affect the accessibility of the antigenic determinants by the antibody molecules.

Detergent-Treatment of Particulate Antigens

In general, detergent-solubilized and detergent-treated particulate antigens were found to bind more antibodies than did untreated particulate antigens (Tables V and VI). These differences could be attributed to "occult" antigenic determinants at hidden sites within the untreated particulate antigens. These sites may have been only partially accessible for antibody binding prior to detergent treatment; however, such determinants could be sufficiently available to stimulate antibody production as a result of macrophage processing of antigens. The treatment of the antigens with Triton X-100 could have exposed hidden antigenic sites. For some unexplained reason, the antibody binding properties of *L. donovani* antigen were not improved by detergent treatment as can

be seen in Table V.

Preabsorption of Antisera

When polyclonal antisera were preabsorbed with either *L. mexicana* or *T. cruzi* antigens, there was a significant reduction in the degree of antibody binding to both of the antigens. These reductions in responses apparently reflected either the relative amounts of reactive sites available or accessibility of the cross-reactive proteins.

There were similarities in monoclonal antibody binding between *Leishmania* sp. (OKD-11) and *T. cruzi*; see Figures 1 and 2. Nevertheless, absorption of the polyclonal anti OKD-11 serum with *T. cruzi* antigen did not significantly reduce the response to its homologous antigen (Fig. 7). It is possible that an insufficient amount of absorbing antigen was used in the test. However, this is not likely because the same amount of antigen produced dramatic changes in binding levels with some of the other antisera. Similarly, *T. cruzi* absorptions of antisera from OKD-11-infected dogs (Arrow and Jeremy) did not reduce responses to the homologous antigen (Figs. 13 and 14). Higher concentrations of absorbing antigen, or longer incubation periods, may have produced greater changes in the amount of antibody binding for these sera.

Absorptions of anti-OKD-11 serum with *T. cruzi*

antigen enhanced its affinity for L. mexicana antigen (Fig. 7). The reason for this increase was not determined. Perhaps serum proteins or antibodies of other classes or specificities blocked or competed for the binding sites; thus, when the competing entities were removed through absorption, antibody binding to L. mexicana was improved.

Ouchterlony Double Gel Diffusion

The Ouchterlony double gel diffusion tests showed that, in general, most of the rabbit antisera cross-reacted with each of the antigens. An exception was the rabbit anti-L. donovani serum which reacted only with its homologous antigen. In every case, the antigen-antibody reactions were represented by single-line precipitin bands (Figs. 16-20).

The antigens which were apparently common to all five organisms studied were indicated by the indirect immunofluorescence antibody tests (Figs. 1 and 2). Undoubtedly, these antigens contributed to the cross-reactions observed in the double gel diffusion tests. In the present study there could have been more than one kind of antigen-antibody complex involved in each band, but the antigenic proteins in each band would have had similar molecular weights because precipitate formed in the same locations. Dwyer (1981) reacted detergent extracts of L. donovani membranes with sera

from patients with cutaneous leishmaniasis and found single lines of precipitin bands. Dwyer attributed the single precipitin band to the presence of a principal antigenic determinant in high concentration within the extracted material. A similar situation could account for the single precipitin bands observed for each of the sera shown in Figures 16-20.

Formation of single precipitin bands in gel diffusion tests is not sufficient criterion to discount the presence of multiple antigens in the detergent extracts. Dwyer (1981) demonstrated the presence of several protein fractions in detergent extracts by using electrophoretic separation. Thus, it is possible that several proteins were present in the detergent extracts described herein, in addition to a principal antigenic determinant. These other proteins were probably present in concentrations that were too small to produce visible precipitin bands.

Similarly, the absence of precipitin bands when antisera were reacted with solubilized antigens (Figs. 16-19) cannot be interpreted as nonidentity between the various antigens. The sensitivity of agar gel diffusion may not have been sufficient to detect protein in low concentrations. FIAX and ELISA tests with their higher sensitivity revealed extensive cross-reaction which is most likely explained by the presence of several types of antigenic determinants. Differences in sensitivity between ELISA and Ouchterlony were demonstrated by Hommel

et al. (1978). They found that 35 of 36 sera from patients with visceral leishmaniasis were positive by the ELISA test, whereas only 18 of 27 sera tested were detected by the Ouchterlony method.

In the present study there was no visible separation of proteins following electrophoresis, probably because of low protein concentrations in the detergent-extracted antigens. The total protein contents of the solubilized antigens were not determined because protein quantitations were hindered by the presence of detergent; addition of detergent solution to Bio-Rad dye solution gave false positive results.

Statistical Comparisons of Serological Measurements

The statistical grouping of the particulate antigens based on quantitative serological data (Tables VII and VIII) showed that the Oklahoma *Leishmania* sp. (OKD-11) was more similar to *L. mexicana* than to *L. donovani*. In fact, *L. donovani* more nearly resembled *T. cruzi* in its serological characteristics. A relationship between the Oklahoma canine isolate and *L. mexicana* from Texas was previously noted through isozyme analyses by Kreutzer et al., (1983). They found that the Oklahoma leishmanial isolate (OKD-11) had an isozyme profile that corresponded closely to that of *L. mexicana mexicana*. Previous studies by Kocan et al. (1983) and by Jackson and Fox

(1981) showed that the Oklahoma canine isolate appeared most similar to visceral leishmanial forms such as _L. donovani_ and _L. infantum_. Kocan et al. used subpellicular microtubule numbers and greatest diameter measurements, whereas Jackson and Fox used a radiorespirometry technic; both methods indicated that the canine _Leishmania_ isolate was not like cutaneous forms. Thus identification of the canine isolate of _Leishmania_ sp. from Oklahoma remains unsettled.

Statistical analysis of antibody responses for polyclonal dog sera using Duncan's Multiple Range Analysis and Tukey's Analysis (Tables XIII and XIV) agreed with known infections in animals with confirmed leishmaniasis or Chaga's disease. Antisera from two dogs (Arrow and Jeremy) which were culture-positive for _Leishmania_ showed similar antibody reaction patterns. A third dog (Carpenter) that was seropositive for _Trypanosoma cruzi_ had reaction patterns that were significantly different from those of the _Leishmania_-infected dogs.

The choice of statistical analytical method was important in relationship groupings of the antisera as to their affinity for the various antigens. Absorption of each antiserum with _L. mexicana_ prior to reactions with all antigens did not affect the statistical groupings dramatically (Table XIII); both methods of statistical analysis revealed similar groupings of the antisera. On the other hand, absorptions with _T. cruzi_ antigen

and analysis of the data by Duncan's Multiple Range Test clearly differentiated the T. cruzi-infected dog serum from those of Leishmania-infected dogs (Table XIV); the grouping obtained from Tukey's Analysis of variance was similar to grouping obtained when the antisera were absorbed with L. mexicana (Table XIII). The difference in the groupings of the dog sera as revealed by Duncan's Multiple Range and Tukey's analyses is attributed to the more liberal evaluations of Duncan's method; smaller differences in treatment means are considered significant by the Duncan's method than by Tukey's method. It is assumed that similar comparisons of cross absorption data have not been done with these organisms because no such reports were found in the literature. Hence this preabsorption study was considered unique.

Particulate antigens used in the present study were not found to be as species-specific as those reported by Constantine and Anthony (1983) and McMahon-Pratt and David (1981). These groups showed that surface antigens of some Leishmania species and T. cruzi that they studied were highly species-specific. The condition of the membrane antigens could have been altered during preparation and storage. Dwyer (1980) beleived that the inclusion of phenylmethyl sulfonyl fluoride, a protease inhibitor, was important in securing the integrity of the membrane antigens during fractionation of the

organisms. This compound was not added to the membrane preparations in the present study. The omission of preservative could have resulted in some proteolytic degradations of antigens. Jaffe et al. (1984) confirmed the usefulness of phenylmethyl sulfonyl fluoride in preparation of antigens. Their method of antigen preparation was patterned after Dwyer (1981). In their study, Jaffe et al. produced sixteen monoclonal antibodies that were specific against *L. donovani* membrane antigens. The preservative presumably helped to maintain the integrity of the *L. donovani*-specific determinants in the antigen preparation.

CHAPTER V

CONCLUSIONS

It was demonstrated in this study that particulate antigens of *Leishmania* species and *Trypanosoma cruzi* may be useful for serodiagnostic purposes. However, serological cross-reactions were observed among the five organisms studied, and quantitative methods are needed to differentiate the organisms.

The ELISA test had greater sensitivity and thus was preferred over FIAX for screening supernatants for hybridoma antibody production. The indirect immunofluorescence antibody tests with monoclonal antibodies showed that antigenic determinants in the pellicular membranes, kinetoplasts and flagella were responsible for some of the serological cross-reactions observed between OKD-11 and *T. cruzi*.

Detergent treatment of particulate antigens generally improved the antibody binding capacity of the antigens except in the case of *L. donovani*. The shaking action contributed to solubilization of the antigen, whereas supernatant proteins from antigen-detergent mixtures that were not shaken did not bind antibodies as well.

The usefulness of preabsorption tests and statistical

analysis on polyclonal antisera was revealed. Graphic comparisons of data from serological reactions for unabsorbed and preabsorbed antisera did not show clearly any relationship groupings. On the other hand, statistical analysis indicated that the canine isolate of *Leishmania* from Oklahoma (OKD-11) and *L. mexicana* antigens were similar. Also, serum from a *T. cruzi*-positive dog showed significantly different affinities for antigens than did the other sera. In addition, sera from *Leishmania*-positive dogs were shown to have similar reactive properties. Thus, statistical comparisons of quantitative serological measurements may be useful for taxonomic classification of leishmanial organisms and identification of species responsible for various forms of leishmanial disease.

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"APPENDIXES"

APPENDIX A

PHOSPHATE BUFFERED SALINE (PBS), TWEEN 20

(pH 7.4)

8.0 gm of Sodium Chloride (NaCl)

0.2 gm of Pottasium Chloride (KCl)

0.2 gm of Pottasium Dihydrogen Phoshate (KH_2PO_4)

2.9 gm of Disodium Hydrogen Phosphate ($Na_2HPO_4.12H_2O$)

0.5 ml of Tween 20

1.0 L of Distilled Water

APPENDIX B

SUBSTRATE SOLUTION FOR PEROXIDASE CONJUGATE

Phosphate-Citrate Buffer

25.7 ml of 0.2M (28.4 gm Na_2HPO_4 / 1,000 ml H_2O) phosphate

24.3 ml of 0.1M (19.2 gm / 1,000 ml) citric acid

50.0 ml of distilled water

Dissolve 40.0 mg orthophenyline diamine (OPD) in 100 ml of of buffer.

Add 40.0 ųl of 30% hydrogen peroxide

VITA

Abiodun Oladayo Adibi

Candidate for the Degreee of

Doctor of Philosophy

Thesis: PARTICULATE ANTIGENS OF *TRYPANOSOMA CRUZI* AND FOUR *LEISHMANIA* SPP: STUDY OF BINDING SPECIFICITIES OF MONOCLONAL AND POLYCLONAL ANTIBODIES

Major Field: Veterinary Parasitology

Biographical:

Personal Data: Born in Ogbomoso, Nigeria, December 24, 1947, the son of Chief and Mrs. Nathaniel A. Adibi.

Education: Graduated from Iwo Baptist High School in December, 1967; received the Bachelor of Science Degree in Biology from Southwest Missouri State University in December 1975; received the Master of Science Degree in Microbiology from East Tennessee State University in December 1978; completed the requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1985.

Professional Experience: Laboratory Technician, State of Missouri Department of Agriculture, Veterinary Diagnostic Laboratory, June, 1976, to December, 1976;

Professional Association: Southwest Association of Parasitologists; Nigerian Research Association.