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COMPARISON OF SOLUBLE ANTIGENS

OF *LEISHMANIA* SPP.

APPROVED BY:

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To ABF: who taught me the joy of teaching science; and who has spent most of her life looking at birds and flowers, looking after students, looking up to God and loving it all.

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SYMBOLS AND ABBREVIATIONS

C	centigrade
CDC	Center for Disease Control
e. g.	for example
et al	and others
FIA	Freund's incomplete adjuvant
Fig.	figure
g	gram or grams
G	gravity
i. e.	that is
L.	*Leishmania*
L - D	Leishman-Donovan
M	molar
mA	milliamperes
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mμ	millimicron
N	normal or Nitrogen
PBS	phosphate buffered saline
pH	potential Hydrogen
Rab.	rabbit
sp.	species (singular)
spp.	species (plural
~	approximately
/	per
μ	micron
2S	2-Stauber
%	percent
#	number
X	times or by
μg	microgram

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COMPARISON OF SOLUBLE ANTIGENS OF *LEISHMANIA* SPP.

CHAPTER I

INTRODUCTION

Leishmania spp., the causative agents of such diseases as kala-azar, espundia, and oriental sore, are flagellated protozoans and probably include some of man's most ancient parasites (81). They share the title of hemoflagellate with the trypanosomes since both genera live in the blood or tissues of man and other animals. These two groups are classed with several other genera in the family *Trypanosomidae* and in the order *Protomastigida*. All other members of the family are also parasitic, infecting insects, lizards or plants. All genera reside in the gut of insects during some stage of their development (2). It is, therefore, believed that the family consisted originally of primitive intestinal parasites of insects and that only later did they become adapted to intracellular life in vertebrates (44).

In cultures containing blood and maintained at room temperature, and in infected insects, all species of *Leishmania* exist in the leptomonad stage. This flagellated form is spindle shaped, 14 to 20 μ long and 1.5 to 3.5 μ wide. A single round nucleus is located in the center of the organism and the oval kinetoplast lies transversely near

the anterior end (21). After this form enters the vertebrate host it undergoes both morphological and physiological alterations to transform into the leishmanial form which is referred to as the Leishman-Donovan (L-D) body in honor of Sir William Leishman who discovered the species L. donovani in 1900 (7) and Charles Donovan who observed L. donovani in connection with the disease kala-azar in 1903 (7). The L-D bodies, also called amastigotes, multiply in the cells of the reticuloendothelial system, particularly the macrophages, where they reproduce until the macrophage bursts and they are released to infect other cells. The ovoid, nonflagellated L-D bodies are approximately 3 μ in diameter. The vesicular nucleus is located laterally and the only remnant of the flagellum is a distinct, dark staining kinetoplast in a rod-like form within the cell (48).

Leishmania chameleonis is an exception to the above rule in that it is found only in the leptomonad form in both the insect vector and in lizards, the vertebrate host (21). The organism is always extracellular and inhabits the cloacal lumen or large intestine of the lizard (2). However, experimentally induced invasion of the epithelial cells of the large intestine has been reported (33). Because of this retention of the leptomonad form in the lizard, Hoare (45) has hypothesized that the Leishmania of lizards serve as evolutionary links through which the Leishmania evolved from intestinal parasites of insects to blood and tissue parasites of man. This theory is supported by the fact that L. adleri has been known to infect not only several genera of lizards, but also hamsters and man (58). Adler and Adler (4) and

Adler et al (5) also found that L. adleri contains some of the same antigens that are found in L. donovani, L. tropica and L. brasiliensis, all of which produce zoonoses.

The most important diseases of man produced by species of Leishmania are kala-azar, espundia, and oriental sore. On rare occasions, transmission of these diseases has been reported to occur from man to man via feces or urine. Recovery of L. donovani from the nasal mucosa of kala-azar patients suggests that mouth to mouth transmission may be possible. One case of congenital infection was reported and one instance of transmission via coitus occurred in England (57). However, the most important mode of transmission is through the sandfly.

The actual identification of the insect vector required many years. In 1907, Patton (2) observed that bed bugs were infected with Leishmania. In 1922, it was suggested that the sandfly of the genus Phlebotomus was the insect vector. In the same year an overlap in the geographic range in India of the disease kala-azar and the distribution of Phlebotomus argentipes was noted. In 1925, the Indian Kala-azar Commission concluded that the bed bug was not a vector. In the Mediterranean region, fleas were suspected as the vector, but this was refuted in 1924. The sandfly, however, could not be proven to be the vector because transmission of kala-azar by infected sandflies had never been reported (57). It was observed that if the sandflies were given a glucose meal after infection, rather than continuous blood meals, the flagellates proliferated so rapidly that they blocked the pharynx. With this type of diet, laboratory transmission of kala-azar

to humans through the bite of the sandfly was reported in 1942 (84). Since that time, examination of the correlation between insect distribution and leishmaniasis has been used as a tool to predict insect vectors.

Since, until recently, only the leptomonad stage could be grown in culture, most of the morphological studies had been done with this form. However, in the 1930's and 1940's, L-D bodies were successfully grown in tissue cultures as described by Heyneman (42). Trager (87) made a major contribution to in vitro culture of Leishmania when he suggested that more exacting nutritional requirements as elevated temperatures might be responsible for failure to maintain the organisms in culture. He maintained L. donovani at 37 C for several hours in an erythrocyte extract medium; however, no growth or reproduction of the organisms occurred. In 1964, Lemma and Schiller (55) successfully cultivated Leishmania in vitro and at the present time, cultural forms of L. mexicana, L. donovani, L. tropica and L. brasiliensis have all been maintained. These cultures have allowed for morphological studies and have provided information concerning the metabolic and biochemical characteristics of the organisms (46).

The entry of Leishmania sp. into the human host is followed by a series of changes in the tissues of the host and in the parasite (80). The pattern of disease produced by the parasite depends upon the interaction of these changes in the host and parasite. Although the pathologic changes in the host differ with each species of Leishmania, since they are all diseases of the reticuloendothelial system, gross hematological abnormalities are induced in every case (40). In addition, there are other pathological alterations peculiar to each

specific disease, and these are summarized in the following paragraphs.

Kala-azar, also called the Black Sickness, Sahib's Disease, Tropical Splenomegaly, Sirkari Disease, Mard el Bicha, Dum-dum Fever, Burdwan Fever and Ponos, is a form of leishmaniasis involving the viscera, particularly the spleen and liver, and is characterized by chronic, high and irregular fever. The infecting organism is Leishmania donovani which was first isolated by Sir William Leishman in 1900 from the spleen of an English soldier who had died of kala-azar in Calcutta (7). In 1903, Charles Donovan discribed the organism in smears he obtained from a splenic puncture of a patient in Madras, India (7). Ross was responsible for naming the species (56).

Kala-azar is endemic in many regions of Asia, Africa, Europe, South America and Central America. During the period 1890-1905 it was responsible for one of the major plagues of southeast Asia. Some authorities have proposed that this strain and some of the strains which cause skin leishmaniasis are all variants of the same species brought over from South or Central America to Asia, Africa and the Mediterranean region in the first century after Columbus' voyages (17). In its early stages, symptoms of the disease often resemble those of typhoid, malaria or dysentery. In some cases, the latter diseases actually precede kala-azar and promote its occurrence. There is evidence that most humans have a high natural resistance to kala-azar and that the parasites are held under control as a latent infection which erupts only when the body's defenses are lowered (21). Although the pathological changes which occur within the host vary slightly among the different geographical forms of the disease, many

of the manifestations of kala-azar remain the same and these have been summarized below.

When the leptomonal stage of L. donovani penetrates the skin of the host via the bite of the sandfly, the organisms are engulfed by macrophages. Within the cytoplasm of these cells the parasite undergoes metamorphosis into the leishmanial stage. The leishmania then multiply slowly and, thus, a quiescent period occurs (30). Even in the same endemic area, the time of incubation varies after the initial infection until actual symptoms of kala-azar occur. However, the usual duration is three to six months. Phagocytic activity increases as the number of leishmania increases. The marked increase in the number of macrophages causes at first a relative, and then an absolute monocytosis and neutropenia. Erythropoiesis is retarded, resulting in an anemia (29). As the total number of neutrophils decreases the hosts defense against pathogenic bacteria also decreases and concurrent disease may occur.

The onset of symptoms may be gradual or sudden. If it is sudden, kala-azar cannot be diagnosed on a clinical basis alone because the symptoms resemble those of other diseases. There is a severe initial fever, often preceded by rigor and vomiting. The fever is usually remittent, showing a double crisis during a 24 hour period similar to that of malaria. Unlike most fevers, there is no pronounced headache or mental lethargy and the appetite remains good even though there is a loss of weight. The weight loss is at first indiscernable due to edematous skin which is stretched and glistening. The fever usually subsides in two weeks. In the chronic form, these changes are not as

abrupt and they may occur over a 90 day period (30). Congestion is one of the causes of the hypertrophy of the spleen. Although a considerable part of the splenic substance is composed of parasites, reticuloendothelial proliferation also occurs (57). The splenic pulp is increased in amount and is very friable, with numerous infarctions. In the acute stage, the capsule is smooth, thickened, and nodular. However, in the chronic form of kala-azar, the splenic capsule is almost entirely cartilaginous. As the endothelial cells and blood vessels of the liver become invaded with organisms, enlargement of the liver also occurs until it protrudes below the costal region of the body.

Ulcerations are typically present in the gastrointestinal tract. These may be due to secondary infections or to the disease itself. There is a proliferation of reticuloendothelial cells in the duodenum and jejunum where the villi hypertrophy and are packed with parasites. The submucosa of the intestine is infiltrated with macrophages, particularly around Peyer's patches and the lymph follicles. The lymph nodes throughout the body become enlarged, especially those of the mesentery. The bone marrow begins to produce large numbers of macrophages, some of which become infected with parasites. In some cases there is so much destruction within the bone marrow that little blood forming tissue remains. The blood becomes acidic and is also characterized by an increase in some of the plasma globulins and a decrease in albumin. The decrease continues with marked emaciation and growing weakness is noted, but surprisingly, death is usually due to secondary infections rather than to kala-azar itself. In some acute cases death may follow in a few weeks, but in more chronic forms several years may

elapse. Faust and Russell (30) state that many asymptomatic cases probably occur, however, untreated patients with the above symptoms rarely survive.

Oriental sore, also called Delhi Boil, Alpeppo Button, Jericho Boil or Bouton de Briska, is a cutaneous leishmaniasis. The disease is caused by Leishmania tropica which was first observed by Cunningham in 1885 (57). Leishmania tropica is morphologically indistinct from L. donovani or the other leishmanias. Like L. donovani, it has endemic foci in Asia, Africa, the Mediterranean, and Latin America. However, L. tropica usually is endemic in different areas of the same region and the two are normally not found together. Although oriental sore was prevalent in Russia before the revolution it has been almost eradicated today. In an effort to eliminate malaria by the regular use of contact insecticides to kill the mosquitoes, the Russian people also killed the sandflies and, therefore, ridded their country of most dermal leishmaniasis (68, 71).

Two clinically different types of oriental sore have been described. One, called the "dry type," has a long incubation period, a long duration, and is characterized by dry papules that seldom rupture. Many leishmania can be found in the papules. The "wet type," in contrast, has a short incubation period, a short duration, and moist, draining ulcers. Immunity follows the first infection, hence children are the most frequently infected. Mothers often immunize their offspring by inoculating them with material from a dry sore. Infection of the "wet type" is often moderated by previous infection of the "dry type" (1).

After the leptomonad form is inoculated by the sandfly into the skin, the organisms are taken up by histiocytes in the dermis. Here, they transform into leishmanial forms and multiply until the macrophage is destroyed and new cells are infected. At this time, there is a proliferation of reticular cells which form a syncytium packed with parasites. This localized hypertrophy of the corneal layer erupts externally as a small, itching, red papule. As the disease progresses, the papule becomes covered with a blister-like layer of epidermis. An invasion of the tissue with lymphocytes, plasma cells, large mononuclear cells, and giant cells then occurs which results in a reduction in the number of parasites. This cellular proliferation is so dense that it often restricts nourishment of the epidermis and the tissue becomes necrotic and the covering becomes thick, brown and moist. As this crust is sloughed off, a clear exudate is discharged. No pus is present unless secondary infection occurs. At this time, scratching may spread the disease and result in the production of satellite lesions, but true metastasis rarely occurs (57).

After the surface of the lesion has been lost, the ulcer appears as a raised, crateriform lesion surrounding a depressed area. The margin of the lesion becomes indurated due to the invasion of fibroblasts. The leishmania become concentrated in the lymph nodes at the base of the lesion.

Although leukocytes are effective in localizing the parasites and preventing infection of the viscera, the open lesion often is

susceptible to bacterial invasion and may enlarge to several inches in diameter. If the disease continues uncomplicated, granulation slowly progresses and scar tissue developes. The scars often result in marked deformity if the lesion is on the face or other extremities.

Espundia, also known as Bubas Braziliana, Pian Bios, Forest Yaws, Bay Sore and Bosch Yaws, is caused by Leishmania brasiliensis. This is a disease of the mucosa which causes disfigurement, particularly of the face. One of the oldest of the leishmaniases, it has been recorded that Spanish conquistadors who entered South America developed lesions of the nares (17, 57). The geographic range of this disease extends from Brazil to Paraguay and northern Argentina. Cases have been reported in Africa, India and China, although it seems to be more prevalent in South America. In this disease the primary dermal lesion is formed in the same manner as that of oriental sore. However, with this parasite some of the organisms escape the macrophages to invade the mucosa. The viscera are not involved, however. The disease is thus differentiated by the frequent presence of numerous ulcers and by deterioration of the mucosa.

The secondary development of espundia, caused by metastasis of the parasites, results in ulcers in the nasal cavity, mouth, larynx, pharynx and vagina. These may appear before the dermal lesions have healed, but often they do not arise until several months or years have elapsed. These secondary lesions may be ulcerative or indurative. There is a marked increase in the endothelial cells of the region and an invasion of plasma cells and macrophages. This hyperplasia causes a blockage of the capillaries which not only restricts nutrition, but

also results in edema. Secondary infections due to bacteria, spirochetes and fungi often occur in the necrotic tissues. If left untreated, the entire surface of the palate, nasal septum and pinna of the ear may erode away. All bone and calcified structures are left intact, a characteristic that distinguishes espundia from syphilis (57).

There are several other variations producing tegumentary leishmaniasis which have slightly different manifestations. "Ulcera de los chicleros" is a tegumentary leishmaniasis found in Mexico, Honduras and Guatemala which is caused by Leishmania mexicana (69). Benign ulcers lead to erythematous lesions, particularly on the external ear. Rarely is there any invasion of the mucosa (59).

Treatments of choice for leishmaniasis are salts of antimony compounds, amphotericin B and cycloguanil pamoate (78). At present, the best method of control is thought to be eradication of the insect vectors (68, 71).

The problems confronting investigators of the immune response in leishmanial infections are common to those of workers involved with other infections produced by intracellular parasites, e.g., tuberculosis and histoplasmosis. An altered immune response would be expected in those diseases caused by organisms which parasitize the cells responsible for antibody production and, indeed, this is the case. In leishmaniasis, skin test reagents and several in vitro serological tests are available, but the results are often confusing (18, 34). The Montenegro reaction is a skin test of the tuberculin type (62). It employs killed promastigotes of any species of Leishmania as antigen. A positive test does not differentiate between past and present infec-

tion and there is no species specificity. Indeed, there is cross reactivity in patients with trypanosomal infections, tuberculosis and leprosy. Many normal individuals in the endemic areas will also show a positive test. The Montenegro reaction is probably most useful in certain cases of espundia where the organisms are difficult to isolate. A positive reaction is rarely seen in kala-azar. In serologic tests, false positives and false negatives, as well as many cross reactivities with seemingly unrelated organisms such as mycobacteria and treponemas seem to be the rule rather than the exception. These cross reactions become more than just a laboratory curiosity when one considers the fact that the endemic areas of the Leishmania are those where multiple infections due to several etiologies are not uncommon. Complement fixation (36, 38), agglutination (37), immunoelectroadsorption (60), and fluorescent antibody tests (15, 28, 79) have all been used for the diagnosis of leishmaniasis, but the cross reactivity and non-specificity mentioned above have been a serious problem. The complement fixation test has been of some diagnostic aid where endemic areas do not overlap (1, 2, 42).

In 1971, Heyneman (42) stated: "The antiserum-culture test is the only procedure at present available for determinig species relationship among the leishmaniae." This procedure, which has come to be known as the "Adler Test," was first reported by Noguchi in 1924 (31, 63, 64) and later revised by Adler in 1963 (3). Locke's solution (85) containing normal serum and agar is dispensed in 2 ml amounts in Kahn tubes. Leishmania antiserum is then added to the tubes to give final concentrations of 1:5 to 1:20,000. The tubes are then inoculated with

heavy suspensions of homologous or heterologous strains of Leishmania. At intervals of several days, cultures with and without antiserum are examined microscopically in wet mount preparations. The method requires careful comparative observations of control cultures versus cultures containing antiserum. The final titer is read as the lowest dilution of immune serum which causes no agglutination of the flagellates. Higher concentrations of heterologous antisera are required to cause the same effects seen with homologous antiserum in lower dilution (3). This method has been used more recently to evaluate fifty isolates of Leishmania spp. (72).

The development of immunity in man is similar in diseases caused by all species of Leishmania. Reinfection is very rare if spontaneous cure occurs. Reinfection has been known to occur if the normal course of the disease is not completed, as in the case following chemotherapy or surgery (49).

In kala-azar, the question arises as to how the parasite can persist in the face of high levels of gamma globulin and immunologically competent cells. Bray (11) suggested that this may be due to in vivo antigenic changes in the organism. This is known to exist in species of Plasmodium and Trypanosoma. Bray was unable to demonstrate antigenic changes, but admitted that his system for detecting them was not ideal. After culture on artificial media, his strains demonstrated no altered capacity for infection. Such alteration would have implied some antigenic change.

Adler (1) has divided the immunological and serological aspects of leishmaniasis into five general categories.

1) Methods of identifying morphologically indistinguishable species of Leishmania. Because strains of Leishmania causing human disease cannot be differentiated on a morphological basis, considerable effort has been made to establish serological methods of identification. Although these methods show some promise, they have not been as easy to standardize as would be desired. For this reason, diseases caused by Leishmania are still diagnosed on the basis of clinical manifestations and epidemiology (2, 9). Subspecies designations have often been based on the same parameters (8).

Animal susceptibility has proven to be equally confusing as a means of identification. Adler (2) believes that this confusion is due to two factors, a) the loss of infectivity by some strains after periods of culture and b) the observation that cutaneous and mucocutaneous leishmaniasis of South and Central America are caused by several distinct species with different host susceptibilities. Based on clinical observations, host specificity and geographic distribution, several workers have concluded that L. brasiliensis is a mixture of species (32, 44, 67, 70).

In 1964, Adler (2) stated: "In the absence of morphological differences between alleged species of Leishmania (except in the case of L. enrietti in the guinea pig), the limitations of clinical data and the variable results of animal inoculations, attempts to differentiate Leishmania spp. by serological methods are the only resort." The first report of serological methods for identification of Leishmania was that of Noguchi in 1924 (63, 64). The method was later extended and perfected by Adler (3). This antiserum-culture method has

been discussed previously in this chapter.

Other serological methods have been used with varying results. Cross reactivities are common. Group specific antigens mask species specific antigens. Khodukin et al (50) were able to identify only L. brasiliensis by the complement fixation technique. Cunha (26) concluded that it was not possible to differentiate the various Leishmania on the basis of agglutination tests due to the presence of a common antigen in all species. More recent workers reported their ability to differentiate organisms isolated from post-kala-azar dermal leishmaniasis and active kala-azar using absorption and agglutination tests (77).

Recently, a hemagglutination test has been developed by Bray and Rahim (16) which employs sheep erythrocytes sensitized with polysaccharide extracts of Leishmania.

Methods utilizing immunoelectrophoresis and gel diffusion are gaining increasing attention and appear to hold some promise. Preliminary work has been reported (76) which indicates a possible means of identification based on diffusion of soluble metabolic factors of Leishmania excreted in vitro against specific antisera. Other work in these areas is discussed in later pages of this chapter.

2) Cellular and humoral factors in leishmanial infections and the sequence of these events in the course of infection. Histiocyte proliferation and secondary round cell and plasma cell infiltration are necessary for spontaneous cure in oriental sore; yet these same responses, when they are seen on a general scale in kala-azar, rather than localized as in oriental sore, rarely cause spontaneous cure even though high levels of circulating antibody may be detected (2).

Heyneman (42) suggests that immunity to visceral leishmaniasis lies in the skin, since the host is protected from reinfection by a sandfly bite. However, these cell-bound antibodies have no effect on the infection once it has passed the dermal barrier. This suggestion is supported by the findings of Manson-Bahr (58). He noted that, in East African kala-azar the leishmanin skin test reaction became positive much sooner if the organisms did not invade the viscera, and that the reaction became positive only after cure. The same was found to be true with forms of leishmaniasis which do not invade the viscera. These findings confirm the skin as the main line of defense against leishmaniasis. If the infected macrophages escape the dermal barrier, the parasites do not come in contact with immunologically competent cells that could produce specific humoral antibody. Heyneman suggests, further, that this may be the key to the mass destruction of cartilagenous tissues in later stages of espundia, when no parasites can be found. The parasites may have altered the macrophages and other tissue components to the point that they, too, become antigenic. This raises the possibility of an autoimmune mechanism in the final, destructive stages of espundia.

3) Cross immunity between species. This would seem to be promising for the production of immunizing agents, since many species exist which are not pathogenic for man, but share antigens with the human pathogens. To date, this has not proven to be particularly significant except with L. tropica, which protects against L. mexicana. However, the reverse is not true (6, 52, 53). Southgate (82) has reported a possible correlation between immunity to kala-azar and transient skin

infections with L. adleri in Kenya.

In 1941, Pessoa (67) reported protection against L. tropica infection through immunization with killed leptomonads. This finding has since been refuted (49). Protection against L. donovani infection via immunization with a ground squirrel strain that does not invade the viscera was reported by Manson-Bahr (56, 58) in a study of a small number of soldier volunteers. He later found this method to be unsatisfactory in field trials involving 1500 persons in the endemic focus where natural infection was used as a challenge. This discrepancy in results may be due to the fact that experimental inoculation of virulent organisms does not always produce disease even in controls or "unprotected individuals" (3).

Functional immunity which will protect the face from oriental sore can be induced by introducing the organisms into an unexposed site (2). Kellina et al (49), however, emphasized the requirement that a highly virulent infection be allowed to proceed through its full course in order that it afford protection against an equally virulent challenge.

4) Delayed hypersensitivity in cutaneous and muco-cutaneous leishmaniasis during infection and after spontaneous cure. As discussed previously, Manson-Bahr (56) noted the development of a positive leishmanin skin test reaction after cure in East African kala-azar and much earlier if there were no invasion of the viscera. This is not true in other forms of disease, such as Indian kala-azar, where visceral involvement is common and evidence of a primary lesion is rare or absent. These observations strengthen the view that skin involvement is necessary for the development of delayed hypersensitivity and subsequent

immunity from reinfection.

5) The efficiency of the immune mechanism in light of the continued and increased production of gamma globulin. Positive complement fixation tests have been reported in patients with kala-azar, but the antibody titers do not correlate with the high levels of gamma globulin being produced. In South American kala-azar, it has been demonstrated that there is no correlation between the levels of serum globulins and specific antibody. Individuals with high serum globulin levels may have low complement fixing antibody titers. Adler (2) observed that serum globulins have no protective value and that, although the immunologically competent cells are stimulated by the parasites and produce high levels of antibody, this antibody is not specific for the organisms. The avidity of this antibody needs further study.

That the Leishmania are highly complex antigenically is unquestionable. Separating out the strain, species and even genus specific antigens would have value from many aspects. Identification of specific disease etiologies could be accomplished with a high degree of reliability. Skin test antigens for each disease would be of use in diagnosis and epidemiologic problems could be greatly lessened. The value of specific antigens to immunotherapy is obvious. Many workers have examined this aspect of leishmanial immunology, approaching the problem in a variety of ways.

Complement fixation, indirect hemagglutination, fluorescent antibody, gel diffusion and antiserum-cultures have all been evaluated. All of these methods have some value in diagnosis and organism identification, however, each has its own limitations. Non-specificity, cross-

reactivity, and lack of practicability continue to be the most serious problems (8, 10, 13, 14, 19, 22, 25, 41, 61, 66).

As stated earlier, the antiserum-culture methods probably allow for the greatest degree of specificity, but these examinations require highly skilled investigators and several months. They are not likely to be of practical value outside the clinical laboratory.

Immunoelectrophoresis and gel diffusion methods, in general, have shown some promise as means of attacking the problem of cross reactivity among the Leishmania. These investigations are still in the preliminary stages and many of the problems are just now being identified. Very little has been done to separate the antigenic components or isolate and identify specific antigens.

One of the earliest demonstrations of the antigenic composition of Leishmania using gel diffusion and immunoelectrophoresis was that of Garcia in 1965 (35). He was able to show four distinct precipitin bands with L. tropica and antiserum from immunized rabbits. One of these components was heat stable at 60 C and to boiling for one hour. Prior to this, Chaffee (20) had reported cross reactivity between L. tropica, L. donovani and L. brasiliensis using the gel diffusion technique. In 1966, Bray and Lainson (14) attempted to confirm and extend this work. They demonstrated shared antigens among all strains, but found each strain to be antigenically distinct. L. donovani shared three or more antigens with other strains. In the same year, Chaves and Ferri (22) used the sera of twenty patients suffering from visceral leishmaniasis to demonstrate lines of precipitation by gel diffusion and immunoelectrophoresis. They also confirmed the increase in IgG

(7S) and IgM (19S) immunoglobulins, which returned to normal values after treatment of the patients with glucantime.

Also in 1966, Schneider and Hertig (75) performed gel diffusion tests with a wide variety of American Leishmania strains and rabbit antisera to the leptomonads. They found precipitin bands in all homologous systems and in some heterologous systems as well. They were able to determine the presence of two distinct serotypes of Leishmania from these Panamanian strains, but discounted the use of specific names for various isolates of Leishmania. Their conclusions were supported by unpublished work of Adler in Bray (12). In more recent work, it has been demonstrated that L. donovani extract gives rise to nine distinct precipitin bands on immunoelectrophoresis (15, 24). These workers also reported the presence of an exoantigen in the culture medium. In 1969, Crook et al (25), working in this laboratory, reported 11 precipitin bands by immunoelectrophoresis using soluble extracts of L. mexicana as antigen and homologous antisera from rabbits.

The studies with gel diffusion and immunoelectrophoresis give strong indication that these methods may well be the key to unraveling some of the problems of leishmaniases and the organisms causing them. It was with this possibility in mind that the present study was initiated. The objectives of the present study were to: 1) prepare suitable soluble antigens from the promastigote stage of L. donovani (two isolates), L. tropica, L. mexicana and L. brasiliensis (two strains), 2) immunize rabbits with each of the antigens in order to obtain high titer, specific antisera, and 3) by the gel diffusion and immunoelectrophoresis techniques, test the antigens with homologous and

heterologous antisera in an attempt to identify each species by its typical precipitin pattern. It was hoped that, by these techniques, species and strain specific antigens might be detected which could be useful in organism identification.

CHAPTER II

MATERIALS AND METHODS

Organisms

The organisms employed in this study were obtained from the following sources:

Leishmania mexicana D-88 was received from Dr. H. Robert Wilson, Bureau of Biological Research, Rutgers University, in 1965. This strain was originally isolated in 1958 by Dr. P. C. C. Garnham from a human case of leishmaniasis in British Honduras. In Dr. Wilson's laboratory, the strain was maintained in hamsters. In this laboratory, the organism has been maintained in Tanabe's medium since its receipt.

Leishmania brasiliensis Bell was received in 1970 from the laboratory of Dr. Emily Bell, University of Cincinnati. In Dr. Bell's laboratory, the organism was maintained on a diphasic medium, N.I.H. blood agar with an overlay of Locke's solution. In this laboratory, the organism has been transferred bi-weekly in Tanabe's medium.

Leishmania brasiliensis CDC was sent to us by Ms. Lois Norman, Center for Disease Control, Atlanta, Georgia. The strain was of Guatamalan origin and had been maintained at CDC since 1951. In this laboratory the organisms were maintained in Tanabe's medium.

Leishmania donovani 2S, Leishmania donovani CDC and Leishmania

tropica were obtained in 1968 from Dr. John Janovy, University of Nebraska. In Dr. Janovy's laboratory, and in this laboratory, the organisms were maintained in Tanabe's medium.

Maintenance of Stock Cultures

Stock cultures were maintained in Tanabe's medium (47). They were incubated at 28 C and transferred bi-weekly. Tanabe's medium was prepared by mixing sterile, defibrinated rabbit blood with an equal volume of sterile distilled water and freeze-thawing the mixture once. Defibrinated rabbit blood was prepared by agitating aseptically drawn blood with sterile glass beads (6mm) in a sterile, 125 ml erlenmeyer flask until a fibrin clot formed around the beads. Distilled water for this, and all other media and reagents was first prepared in a stainless steel still, demineralized (Crystalab Deiminizer, model CL-5, using Deeminite L-10 as the ion exchange resin), and then distilled in a glass still.

The sterile mixture of defibrinated rabbit blood and water was then added to an equal volume of a sterile, aqueous solution containing 1.3% sodium citrate and 1.1% sodium chloride. This resultant mixture was dispensed in 2 ml volumes in sterile, screw-cap tubes and stored at 4 C until used. Transfers of cultures were made by withdrawing approximately 0.5 ml of medium from a growing culture with a capillary pipet and placing it into a tube of sterile medium that had been warmed to room temperature.

Batch Culture

Twenty liter carboys and six liter flasks were used for batch culture of the organisms. Each vessel was fitted with a rubber stopper containing one hole to accommodate a four inch needle sterilizing tube. A #12 rubber stopper was used for each carboy and a #10 stopper for each flask. The bottom of the needle sterilizing tube was removed so that it was open at both ends.

One end of a three foot length of dialysis tubing (Curtain #077-040, 0.00072 inch thickness, 1 7/8 inch inflated diameter) was affixed with heavy cord to the constriction in the needle sterilizing tube such that materials introduced into the tube would enter the dialysis tubing. The other end of the dialysis tubing was knotted and tied with cord to form a bag. A small slit was made in the top of the dialysis tubing to allow for pressure equalization when liquids were introduced into the bag.

Locke's solution (85), referred to as "Solution A" in Table 1, was placed in the vessel, 4 liters for each twenty liter carboy or 2 liters for each 6 liter flask. A mixture of beef extract, yeast extract, peptone and calcium chloride, referred to as "Solution B" in Table 1, was placed inside the dialysis bag, 100 ml for each liter of solution "A" in the vessel.

The needle sterilizing tube was then fitted with a cotton plug. A cap to cover the top of the vessel was fashioned from aluminum foil. This apparatus was then autoclaved at twenty pounds pressure, 121 C, with time varying according to the volume of solution. After

TABLE 1.--Dialysate medium for growing Leishmania.

Solution "A" (Outside the dialysis bag)		Solution "B" (Inside the dialysis bag)	
KCl	0.07 g	Beef Extract	25 g
NaCl	0.80 g	Yeast Extract	15 g
KH_2PO_4	0.10 g	Peptone	40 g
Dextrose	0.08 g	$CaCl_2$	0.35 g
Distilled H_2O	1000 ml	Distilled H_2O	1000 ml

autoclaving, the containers were allowed to cool and were incubated at room temperature for three days to check for sterility. See photograph in Figure 1. To the solution inside the bag was then added pooled, freeze-thawed, heat inactivated (56 C for 30 min) human blood in the ratio of 100 - 125 ml per liter of solution in the vessel.

The inoculum consisted of 50 ml per liter of a full grown culture of the organism. Cultures to be used for inocula were grown in 1 liter flasks. Inoculation was made by lifting the stopper slightly and introducing the organisms into the solution in the vessel. Depending on the density of the inoculum, growth usually reached a peak in six to ten days. Incubation was at room temperature.

Preparation of Antigen

The organisms were harvested by centrifugation at 7000 X G and 5 C in a Sorval centriguge, model RC2-B, and using the Sorval KSB continuous flow system (Ivan Sorval Inc. Norwalk, Conn.). The flow rate was adjusted to approximately 2 liters per hour. The packed cells were then washed three times in phosphate buffered saline (PBS) and frozen at -20 C until sufficient material was collected. The PBS was prepared as follows.

NaCl	8.00 g
KCl	0.20 g
$Na_2HPO_4 \cdot 12\ H_2O$	2.89 g
KH_2PO	0.20 g
H_2O	1000 ml

The solution was autoclaved for 15 min at 121 C and 15 pounds pressure.

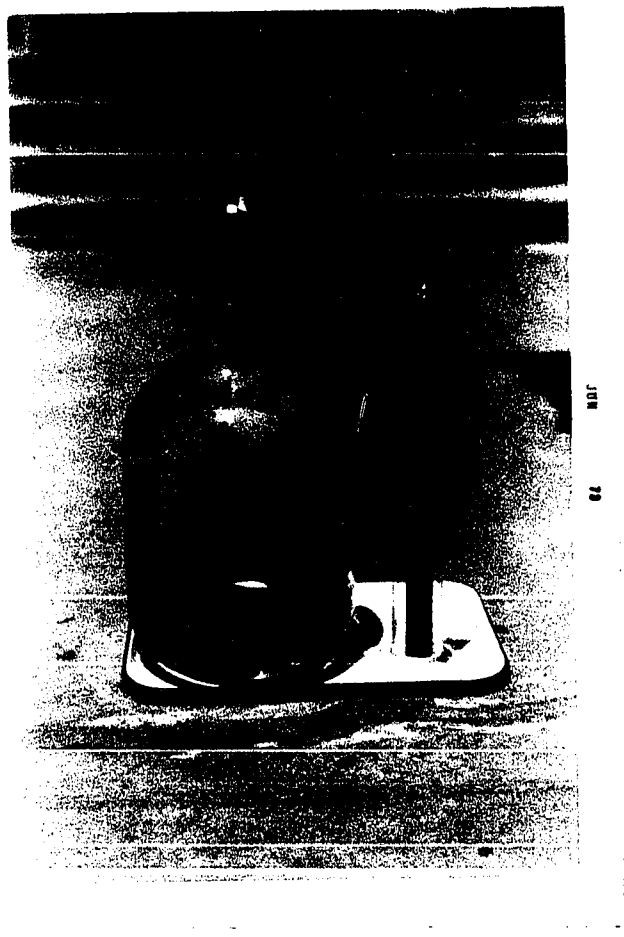


Figure 1 Dialysate apparatus for growing Leishmania in batch culture.

Lysis of the cells was initiated by freeze-thawing twice at -20 C and room temperature in a five fold volume of PBS. The process was completed by sonication for 10 min at setting four using a Branson Sonifier, model S-75, (Heat Systems Inc., 38 East Mall, Plainview Long Island, N.Y.) with an ice bath surrounding the cup.

After sonication, the suspension was centrifuged at 10,000 X G for 20 min at 5 C. The supernatant fluid was dialyzed against three changes of triple distilled water at 5 C for 24 hours each time. The material was then lyophilized using the Virtis lyophilizer, model 10-100D. The resultant fluffy, buff colored material was stored at -20 C and used later as soluble antigen.

Nitrogen Determination

Kjeldahl nitrogen determinations were performed on each of four antigen preparations according to the method of Lang (54) as follows: Antigen samples containing 1.5 - 2.7 mg dry weight of material were placed in digestion tubes together with 0.20 ml of digestion mixture (40 g potassium sulfate, 2 ml selenium oxychloride, and 250 ml water), 0.2 ml of 30% hydrogen peroxide, and distilled water to a volume of 2 ml. Standards were prepared by dissolving 1.179 g of anhydrous ammonium sulfate in 250 ml of 0.2 N sulfuric acid to give a final concentration of 1000 µg nitrogen per ml. The standard solution was then diluted to obtain samples containing 200, 150, 100 and 50 µg of nitrogen per ml. The standards were treated in the same manner as the unknown samples.

Digestion of the samples was conducted over a flame and was usually complete in two hours. The tubes were then removed from the

flame, cooled to room temperature, and placed in an ice bath. Next, the volume was adjusted to 10 ml with distilled water and three ml was transferred to another tube. To this tube was added 1 ml of distilled water and 2 ml of Nessler's reagent. The solutions were mixed and allowed to stand for 10 min. Nessler's reagent was prepared by dissolving 68 g of Nessler's granules (Tenso-Lab, Irvington-on-Hudson, N.Y.) in 100 ml of distilled water, adding 850 ml of 10% sodium hydroxide solution, and adjusting the final volume to one liter with distilled water.

Samples were read at 500 mμ using a Bausch & Lomb Spectronic "20" (Bausch & Lomb, Rochester, N.Y.).

Preparation of Antiserum

Twenty young adult, male, albino rabbits were purchased from Pel-Freez (Pel-Freez Biologicals Inc., Rogers, Arkansas 72756). Since four different antigen preparations were to be used, the animals were divided into four groups and marked by tatooing in the right ear.

The schedule of immunization, modified from that of Crook *et al* (25), is shown in Table 2.

Intravenous injections were made in the marginal ear vein and the antigen was suspended in PBS. Subcutaneous injections were made in the flanks and the nape of the neck and the antigen was suspended in PBS and emulsified in Freund's incomplete adjuvant (FIA) (Difco #0639-60), using a Sorval Omnimixer with a micro attachment (Ivan Sorval Inc., Norwalk, Conn.). The cup was surrounded by an ice bath during the emulsification procedure to prevent any inactivation due to the generation of heat. Emulsification was complete in one to two minutes.

TABLE 2.--Schedule of immunization.

Day	Total Dose mg (dry weight) of antigen	Diluent ml	Injection Sites	Route of Injection and Amount
0	2.0	1.0 PBS[a] & 1.0 FIA[b]	Both flanks Nape of neck	Subcutaneous-0.5 ml per flank, 1.0 ml in neck
7	2.0	0.5 PBS	Marginal ear vein	Intravenous-0.5 ml
14	2.0	0.5 PBS & 0.5 FIA	Both flanks	Subcutaneous-0.5 ml per flank
21	2.0	0.5 PBS & 0.5 FIA	Nape of neck	Subcutaneous-1.0 ml
55	2.0	0.5 PBS & 0.5 FIA	Nape of neck	Subcutaneous-1.0 ml
97	2.0	1.0 PBS & 1.0 FIA	Both flanks	Subcutaneous-1.0 ml per flank
187	5.0	0.5 PBS	Flank	Subcutaneous-0.5 ml
211	5.0	0.5 PBS	Flank	Subcutaneous-0.5 ml
232	5.0	0.5 PBS	Flank	Subcutaneous-0.5 ml

[a]PBS - phosphate buffered saline

[b]FIA - Freund's incomplete adjuvant

Prior to immunization, all rabbits were bled by cardiac puncture or from the median ear artery to obtain a control serum sample. At various intervals after immunization the animals were again bled. One to five ml of blood was taken each time and allowed to clot. The serum was withdrawn and stored at -20 C until used. The samples were not pooled.

Immunoelectrophoresis

Immunoelectrophoretic analysis was performed using LKB 6800A immunoelectrophoresis equipment (LKB-Produkter AB, Box 12220, Stockholm 12, Sweden) according to the method described in the manual for this equipment, with certain modifications described below. The technique described in the manual refers to the modifications introduced by Scheidegger (74) of the method first described by Grabar and Williams (39).

One bottle of Veronal buffered salts (LKB 3276-VB10) was dissolved in 1500 ml of distilled water (pH 8.6, ionic strength 0.1). Veronal buffered salts contain 0.05 M sodium diethyl barbiturate, 0.01 M diethyl barbituric acid and 0.05 M sodium acetate. This volume of solution provided a sufficient quantity for three operations of the equipment and for the preparation of the agar required. The buffer was stored at 5 C until it was used. Special Agar-Noble (Difco #0142-01) was used for coating the slides. For this purpose, 1 part of agar was mixed with 25 parts of Veronal buffered salts solution and 75 parts of distilled water. Merthiolate (1 : 10,000) was added as a preservative.

Precleaned glass slides (LKB 6890-03), 76 X 26 X 1 mm, were

used. A 1 : 10 dilution of the agar mixture was painted onto the slides and allowed to dry completely. After the slides were completely dry, the 1% agar solution was added in the amount of 10 ml per side of the slide holder (i. e., per three slides). The process was repeated until each holder was filled. When the agar had solidified, the slide holders were placed in a moist chamber and held at 5 C for at least 12, but not more than 72 hours.

Wells and troughs were cut in the agar using a gel punch (LKB 6808A). Various patterns were evaluated, however, most determinations were made with a trough (1 X 65 mm) in the center of the slide and wells (1 mm diameter) 10 mm apart on either side of the trough. Templates (LKB 6811B) were used for this purpose. The wells were placed off-center toward the negative pole of the apparatus (15 mm from the end of the trough), since most of the components were found to migrate to the anode. This arrangement of trough and wells was used for analyzing each antiserum with its homologous antigen. Other arrangements, such as two troughs and three wells, were used in the comparison studies and will be discussed more fully later.

The plugs of agar were removed from the wells and they were filled with 5 - 10% antigen solution prepared in PBS. Higher concentrations of antigen did not appreciably alter the results. The antigen was then electrophoresed at 50mA, at 240 volts, for 1 hour using the LKB power supply (LKB-Type 3290B). After electrophoresis the slide trays were held at 5 C for a few minutes to allow cooling so the agar could be more easily removed from the troughs. Undiluted antiserum was then placed in each trough and the trays were then stored at room

temperature in the moist chamber until precipitin bands could be visualized. This usually required 36 - 48 hours. After the precipitin bands were formed, the slides are immersed in 1% sodium chloride solution for six hours, then in fresh 1% sodium chloride solution for 16 hours, and finally in distilled water for one hour. Next, the slides were covered with filter paper strips to insure even drying, which was usually complete in 48 hours at room temperature. After drying, the slides were stained in Amido Schwartz 10-B. The staining solution was prepared by dissolving nine grams of Amido Schwartz 10-B in 1500 ml of rinsing solution (45 parts methyl alcohol, 10 parts glacial acetic acid, and 45 parts distilled water). Staining was accomplished in five to ten minutes and the slides were then rinsed in four changes of the rinsing solution. Slides were observed immediately and drawings of the precipitin bands were made.

In certain of the studies, heterologous antisera were reacted against a single antigen and bands of identity were sought. For this purpose, two troughs were cut on either side of a well. One trough was then filled with homologous antiserum and the other trough with heterologous antiserum.

Immunodiffusion (65)

Microimmunodiffusion studies were performed on microscope slides using the same agar as that used for immunoelectrophoresis. A gel cutter (LKB 6865A) was used which provides four wells, 5 mm from and surrounding a center well. All wells were 2.5 mm in diameter. A 10% antigen solution in PBS and undiluted antisera were used. Precipitin

bands were formed in 36 - 48 hours. The agar was then allowed to dehydrate at room temperature after wetted filter paper strips were applied. The slides were stained with Amido Schwartz 10-B. Various combinations of antigens and antisera were compared using this arrangement, and will be discussed in the Results Section.

CHAPTER III

RESULTS

Batch Cultures

Because of the occasional tendency of *Leishmania* to form clumps or rosettes in liquid culture, it was not feasible to make accurate counts of the organisms in batch cultures. Turbidity of the medium was used as an index of the amount of growth. Cultures usually yielded between 10^5 and 10^7 organisms per ml. This method proved quite satisfactory for growing all species and strains of *Leishmania* used in this study except *L. brasiliensis*. Contamination was sometimes a problem because no antibiotics were used in the medium.

Preparation of Antigen

Microscopic examination of the cultures revealed only flagellated forms of *Leishmania*. Although older cultures will occasionally show intermediate, aflagellated forms, the batch cultures were not incubated long enough for the development of such forms.

The results of the micro-Kjeldahl nitrogen determinations given on the next page are expressed as µg of nitrogen per mg of lyophylized antigen material.

L. donovani CDC . . . . . 71.3 µg N/mg

L. donovani 2S . . . . . 60.0 µg N/mg

L. tropica . . . . . 55.5 µg N/mg

L. mexicana . . . . . 60.7 µg N/mg

Preparation of Antisera

The immunological responses of individual rabbits were quite variable, however, no rabbit failed completely to respond to antigenic stimulation. Some animals expired during the course of the study, but each group contained a minimum of three animals at the end of the study (day 253).

Immunoelectrophoresis

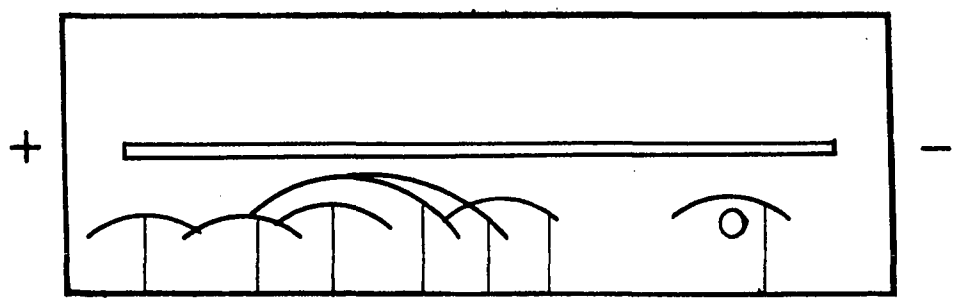
The results of immunoelectrophoretic studies of the four different antigens and their homologous antisera are presented in tabular form. These tables have been divided into four groups, one group for each antigen. Each table represents the results from a single rabbit in that group throughout the study, and a composite table is included to compare all rabbits in a group on any given day. The drawings of the immunoelectrophoresis slides are $1^{1}/_{2}$ X scale and every effort was made to depict the precipitin bands in the exact relationship to each other as they were on the original slides.

The bands are numbered in reference to the composite slide which shows all bands within any group. For this reason, no animal within a group will show all possible bands seen in that group. In order to conserve space, certain days are not shown in the tables, but

the days shown are a very close representation of the overall results from day 0 of the immunization schedule to day 253 when the study was terminated. Controls consisted of various high titer sera reacted with 1X and 20X concentrated medium that had been dialyzed against pooled, postdated human blood. All controls were negative.

Tables 3 through 7 contain the results obtained with sera from four rabbits immunized with L. donovani 2S antigen. Examination of Table 7 suggests the presence of a total of eleven different bands against this antigen in these four animals. The earliest response was seen with rabbit #04, three weeks after the first injection. Two weeks later, rabbits #03 and #05 showed their first response, and in the eighth week the last rabbit, #01, began to show detectable precipitins. The earliest bands to appear in all animals in this group were bands six and ten, with band two following soon thereafter. These three bands generally persisted throughout the study, except in rabbit #03 where band two appeared only once, on day 194. Band seven was consistent in only one rabbit, and appeared but briefly, or not at all, in the others. Bands one, three, five, and nine were seen in only one rabbit each, and not consistently. Band eight appeared in two rabbits, but it also waned. Table 7 is a condensation and comparison of the information shown in Tables 3 through 6. The appearance of a number in a column is indication of the presence of the band corresponding to that number on the drawing. Booster injections later in the schedule stimulated additional bands in some cases or caused the reappearance of bands that had disappeared.

TABLE 3.--Immunoelectrophoresis of L. donovani 2S antigen against homologous antisera from rabbit number 01.

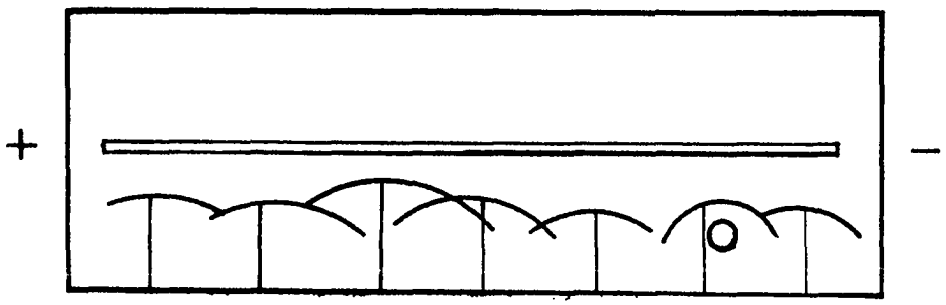


DAY	1	2	4	5	6	7	10
[a]→14							
→21							
34							
→55					+[b]		
62	+	+		+	+		+
69		+			+		+
→76					+		
125	+	+	+	+	+	+	
156					+		
→180		+			+		+
194		+	+		+		+
201		+	+	+	+		+
→211		+	+	+	+		
218		+		+	+		+
→225		+		+	+		+
239	+	+	+		+		+
246		+	+	+	+		+
253	+	+	+		+		+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 4.--Immunoelectrophoresis of L. donovani 2S antigen against homologous antisera from rabbit number 03.

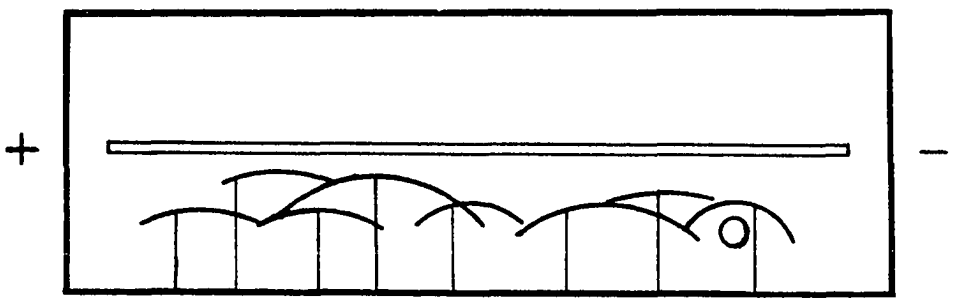


DAY	2	4	6	7	8	10	11
[a]→14							
→21							
34			[b]+	+		+	
→55			+	+		+	
62		+	+	+		+	
76		+	+			+	
→ 97		+	+			+	
125			+	+		+	
156		+	+	+		+	
180		+	+	+		+	
→194	+	+	+	+		+	
201		+	+	+			
→211			+			+	
218		+	+	+	+	+	+
225		+	+	+		+	
→239		+	+	+		+	
246		+	+	+		+	+
253		+	+	+		+	+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 5.--Immunoelectrophoresis of L. donovani 2S antigen against homologous antisera from rabbit number 04.

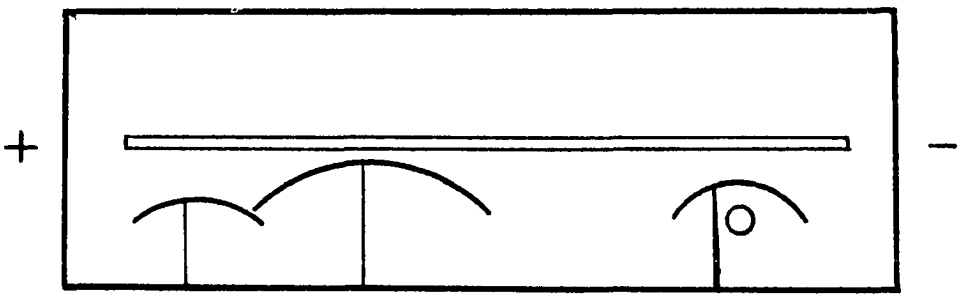


DAY	2	3	4	6	7	8	9	10
[a]→14								
→21				+ [b]				+
34	+		+	+				+
→55	+			+				+
62	+			+				+
76	+			+				+
→97	+			+				+
125	+			+				+
156	+			+	+			+
→180	+			+				+
194	+		+	+	+	+	+	+
201	+			+		+	+	+
→211	+	+		+		+		+
218	+			+				+
→225	+			+				+
239	+			+				+
246	+	+	+	+				+
256	+		+	+				+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 6.--Immunoelectrophoresis of L. donovani 2S antigen against homologous antisera from rabbit number 05.



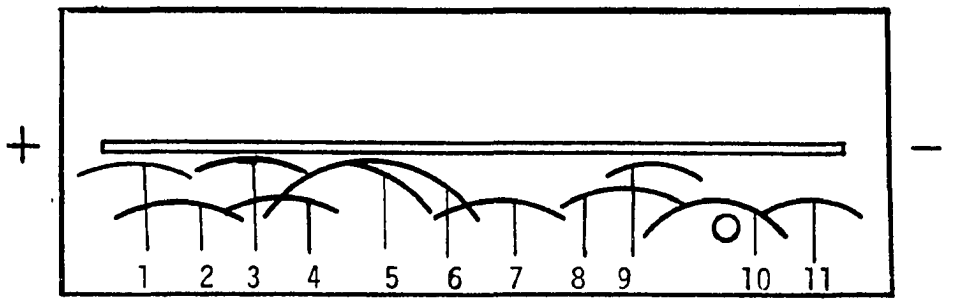
DAY	2	6	10
→[a]14			
→21			
34	+[b]	+	+
41	+	+	+
48	+	+	+
→55	+	+	+
62	+	+	+
69	+	+	+
76	+	+	+
83	+	+	
→97	+	+	+
104	+	+	+
111	+	+	
125	+		
146	+		
156	+	+	
166 †[c]	+	+	

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

[c] Cross indicates mortality.

TABLE 7.--Immunoelectrophoresis of L. donovani 2S antigen against homologous antisera. Composite representation of all precipitin bands from all rabbits.



Rabbit Number

DAY	01	03	04	05
→[a] 14	0	0	0	0
→21	0	0	6,10[b]	0
34	0	6,7,10	2,4,6,10	2,6,10
→55	6	6,7,10	2,6,10	2,6,10
62	1,2,5,6,10	4,6,7,10	2,6,10	2,6,10
69	2,6,10	4,6,10	2,6,10	2,6,10
→76	6	4,6,10	2,6,10	2,6,10
125	1,2,4,5,6,7	6,7,10	2,6,10	2
156	6	4,6,7,10	2,6,7,10	2,6
→180	2,6,10	4,6,7,10	2,6,10	†[c]
194	2,4,6,10	2,4,6,7,10	2,4,6,7,8,9,10	
201	2,4,5,6,10	4,6,7	2,6,8,9,10	
→211	2,4,5,6	6,10	2,3,6,8,10	
218	2,5,6,10	4,6,7,8,10,11	2,6,10	
→225	2,5,6,10	4,6,7,10	2,6,10	
239	1,2,4,6,10	4,6,7,10	2,6,10	
246	2,5,6,10	4,6,7,10,11	2,3,4,6,10	
253	1,2,4,6,10	4,6,7,10,11	2,4,6,10	

[a] Arrows indicate days of inoculation.

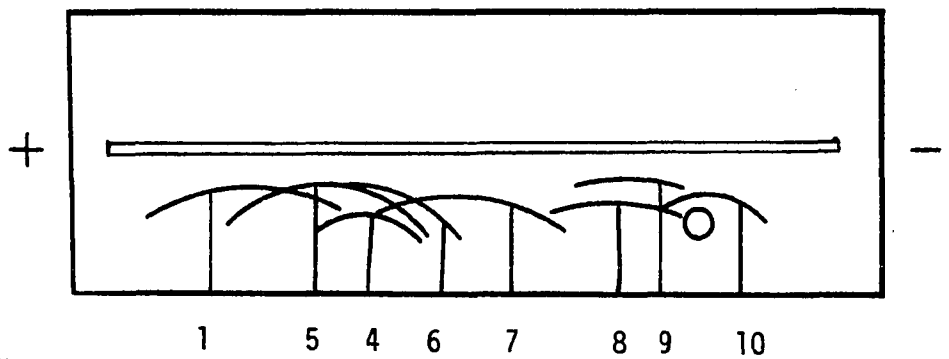
[b] Numbers refer to presence of that band on the indicated day.

[c] Cross indicates mortality.

Tables 8 through 12 concern animals that received L. donovani CDC as antigenic stimulation. As in the proceeding group, eleven precipitin bands can be seen. Bands six through eleven appear to correspond very closely to those seen in animals immunized with L. donovani 2S. Bands one through five correspond more closely than preliminary observation would suggest. If one recognizes the fact that band one of L. donovani 2S is not seen in L. donovani CDC, then the remaining bands are more closely similar even though their numbers are different. With L. donovani CDC, a response was first noted on day fourteen in two rabbits, and followed more slowly in the other two animals. Bands six and seven seem to be the most persistent and were the first to appear, but bands one and eight were seen fairly consistently also. Rabbits #1 and #6 showed only two bands until they were boosted on day 187, when they responded with five to six bands.

Results obtained with rabbits receiving soluble extract of L. mexicana are seen in Tables 13 through 18. Two of the rabbits (#11 and #15) died rather early in the study, on day 166. One of these, #15, had been exhibiting four to six bands, which was maximal for this group and was not subsequently reached with any other animal, even after boosting. Response began on day 14 in one animal, (#14), and was present in all animals by day 34. The first bands to appear were bands four and eight. They persisted through the end of the study. Band four corresponds in position to band six in both groups of animals which had received L. donovani. Band eight was at the well, and corresponds in position to band ten in the L. donovani animals. Bands two, three, five and seven appeared in more than one animal, but were not

TABLE 8.--Immunoelectrophoresis of L. donovani CDC antigen against homologous antisera from rabbit number 1.

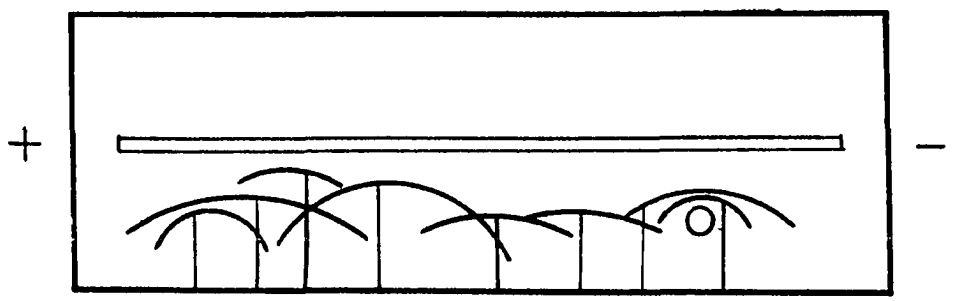


DAY	1	5	4	6	7	8	9	10
[a]→14				+[b]				
→ 34								
→55				+				
76				+				
→ 97				+	+	+		
111				+	+			
125								
146				+	+			
156				+	+			
166				+				
180				+				
→ 201	+			+	+	+	+	+
→211	+			+	+			+
218	+			+	+			
225	+		+	+	+			
→ 239		+		+	+			
246		+	+	+	+			
253	+			+	+	+		

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 9.--Immunoelectrophoresis of L. donovani CDC antigen against homologous antisera from rabbit number 3.

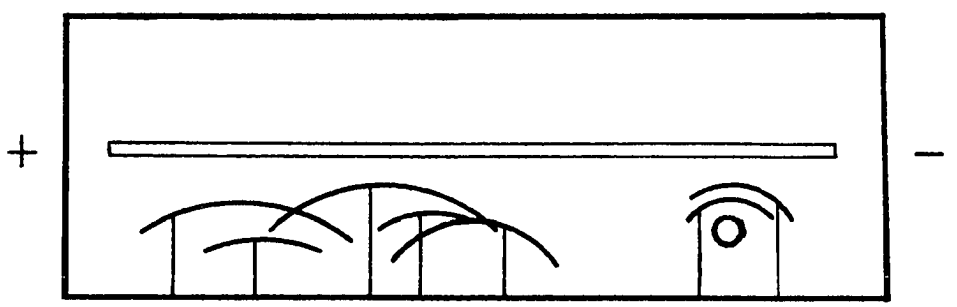


DAY	2	1	3	6	7	8	9	10
→[a] 14		+[b]		+	+			
→ 34				+				
→55				+		+		+
76		+	+	+		+	+	+
→97		+		+	+		+	+
111				+	+	+		+
125		+		+			+	
146				+	+	+	+	
156		+		+	+	+	+	
166		+		+	+		+	
→180		+		+	+	+	+	
201				+	+			
→211		+		+	+		+	+
218		+		+	+	+	+	
→225		+		+	+	+	+	
239		+		+	+	+	+	
246	+	+		+	+	+	+	
253		+		+	+	+	+	

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 10.--Immunoelectrophoresis of L. donovani CDC antigen against homologous antisera from rabbit number 4.



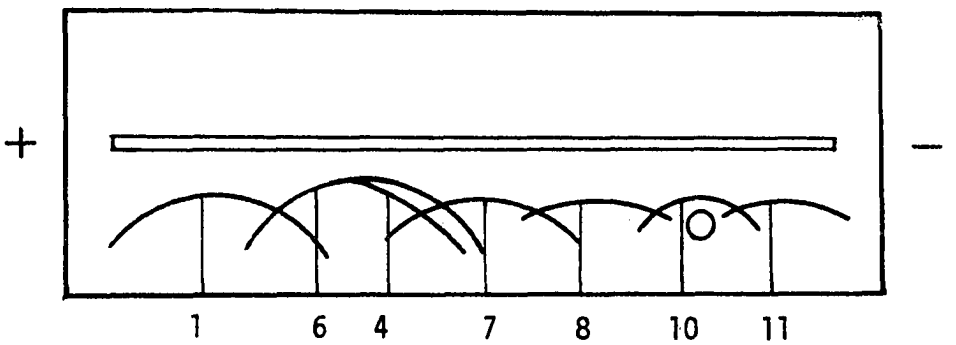
DAY	1	2	6	5	7	10	9
→[a]14							
→21			+[b]				
27			+				
34			+				
41			+				
48			+				
→55			+				
62	+	+	+			+	+
69	+		+		+	+	
76	+		+			+	+
83	+		+		+	+	
→97	+		+		+	+	
104	+		+		+	+	
111	+		+	+	+	+	
125	+		+	+	+	+	
132	+		+	+			
146	+		+	+	+	+	
156 †[c]	+		+		+		

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

[c] Cross indicates mortality.

TABLE 11.--Immunoelectrophoresis of L. donovani CDC antigen against homologous antisera from rabbit number 6.

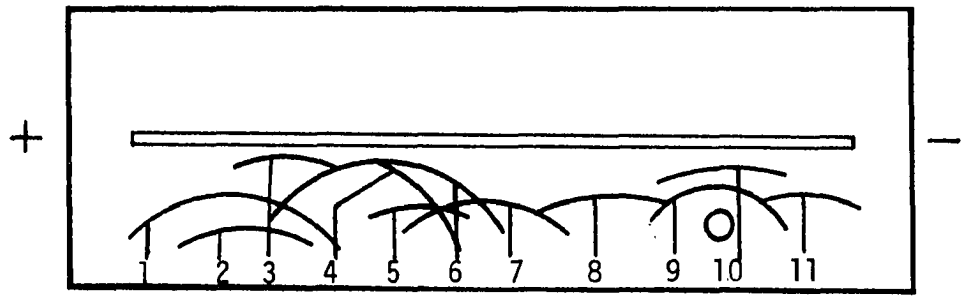


DAY	1	6	4	7	8	10	11
→[a] 14							
→ 34							
→ 55							
76		+[b]		+			
→ 97		+		+			
111				+	+		
125		+					
146		+					
156		+					
166		+					
→ 180		+					
201	+	+	+	+			
→211		+		+		+	
218	+	+		+	+		
→ 225		+		+	+		
239							
246	+	+		+	+	+	+
253	+	+		+	+		+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 12.--Immunoelectrophoresis of L. donovani CDC antigen against homologous antisera. Composite representation of all precipitin bands from all rabbits.



Rabbit Number

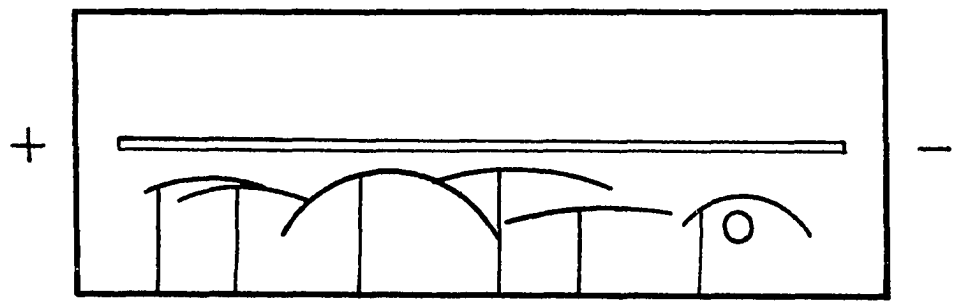
DAY	1	3	4	6
→[a] 14	6[b]	1,6,7	0	0
→ 34	6	6	6	0
→ 55	6	6,8,10	6	0
76	6	1,3,6,8,9,10	1,6,9,10	6,7
→ 97	6,7,8	1,6,7,8,10	1,6,7,10	6,7
111	6,7	6,7,8,10	1,5,6,7,10	7,8
125	6,7	1,6,9	1,5,6,7,10	6
146	6,7	6,7,8,9	1,5,6,7,10	6
156	6,7	1,6,7,8,9	1,6,7	6
166	6	1,6,7,9	†[c]	6
→ 180	6	1,6,7,8,9		6
201	1,6,7,8,9,10	6,7		1,6,4,7
→ 211	1,6,7,10	1,6,7,9,10		6,7,10
218	1,6,7	1,6,7,8,9		1,6,7,8
→ 225	1,4,6,7	1,6,7,8,9		6,7,8
239	5,6,7	1,6,7,8,9		6,7,8
246	4,5,6,7	1,2,6,7,8,9		1,6,7,8,10,11
253	1,6,7,8	1,6,7,8,9		1,6,7,8,11

[a] Arrows indicate days of inoculation.

[b] Numbers refer to presence of that band on the indicated day.

[c] Cross indicates mortality.

TABLE 13.--Immunoelectrophoresis of L. mexicana D-88 antigen against homologous antisera from rabbit number 11.



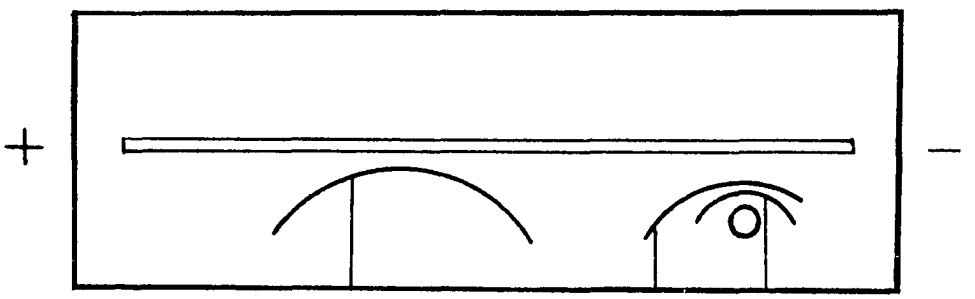
DAY	1	2	4	5	6	8
→[a] 14						
→ 27			+[b]			+
34			+			+
41		+	+	+		+
48	+	+	+			+
→ 55		+	+			+
62	+	+	+	+		+
69			+	+	+	+
76		+	+	+		+
83			+	+		+
→ 97			+	+		+
104			+			+
111			+			
125			+			+
132						
146			+			
156			+			
166 †[c]						

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

[c] Cross indicates mortality.

TABLE 14.--Immunoelectrophoresis of L. mexicana D-88 antigen against homologous antisera from rabbit number 13.

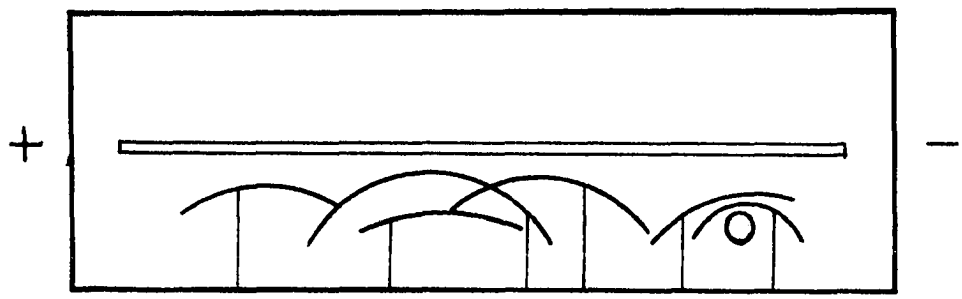


DAY	4	7	8
→[a] 14			
→			
34	+ [b]		+
→ 55	+		+
76	+		+
→ 97	+		+
111	+		+
125	+		+
146	+		+
156	+		+
166	+		+
→ 180	+		+
201	+		+
→ 211	+		+
218	+	+	+
→ 225	+	+	+
239	+		+
246	+		+
253	+		+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 15.--Immunoelectrophoresis of L. mexicana D-88 antigen against homologous antisera from rabbit number 14.

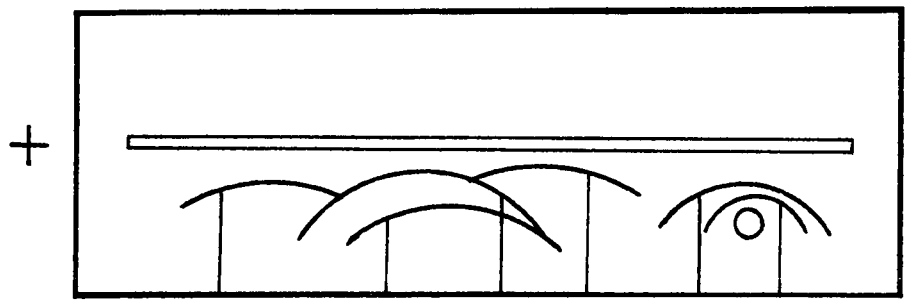


DAY	2	3	4	5	7	8
[a] → 14			+[b]			
→ 34			+			+
→ 55		+	+			+
76		+	+			+
→ 97		+	+			+
111	+	+	+			
125			+			+
146			+	+		
156			+			+
166			+			+
→ 180			+			
201			+			+
→ 211	+		+			+
215			+		+	+
→ 225			+		+	+
239			+			+
246			+		+	+
253			+			+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 16.--Immunoelectrophoresis of L. mexicana D-88 antigen against homologous antisera from rabbit number 15.



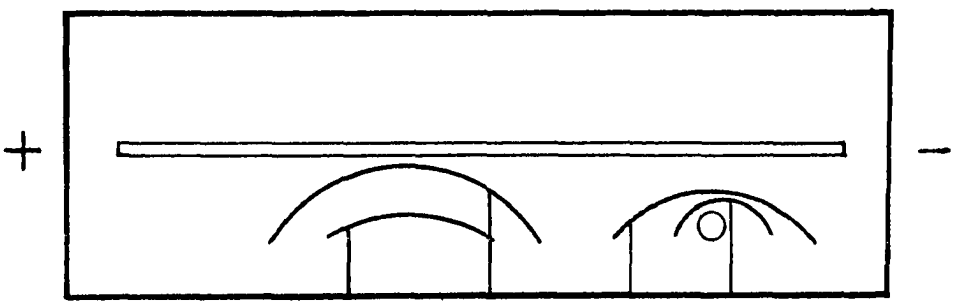
DAY	2	3	4	5	7	8
[a] → 14						
27		+[b]	+			+
34		+	+			+
41			+			+
48	+	+	+			+
→ 55	+	+	+			+
62	+	+	+			+
69	+		+			+
76	+	+	+			+
83	+	+	+			+
→ 97	+	+	+	+	+	+
104	+	+	+	+		+
111	+		+	+		+
125	+		+	+		+
132	+		+	+		+
146	+		+	+		+
156	+		+	+		+
166 +[c]	+		+	+		+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

[c] Cross indicates mortality.

TABLE 17.--Immunoelectrophoresis of L. mexicana D-88 antigen against homologous antisera from rabbit number 16.

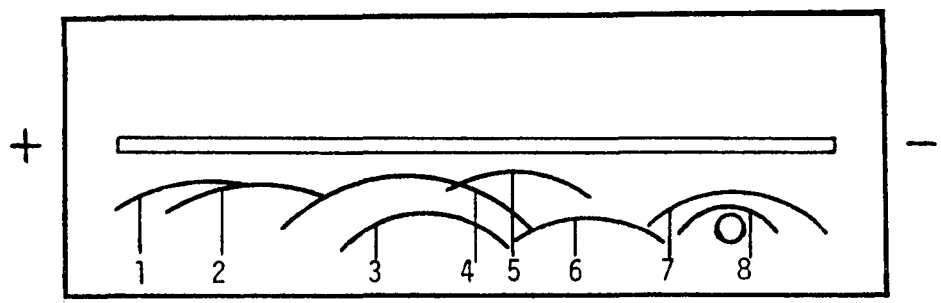


DAY	3	4	7	8
[a]→ 14				
→ 34				+[b]
→ 55				+
76		+		+
→ 97				+
111		+		+
125				
146		+		
156		+		
166		+		
→180		+		
194		+		+
201	+	+		+
→211	+	+		+
218		+	+	+
→225		+	+	+
239	+	+	+	+
246	+	+	+	+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 18.--Immunoelectrophoresis of L. mexicana D-88 antigen against homologous antisera. Composite representation of all precipitin bands from all rabbits.



	Rabbit Number				
DAY	11	13	14	15	16
→14	0	0	4[b]	0	0
34	4,8	4,8	4,8	3,4,8	8
→55	2,4,8	4,8	3,4,8	2,3,4,8	8
76	2,4,5,8	4,8	3,4,8	2,3,4,8	4,8
→97	4,5,8	4,8	3,4,8	2,3,4,5,7,8	8
111	4	4,8	2,3,4	2,4,5,8	4,8
125	4,8	4,8	4,8	2,4,5,8	4
146	4	4,8	4,5	2,4,5,8	4
156	4	4,8	4,8	2,4,5,8	4
166	4	4,8	4,8	2,4,5,8	4
→180	†[c]	4,8	4	†	4
201		4,8	4,8		3,4,8
→211		4,8	2,4,8		3,4,8
218		4,7,8	4,7,8		4,7,8
→225		4,7,8	4,7,8		4,7,8
239		4,8	4,8		3,4,7,8
246		4,8	4,7,8		3,4,7,8
253		4,8	4,8		†

[a] Arrows indicate days of inoculation.

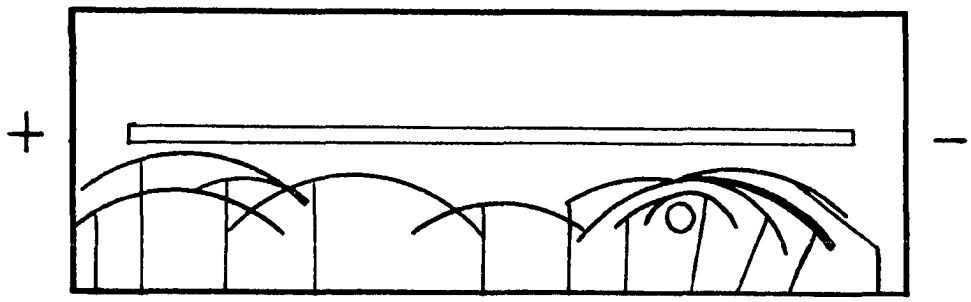
[b] Numbers refer to presence of that band on the indicated day.

[c] Cross indicates mortality.

always persistent. Band one appeared only twice in rabbit #11 and band six appeared only once in this same rabbit. These two bands did not appear in any other animals in this group. Although both bands are shown in Table 18, the days of appearance are not indicated. This group of animals is the only group in which booster injections seem to have had little or no effect on most of the animals. A possible exception is #16 which showed some enhancement after boosting on day 180.

The group of rabbits receiving L. tropica gave the greatest response of any group in this study (Tables 19 - 23). Two of the animals (#44 and #55) died during the study, but the three remaining animals showed a maximum of nine bands. Four of the five rabbits were responding on day 14 with one to five bands. Band four compares in position with band four of the animals which received L. mexicana and band six of the animals which received L. donovani. It appeared early and persisted. This band is the most consistent one seen in all of the animals. The most striking difference this group exhibits in comparison with the other groups is the activity surrounding the antigen well. In the other groups, a maximum of two bands were seen surrounding the well. In this group as many as six bands could be seen surrounding the well. This phenomena made delineation of the bands in this area somewhat difficult.

TABLE 19.--Immunoelectrophoresis of L. tropica antigen against homologous antisera from rabbit number 00.

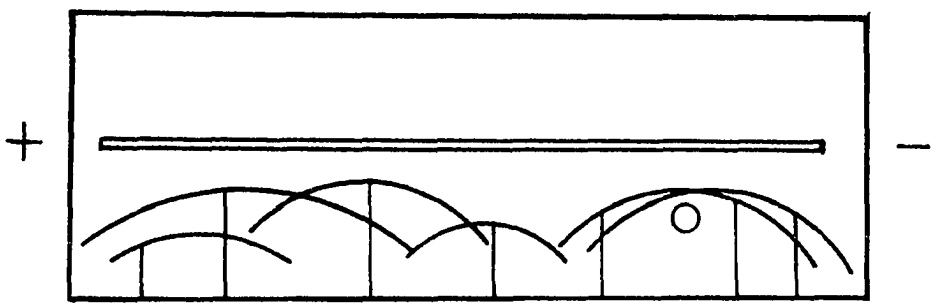


DAY	1	2	3	4	5	6	8	9	10	7	11
[a]→14				+[b]		+		+			
→21	+			+		+		+			
34	+	+		+		+		+		+	
→55	+			+		+		+		+	
62	+			+		+				+	
69	+			+		+				+	
76	+			+		+		+		+	
→97	+			+		+	+	+		+	+
125	+	+						+	+	+	
156	+			+					+	+	
180	+	+		+	+	+				+	
→ 194	+	+		+					+	+	+
201	+	+		+		+			+	+	+
→211	+	+		+		+			+	+	+
→225	+	+		+		+			+	+	+
239	+	+	+	+		+			+	+	+
246	+	+	+	+		+			+	+	+
253	+	+	+	+		+			+	+	+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 20.--Immunoelectrophoresis of L. tropica antigen against homologous antisera from rabbit number 33.

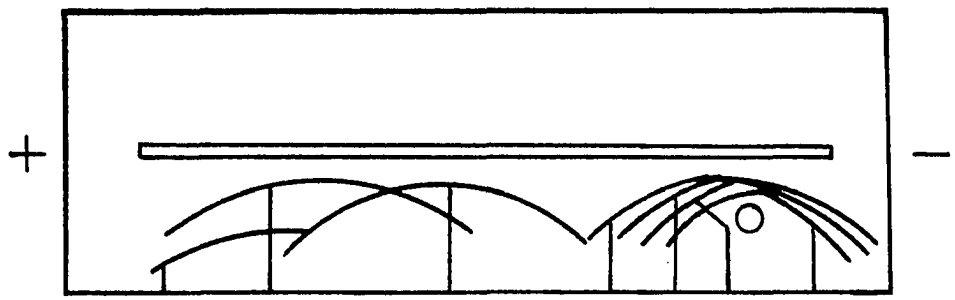


DAY	1	2	4	5	6	10	11
→[a]14	+[b]		+	+	+	+	
→21		+	+	+		+	+
34		+	+	+	+		
→55		+	+		+		+
62	+	+	+		+		+
69	+	+	+			+	+
76	+		+		+	+	
→97		+	+		+	+	
125	+		+		+	+	
156	+		+		+	+	
180		+	+		+	+	
→194	+	+	+		+	+	
→211		+	+		+		+
225	+	+	+		+	+	+
→239	+	+	+		+	+	+
246	+	+	+		+	+	+
253	+	+	+		+		+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 21.--Immunoelectrophoresis of L. tropica antigen against homologous antisera from rabbits number 44 and 55.



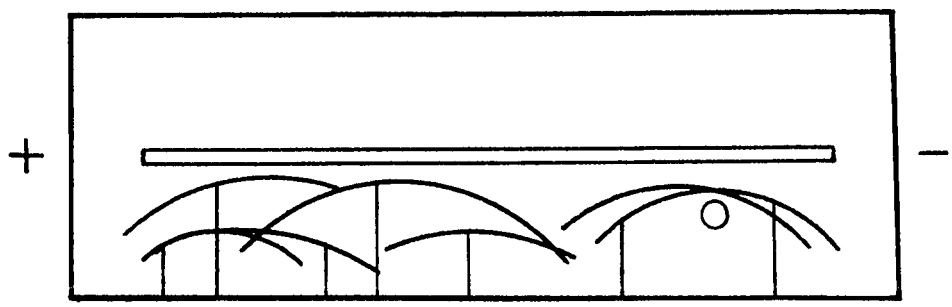
Rabbit #44 DAY	1	2	4	6	7	8	10
[a]→14 →				+[b]			
34			+	+		+	
→55		+	+	+		+	
69		+	+	+	+	+	+
76		+	+	+	+	+	+
83		+	+	+		+	
→97		+	+	+	+		+
104		+	+	+	+	+	+
111		+	+	+	+	+	+
125		+	+	+	+	+	
132		+	+	+		+	
146		+	+	+		+	+

Rabbit #55 DAY	1	2	4	6	7	8	10
→→34				+	+		
→55		+	+	+	+		
62				+	+		
69	+	+	+	+	+		
76	+	+	+	+	+		

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 22.--Immunoelectrophoresis of L. tropica antigen against homologous antisera from rabbit number 66.

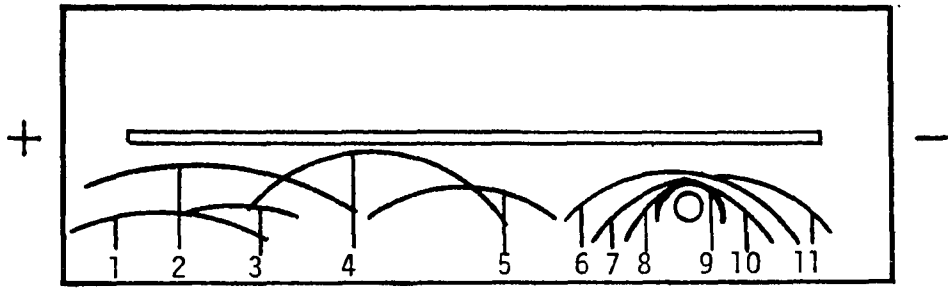


DAY	1	2	3	4	5	6	7 + 11
[a]→14						+[b]	
→21						+	
34				+	+	+	+
→55						+	
62		+		+		+	
76						+	+
83				+		+	+
→97				+		+	
125		+		+		+	+
156				+		+	
→180				+		+	
194	+	+		+	+	+	+
201				+	+	+	+
→211	+	+		+		+	
218	+	+		+		+	+
→225	+	+	+	+		+	+
239	+	+	+	+		+	+
246	+	+		+		+	+

[a] Arrows indicate days of inoculation.

[b] Presence of precipitin band on a particular day indicated by +.

TABLE 23.--Immunoelectrophoresis of L. tropica antigen against homologous antisera. Composite representation of all precipitin bands from rabbits number 00, 33, and 66.



Rabbit Number

DAY	00	33	66
[a]→14	4,6,9[b]	1,4,5,6,10	6
→21	1,4,6,9	2,4,5,10,11	6
34	1,2,4,6,7,9	2,4,5,6	4,5,6,7,11
→55	1,4,6,7,9	2,4,6,11	6
62	1,4,6,7	1,2,4,6,11	2,4,6
69	1,4,6,7	1,2,4,10,11	
76	1,4,6,7,9	1,4,6,10	6,7,11
→97	1,4,6,7,8,9,11	2,4,6,10	4,6
125	1,2,7,9,10	1,4,6,10	2,4,6,7,11
156	1,4,7,10	1,4,6,10	4,6
→180	1,2,4,5,6,7	2,4,6,10	4,6
194	1,2,4,7,10,11	1,2,4,6,10	1,2,4,5,6,7,11
201	1,2,4,6,7,10,11	2,4,6,11	4,5,6,7,11
→211	1,2,4,6,7,10,11	1,2,4,6,10,11	1,2,4,6
→225	1,2,4,6,7,10,11	1,2,4,6,10,11	1,2,3,4,6,7,11
239	1,2,3,4,6,7,9,10,11	1,2,4,6,10,11	1,2,3,4,6,7,11
246	1,2,3,4,6,7,9,10,11	1,2,4,6,10,11	1,2,4,6,7,11
253	1,2,3,4,6,7,9,10,11	1,2,4,6,11	†[c]

[a] Arrows indicate days of inoculation.

[b] Numbers refer to presence of that band on the indicated day.

[c] Cross indicates mortality.

Double Diffusion in Gel

The double diffusion results are presented in a series of sets. Each of the first four sets depicts a comparison of one antigen with a series of antisera. The examples shown do not represent all animals or serum samples examined. The samples selected are those which appeared to provide the greatest amount of information. All sera were taken on day 253. Animal identification is shown on each figure.

Figures 2 through 5 comprise the first set. Each figure in this set is a comparison of two antisera with L. tropica antigen. In Figure 2, three distinct bands are seen between the antigen well and both antisera. The bands in both cases appear to be identically situated, but partial identity is noted only between the central bands and the bands closest to the serum wells. In Figure 3, one can see a band of identity between the two strains of L. donovani. Also, one band between L. tropica antigen and antiserum to L. donovani 2S from rabbit #04 appears with a spur of partial identity and one of non-identity to anti-L. donovani CDC serum from rabbit #6. Figure 4 is a representation of the results with homologous antiserum to L. tropica compared with anti-L. donovani CDC serum. Two bands are in similar position, but they have not fused. An additional band is present with the heterologous antiserum that is not present with homologous serum. One can see in Figure 5 a band of identity between L. donovani CDC and L. mexicana, and two additional bands with the latter.

Figures 6 through 9 present comparisons of several antisera against L. mexicana antigen. In Figure 6, two well delineated bands of

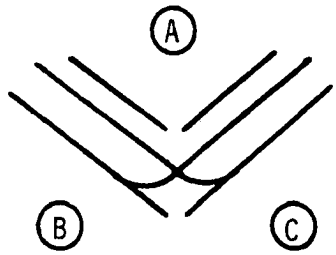


Fig. 2
A - L. tropica antigen
B - Anti-L. mexicana, Rab.#13
C - Anti-L. donovani CDC, Rab.#3

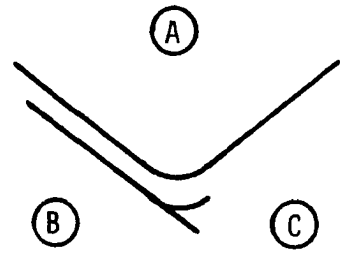


Fig. 3
A - L. tropica antigen
B - Anti-L. donovani 2S, Rab.#04
C - Anti-L. donovani CDC,Rab.#6

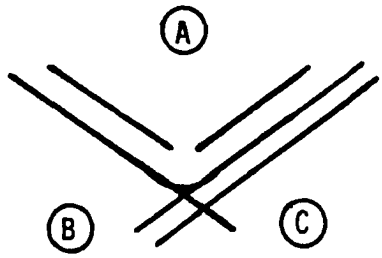


Fig. 4
A - L. tropica antigen
B - Anti-L. tropica, Rab.#00
C - Anti-L. donovani CDC, Rab.#3

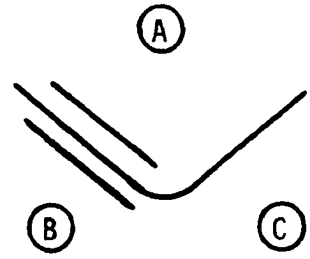


Fig. 5
A - L. tropica antigen
B - Anti-L. mexicana, Rab.#14
C - Anti-L. donovani CDC, Rab.#6

Figures 2-5.--Double diffusion of L. tropica antigen against homologous and heterologous antisera.

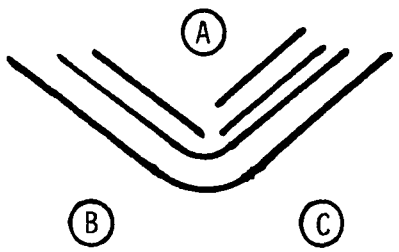


Fig. 6

A - L. mexicana antigen

B - Anti-L. mexicana, Rab. #13

C - Anti-L. donovani 2S, Rab.#04

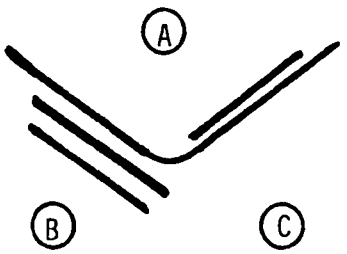


Fig. 7

A - L. mexicana antigen

B - Anti-L. donovani CDC, Rab.#3

C - Anti-L. donovani 2S, Rab.#01

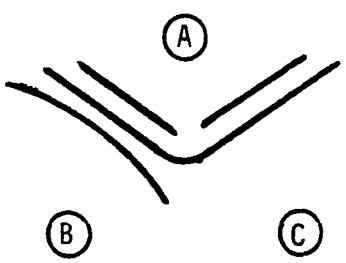


Fig. 8

A - L. mexicana antigen

B - Anti-L. mexicana, Rab.#14

C - Anti-L. donovani 2S, Rab.#01

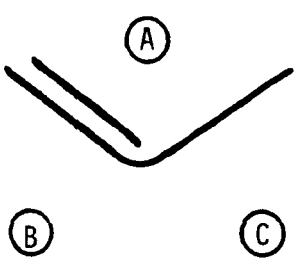


Fig. 9

A - L. mexicana antigen

B - Anti-L. tropica, Rab.#00

C - Anti-L. tropica, Rab.#33

Figures 6-9.--Double diffusion of L. mexicana antigen against homologous and heterologous antisera

identity can be seen between L. mexicana and L. donovani 2S, with additional non-identical bands present for each. Results obtained with different rabbits which had received the same antigenic stimulation are shown in Figure 8. Only one band of identity is seen here. The two strains of L. donovani, compared in Figure 7, show one band of identity, and one additional band with strain 2S and two additional bands with strain CDC. Figure 9 is a comparison of the sera from two rabbits which had received L. tropica antigen. Only one band of identity is seen.

Figures 10 through 13 compare various antisera with L. donovani CDC antigen. Figures 10 and 11 both are a comparison of antisera to strains of L. donovani and L. tropica. There is one band of identity in both instances, and one additional band with each strain of L. donovani. In a comparison of L. mexicana and L. tropica antisera (Fig. 12), one circular band of identity very close to the antigen well can be seen. One additional band each for L. mexicana and L. tropica is also visible in similar position, but they are not fused. In Figure 13, which is a comparison of L. donovani CDC and L. mexicana antisera, no identity if seen, although one band for each appears in similar position.

In Figure 14 through 17 the antigen is from L. donovani 2S. Figure 14 is a comparison of L. donovani 2S and L. mexicana antisera. Two bands of identity can be seen. This Figure is similar to Figure 6 which also showed bands of identity when L. mexicana was the antigen and anti-L. mexicana and anti-L. donovani 2S were in the serum wells. In Figures 15 and 17 a comparison is made between antisera to L. donovani, L. tropica and L. mexicana. Each has one band of identity and an

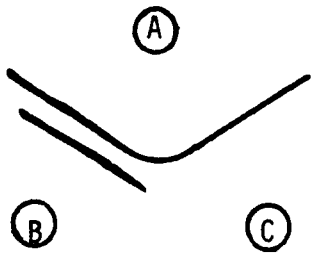


Fig. 10

A - L. donovani CDC antigen

B - Anti-L. donovani CDC, Rab.#3

C - Anti-L. tropica, Rab.#00

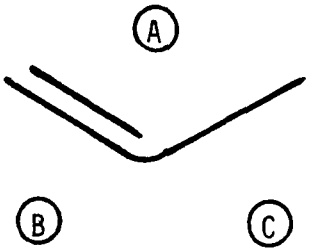


Fig. 11

A - L. donovani CDC antigen

B - Anti-L. donovani 2S, Rab.#04

C - Anti-L. tropica, Rab.#33

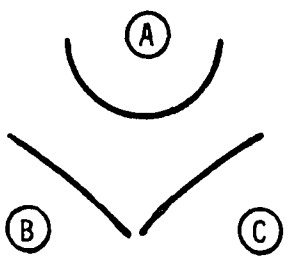


Fig. 12

A - L. donovani CDC antigen

B - Anti-L. mexicana, Rab.#11

C - Anti-L. tropica, Rab.#33

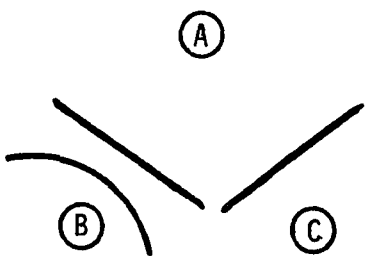


Fig. 13

A - L. donovani CDC antigen

B - Anti-L. donovani CDC, Rab.#3

C - Anti-L. mexicana, Rab.#13

Figures 10 - 13.- Double diffusion of L. donovani CDC antigen against homologous and heterologous antisera

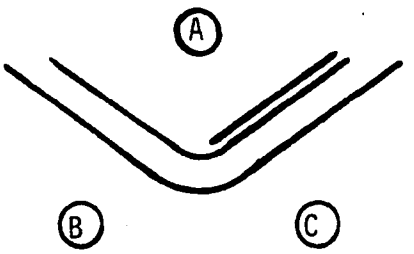


Fig. 14

A - L. donovani 2S antigen

B - Anti-L. donovani CDC, Rab. #3

C - Anti-L. mexicana, Rab. #14

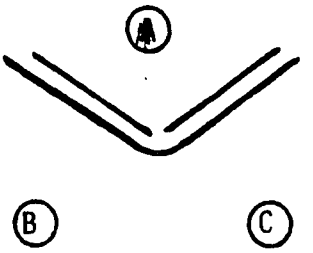


Fig. 15

A - L. donovani 2S antigen

B - Anti-L. mexicana, Rab. #13

C - Anti-L. donovani 2S, Rab. #01

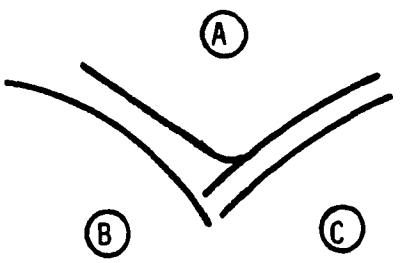


Fig. 16

A - L. donovani 2S antigen

B - Anti-L. tropica, Rab. #33

C - Anti-L. mexicana, Rab. #13

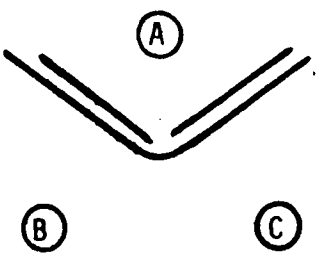


Fig. 17

A - L. donovani 2S antigen

B - Anti-L. donovani CDC, Rab. #6

C - Anti-L. tropica, Rab. #00

Figures 14 - 17.--Double diffusion of L. donovani 2S antigen against homologous and heterologous antisera

additional band in similar position but not fused. As shown in Figure 16, two bands occur when anti-L. tropica or anti-L. mexicana serum react with L. donovani 2S antigen. A partial identity exists in one of these bands (anti-L. mexicana).

The results of the second group of gel diffusion studies are presented in Figures 18 through 21. In this case, Leishmania spp. antiserum, in the central well, is compared with all four soluble leishmanial antigen preparations.

Figure 18 is an illustration of the results seen when anti-L. donovani 2S serum was reacted with all four leishmanial antigens. Homologous antigen exhibited four distinct bands, one being of partial identity with L. tropica antigen. One band of identity and one spur of partial identity are seen between L. donovani CDC and L. mexicana.

In Figure 19 will be seen results obtained with L. donovani CDC antiserum. The results are similar to those seen in Figure 18, with two exceptions: 1) there are two bands of identity between L. mexicana and L. donovani CDC, and 2) one band of identity is noted between L. mexicana and L. tropica.

A continuous band of identity among all four antigens, when they were diffused against L. tropica antiserum, is seen in Figure 20.

In Figure 21, which presents the results obtained with L. mexicana antiserum, one band of identity is observed with all antigens. One additional band of identity and one of partial identity are seen with L. mexicana and both strains of L. donovani. Two bands of identity appear with the two strains of L. donovani, and one additional band of partial identity between L. tropica and L. donovani 2S.

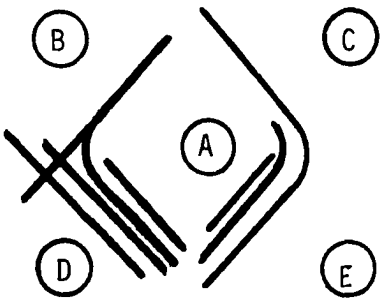


Fig. 18

A - Anti-L. donovani 2S Rab. #04

B - L. tropica

C - L. mexicana

D - L. donovani 2S

E - L. donovani CDC

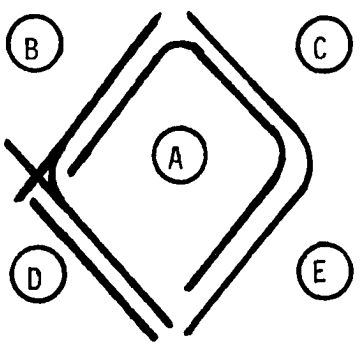


Fig. 19

A - Anti-L. donovani CDC Rab. #3

B - L. tropica

C - L. mexicana

D - L. donovani 2S

E - L. donovani CDC

Figures 18 & 19.--Double diffusion of anti-Leishmania serum against all four leishmanial antigen preparations

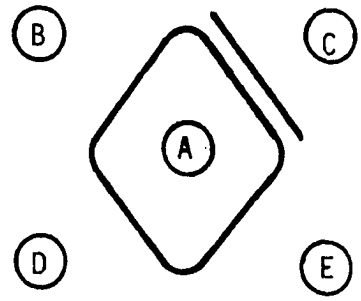


Fig. 20

A - Anti-L. tropica, Rab.#33

B - L. tropica antigen

C - L. mexicana antigen

D - L. donovani 2S antigen

E - L. donovani CDC antigen

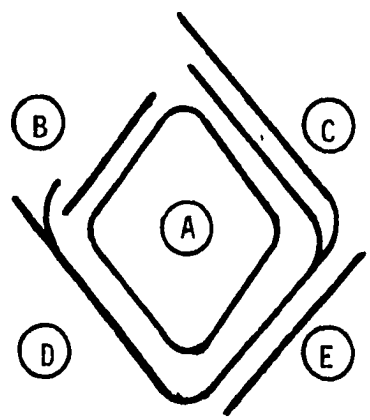


Fig. 21

A - Anti-L. mexicana, Rab.#14

B - L. tropica antigen

C - L. mexicana antigen

D - L. donovani 2S antigen

E - L. donovani CDC antigen

Figures 20 & 21.--Double diffusion of anti-Leishmania sera against all four leishmanial antigen preparations

Immunoelectrophoretic Comparison of Soluble Leishmanial Antigens with Homologous and Heterologous Antisera

In the studies depicted in Figures 22 through 33, a single antigen was reacted with one homologous and one heterologous antiserum, using immunoelectrophoresis. The figures are grouped such that all figures on any page compare a single antigen with each of the different antisera. Again, the well was placed off-center toward the cathode in order to obtain maximum separation, since no components migrated to the cathode.

Figures 22 through 24 represent the results obtained with L. tropica antigen and antiserum against each of the four antigens. Homologous antisera produced four or five precipitin bands, depending on the rabbit from which the serum was taken. L. donovani CDC antiserum exhibited two bands that compared favorably in position with two of the bands seen with homologous antiserum (Fig. 22). In Figure 23, L. donovani 2S antiserum shows only one of these bands. Using L. mexicana antiserum, as seen in Figure 24, four bands are noted which compare very closely in position with the four bands seen with homologous antiserum. No fusion of bands was noted in this group.

Figures 25, 26, and 27 represent a comparison of L. donovani 2S antigen. Similar results are seen in all three examples. All antisera tested exhibited a fused band which surrounded the antigen well and an additional band which fused only in the case of L. tropica antiserum (Fig. 25). L. donovani CDC antiserum produced one additional band which can be seen in Figure 27.

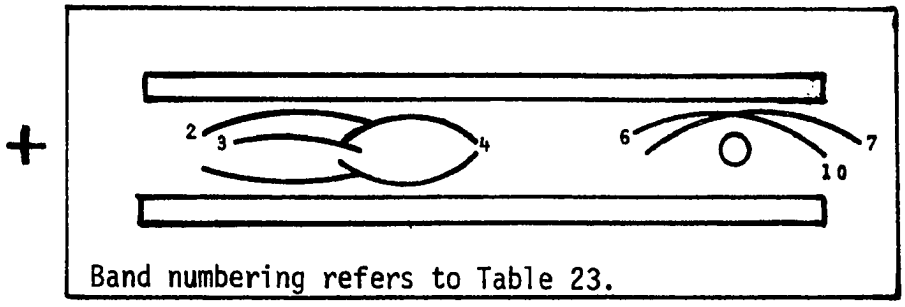


Figure 22

Top trough - L. tropica antiserum, Rab. #00, day 253
Well - L. tropica antigen
Bottom trough - L. donovani CDC antiserum, Rab. #3, day 211

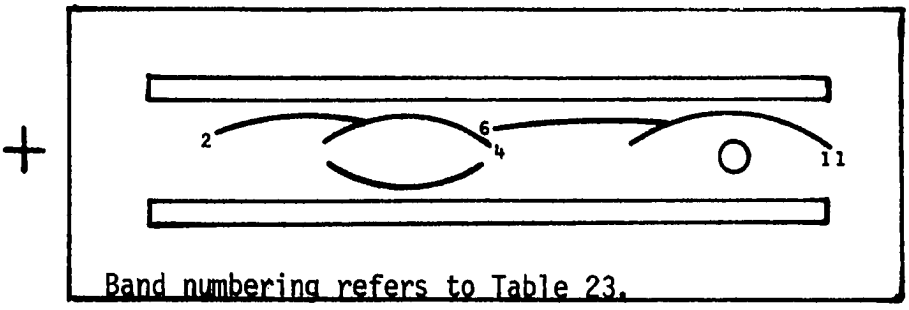


Figure 23

Top trough - L. tropica antiserum, Rab. #33, day 253
Well - L. tropica antigen
Bottom trough - L. donovani 2S antiserum, Rab. #01, day 246

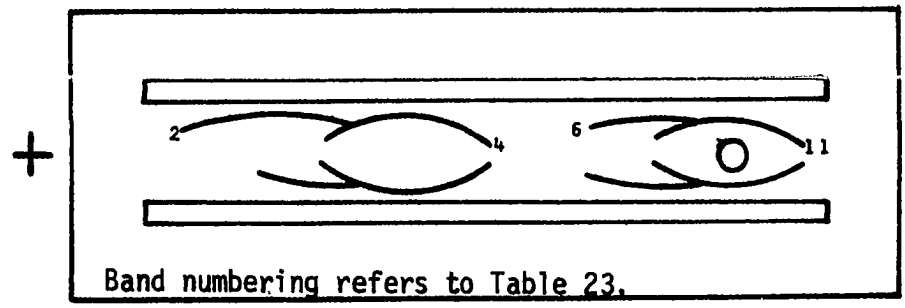


Figure 24

Top trough - L. tropica antiserum, Rab. #33, day 253
Well - L. tropica antigen
Bottom trough - L. mexicana antiserum, Rab. #14, day 239

Figures 22 - 24.--Immunoelectrophoretic comparison of L. tropica antigen with homologous and heterologous leishmanial antisera

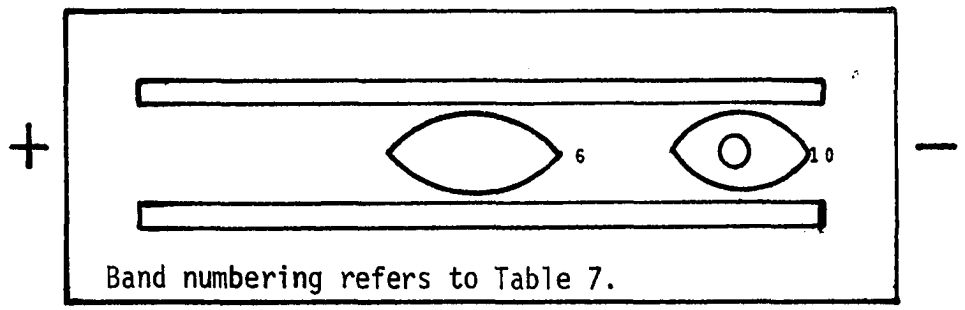


Figure 25

Top trough - L. donovani 2S antiserum, Rab. #03, day 253
Well - L. donovani 2S antigen
Bottom trough - L. tropica antiserum, Rab. #00, day 253

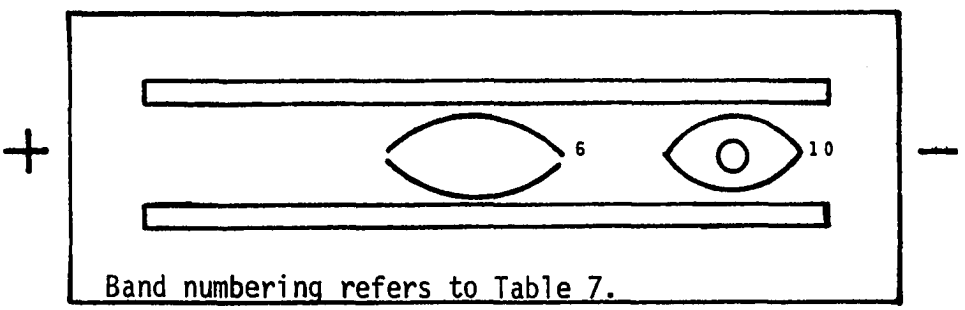


Figure 26

Top trough - L. donovani 2S antiserum, Rab. #04, day 253
Well - L. donovani 2S antigen
Bottom trough - L. mexicana antiserum, Rab. #14, day 253

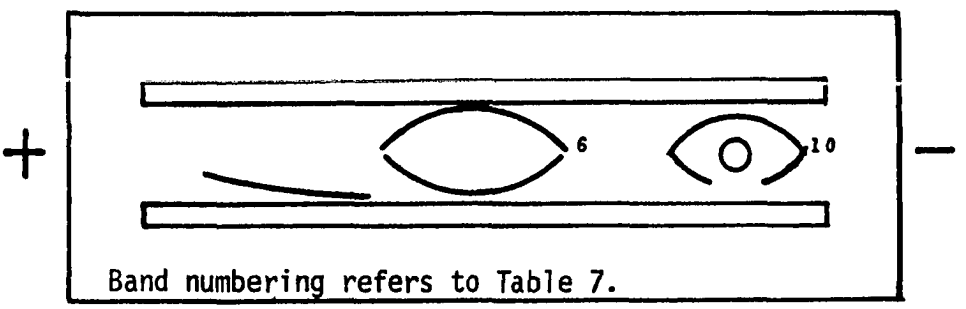


Figure 27

Top trough - L. donovani 2S antiserum, Rab. #04, day 253
Well - L. donovani 2S antigen
Bottom trough - L. donovani CDC antiserum, Rab. #3, day 253

Figures 25 - 27.--Immunoelectrophoretic comparison of L. donovani 2S antigen with homologous and heterologous leishmanial antisera

In Figures 28 - 30, which represent reactions of L. mexicana antigen with antisera against the four leishmanial antigens, two bands are observed in each figure. One band surrounds the antigen well and is fused, except in the case of L. donovani 2S (Fig. 29). Another pair of bands is seen about midway on the slide. They are in juxtaposition, but they are not fused.

Figures 31 - 33 represent comparisons using L. donovani CDC as antigen. One can see, in Figure 31, one band produced with L. tropica antiserum as compared with four bands seen with homologous antiserum. No bands were observed at the well. When L. mexicana antiserum was used (Fig. 32), and homologous antiserum from a different rabbit, a set of bands appeared surrounding the well. The central band is again seen but it is not fused. With L. donovani 2S antiserum, the remnants of a band can be seen surrounding the well (Fig. 33). The central band is present, with an additional band on either side.

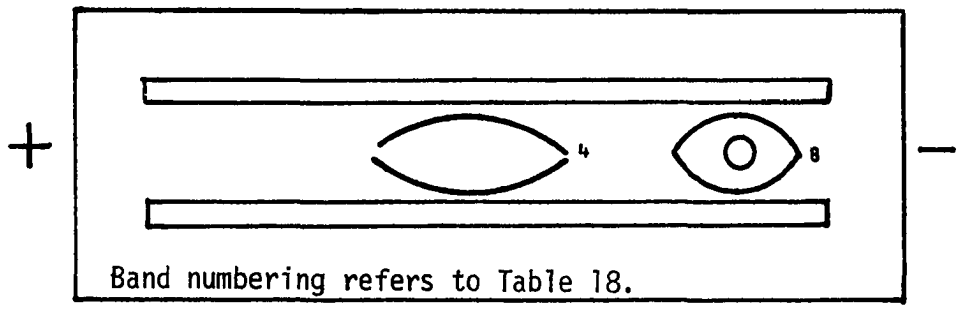


Figure 28

Top trough - L. mexicana antiserum, Rab. #13, day 253
Well - L. mexicana antigen
Bottom trough - L. tropica antiserum, Rab. #00, day 253

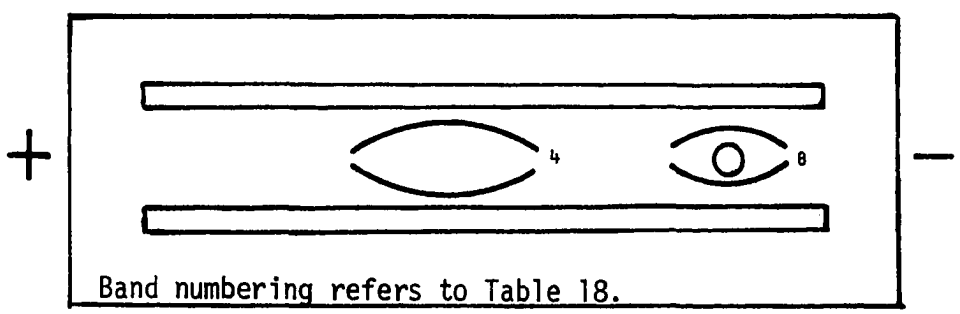


Figure 29

Top trough - L. mexicana antiserum, Rab. #14, day 253
Well - L. mexicana antigen
Bottom trough - L. donovani 2S,antiserum, Rab. #04, day 253

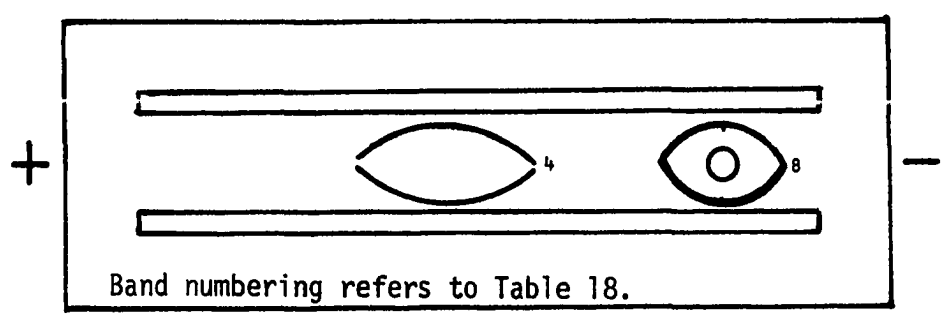


Figure 30

Top trough - L. mexicana antiserum, Rab. #14, day 253
Well - L. mexicana antigen
Bottom trough - L. donovani CDC antiserum, Rab. #3, day 253

Figures 28 - 30.--Immunoelectrophoretic comparison of L. mexicana antigen with homologous and heterologous antisera

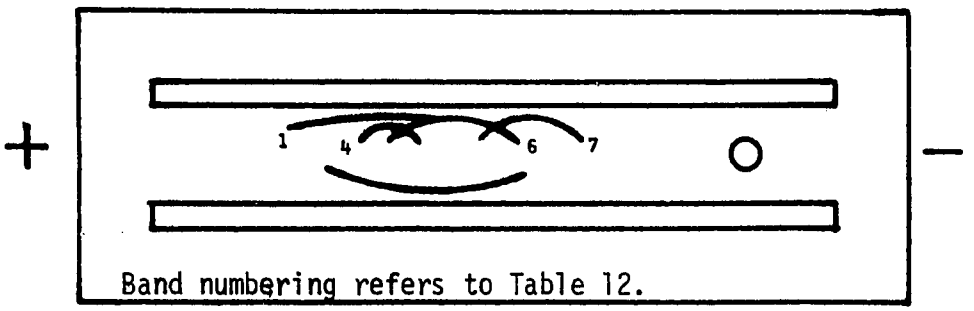


Figure 31

Top trough - L. donovani CDC antiserum, Rab. #1, day 253
Well - L. donovani CDC antigen
Bottom trough - L. tropica antiserum, Rab. #33, day 253

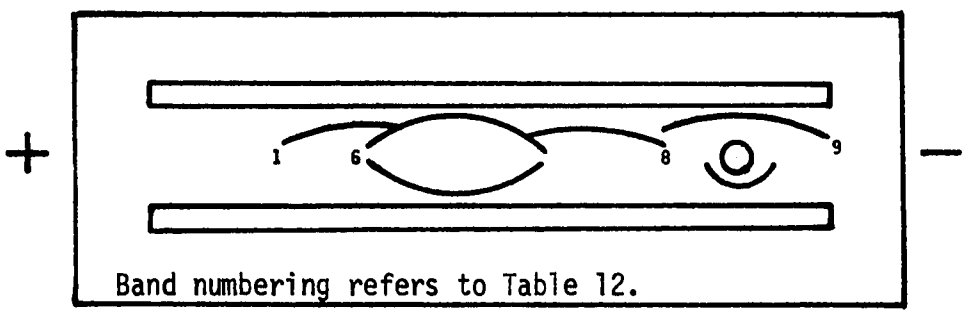


Figure 32

Top trough - L. donovani CDC antiserum, Rab. #3, day 253
Well - L. donovani CDC antigen
Bottom trough - L. mexicana antiserum, Rab. #13, day 253

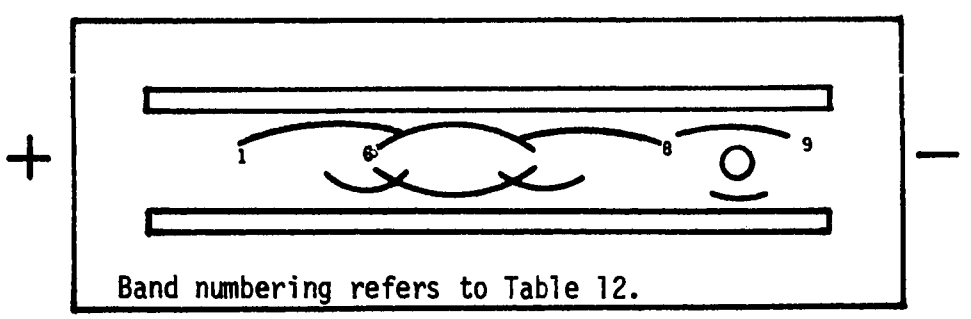


Figure 33

Top trough - L. donovani CDC antiserum, Rab. #3, day 253
Well - L. donovani CDC antigen
Bottom trough - L. donovani 2S antiserum, Rab. #01, day 253

Figures 31 - 33.--Immunoelectrophoretic comparison of L. donovani CDC antigen with homologous and heterologous antisera

CHAPTER IV

DISCUSSION

In 1970, Schaefer et al (73) reported the successful cultivation of several species of Leishmania using a chemostat. The best yields were obtained with L. tropica and the poorest with L. brasiliensis. In 1972, Dwyer (27) reported the successful growth of L. donovani in large quantities using a monophasic medium. With both of these methods, there is a disadvantage in that the media contain blood, serum proteins or both. This could be a potential problem when immunological studies are to be performed.

Crook et al (25) initiated studies of the growth of Leishmania using dialysate culture preparations in this laboratory, thereby isolating the organisms from the blood and serum proteins. They reported success with L. mexicana in this dialysate culture.

In the present study, significant yields were obtained with all species and strains of Leishmania except L. brasiliensis. In the studies referred to previously, the various workers also reported greater difficulty in growing L. brasiliensis, perhaps due to its more fastidious nature. It is possible that longer periods of adaptation to the medium may be required for this organism, since it did grow, but to only a limited extent ($\sim 10^4$ organisms/ml). These results would seem to indicate that the dialysate culture technique is the best method

for growing promastigotes of Leishmania, especially for immunological experimentation. These results also tend to lend support to the hypothesis concerning the small, dialyzable nature of the growth factor in serum and blood proteins which is necessary for growth of these organisms.

The first completely defined medium for the growth of Leishmania was that reported by Trager (86). This medium supported the growth of L. tarentolae, a lizard strain, but would not support the growth of a human strain, L. donovani. This suggested a more fastidious nature of the human parasites. Also noted was the fact that less restrictive requirements were necessary the longer the organisms were maintained on artificial media. This is significant in assuming that the organisms are adapting under these cultural conditions and also that many strains lose infectivity after varying periods of time in culture on artificial media (2).

Early work with Trypanosoma cruzi showed that all factors in blood could be substituted, except albumin (23). The specific function of albumin was traced to its ability to bind and detoxify oleic acid, an essential lipid. Crystalline albumin was not functional unless minute quantities of oleic acid were added. This does not seem applicable with the Leishmania since the albumin should not be able to traverse the dialysis membrane.

To date, the exact nature of the growth factor or factors present in blood remains unknown. However, studies of the metabolic pathways in Leishmania respiration are in progress and should shed some light on this problem.

Krassner and Flory (51) have recently shown a high rate of proline uptake by promastigotes of L. donovani. This compound is known to exist in high concentration in hemolymph. These authors have proposed a metabolic pathway for proline oxidation. Of related interest is the discovery that Trypanosoma rhodesiense does not oxidize proline in the bloodstream, but oxidizes it at a high rate in culture (83).

In the present studies the methods employed for antigen preparation and subsequent immunization gave good results. The antigen preparations were assumed to be free of any contaminating proteins, since controls were consistently negative. Nitrogen contents ranged from 71.3 µg N/mg for L. donovani CDC to 55.5 µg N/mg for L. tropica, which is consistent with the 61.0 µg N/mg reported by Crook et al (25) for L. mexicana. It is interesting to note that the antigen with the lowest nitrogen content, L. tropica, gave the largest number of bands on immunoelectrophoresis. Obviously, the nitrogen content indicates nothing as to the different kinds of antigens present.

Less than half of the animals gave any detectable immune response on day 14, while all but two animals were responding by day 34. One rabbit (#33) receiving L. tropica antigen was showing five bands on day 14. This particular animal never showed more than six bands at one time. Therefore, day 14 was very near the maximum response period for this animal. Booster injections were given several times throughout the 253 day immunization schedule. In most instances, the results of boosting were quite dramatic. Several animals that were exhibiting only one or two bands began showing six or seven bands after additional

stimulation later in the immunization schedule.

No rabbit failed completely to respond to the antigenic stimulation, although several animals showed only one or two bands throughout most of the study. The maximum response as a group was seen with animals which received L. tropica, where as many as nine bands were seen at one time and an average of five or six was seen. The minimum response as a group was observed in animals immunized with L. mexicana. The maximum number of bands seen at one time was six, with most animals exhibiting only one to three bands throughout the study.

If the four composite immunoelectrophoretic tables, (Tables 7, 12, 18 and 23) are compared, certain similarities and differences in strains and species become apparent. The results must be analyzed on a qualitative as well as a quantitative basis. In other words, the presence of a band is the most obvious observation to be made, but it should also be noted how many rabbits exhibited that band and how consistently it was present. With these criteria in mind, the most striking observation concerns the presence of a band about half way between the antigen well and the anode. In both strains of L. donovani this band is labeled number six and in L. mexicana and L. tropica it is number four. The position of this band is quite similar with all antigen preparations. It apparently represents a strong antigenic component, since it was the first band to appear and was seen the most consistently throughout the study. It also persisted to the end of the study. Further support for this observation can be seen in the comparative electrophoretic studies (Figs. 22 - 33). Every combination

of two antisera tested against each antigen demonstrated a band at about this same location. In most cases, the bands did not coalesce, but the positions were identical to each other. This band then represents a common antigen among all species of Leishmania tested.

In Figures 25 - 27, L. donovani 2S antigen showed an additional band of identity surrounding the well. These bands coalesced and were present in sera from all of the other three groups. These results seem to indicate the presence of two components in all antisera tested that reacted with L. donovani 2S antigen. Virtually the same picture was seen when L. mexicana was tested against heterologous antisera (Figs. 28 - 30). Two bands of identity were seen, one surrounding the well and the other midway on the slide. These were the only two bands that appeared with homologous antisera to L. mexicana, but on day 253 only two bands were seen in the original homologous studies shown in Table 18. Even though the rabbits receiving L. mexicana antigen responded with fewer bands than other animals, certain sera contained more than the two components demonstrable on day 253. It must be pointed out that some of these sera were not available for the comparative studies, since they had been exhausted in the equilibration of the homologous antigen-antibody immunoelectrophoresis examinations. For this reason all of the comparative studies employed sera from day 253, when the animals were sacrificed and a larger serum sample was collected.

The obvious and expected similarity between the two strains of L. donovani can be seen in Figure 33. These four bands account for all but one of the five bands seen in the original homologous antigen-

antibody comparisons. In only one other situation were as many as four similarities noted. In Figure 24, with L. tropica antigen and L. mexicana antiserum, four bands were also noted in similar positions. This observation lends support to the suggestion of a close relationship between L. tropica and L. mexicana (2).

In Figures 22 and 23, L. tropica antigen produced one or two bands surrounding the well, depending on the rabbit source, when it was reacted with homologous antiserum. No cross reactivity was seen at this site with either strain of L. donovani. This may well represent a means of distinguishing L. tropica from L. donovani. Another striking observation concerning L. tropica was the activity surrounding the well. At least six bands or spurs were noted with this antigen, which is significantly more than was seen with any other system tested.

In Tables 7 and 12, one band (number 11), appearing with both strains of L. donovani, migrated to the cathode. Although it was seen in only one rabbit in each group, it may well represent a species specific antigen for L. donovani since it was seen in no other species tested.

In the comparison electrophoretic studies, one or two of the bands which had been seen in the original homologous studies were occasionally not seen. The homologous antiserum studies were performed several months before the comparison studies. Although every attempt was made to duplicate experimental conditions, it was not possible to control all the variables. Hirschfeld (43) has reported the results of repeated analyses of human serum samples. These tests were performed under identical conditions as far as possible but they demonstrated quite

a great deal of variability, prompting the author to state:

> Immunodiffusion techniques in general and micro-techniques in particular are very sensitive to small quantitative variations in the amounts of reactants added to the wells. This effect may give non-reproducible findings according to the 'either-or' principle, that is to say, sometimes precipitates appear and sometimes they do not. Moreover, the reliability of a test performed on a particular biological sample is often very high, but it may also be very low, despite its being made with the same technique by one and the same worker and under identical experimental conditions.

A reverse of the above situation is seen in Figure 24, where L. tropica antigen was reacted with homologous and L. mexicana antiserum. Four bands were seen with both sera and they matched each other very closely. Oddly enough, this same L. mexicana antiserum showed only two bands when it was reacted with homologous antigen (Fig. 29). It is possible that the L. mexicana antigen contained a sufficient amount of these two components to stimulate the formation of antibody, but not enough to precipitate, whereas L. tropica antigen contained these same components in higher concentration. It has already been pointed out that L. tropica stimulated the best immune response of the four antigen preparations, if the number of bands is used an an index.

Examination of the double diffusion results (Figs. 2 - 21) suggests several general conclusions. With rare exceptions, the number of bands seen was less than in the immunoelectrophoresis studies. This is not surprising since double diffusion allows for separation of antigenic components based entirely on diffusion rate, whereas immuno-

electrophoresis introduces, in addition, the factor of electrical charge. One notable exception to this observation occurred with L. mexicana. Antigen from L. mexicana exhibited only two bands by immunoelectrophoresis with homologous antiserum collected on day 253 (Table 18), but in Figures 6, 8 and 21, three distinct bands could be seen with L. mexicana antigen and homologous antisera. This additional band could be accounted for on the basis of concentration, since the wells used in double diffusion tests were larger than those used in immunoelectrophoresis.

L. tropica gave the largest number of bands in immunoelectrophoresis and L. mexicana gave the least, but this trend seems to be reversed in diffusion studies (Figs. 20 and 21). This, too, may be a matter of concentration in that L. tropica may stimulate antibody formation against a larger number of antigenic components, but L. mexicana may well stimulate a greater concentration of antibody to a smaller number of determinants. These two figures also present the best evidence for at least one common antigenic component in all Leishmania tested, since a complete coalescence is seen with both antisera when compared with all four antigens. Indeed, Figure 21 shows three bands of identity between L. mexicana and L. donovani CDC.

Recent work with L. donovani 2S using methods similar to those employed in the present study has shown nine precipitin bands on immunoelectrophoresis (24). Although the number of precipitin bands is similar to that seen in our study, the authors reported five components which migrated to the cathode. In the present studies, only one component of L. donovani 2S migrated to the cathode. The other organ-

isms contained none.

In comparing several isolates of Leishmania by double diffusion in gel, Bray and Lainson (14) found that L. mexicana and L. tropica shared only one antigen out of four or five antigens demonstrated. In the present study, as many as three shared bands were demonstrated between L. tropica and L. mexicana. Bray and Lainson (14) also found that L. donovani shared as many as three antigens with all other strains tested, but all the strains appeared to be antigenically distinct. The present study demonstrated as many as four antigens shared by L. donovani and other strains and confirmed the antigenically distinct nature of all antigen preparations.

Using gel diffusion and immunoelectrophoresis, Garcia (35) demonstrated four antigenic components in L. tropica. The present studies revealed three bands by gel diffusion, but a maximum of eleven bands by immunoelectrophoresis.

Schneider and Hertig (75) compared five human isolates and eight sandfly isolates of Panamanian Leishmania by double diffusion. They were able to divide them into two groups on the basis of shared antigens. Some strains shared as many as five antigens. This compares well with the number of antigens detected in the present study. A comparison cannot be made with their work, however, since they did not utilize species designations.

Schnur et al (76) have reported a possible means of serotyping of Leishmania using as antigens metabolic factors excreted in the culture medium. In gel diffusion, these antigens cross reacted strongly with antisera against the same serotype, but very little with anti-

sera against a different serotype. They employed two isolates of L. donovani and four isolates of L. tropica. If this secreted antigenic material is present in the medium, it is probably also present in the cells, and is likely represented by one or more of the bands which were noted in the present study. This possibility has been confirmed by other workers (14, 24).

Crook et al (25), working in this laboratory with soluble antigen of L. mexicana, were able to demonstrate eleven distinct bands on immunoelectrophoresis. The results in the present study are similar, but only eight were demonstrated. In the work of Crook et al , several bands were at the origin and one migrated to the cathode. In the present study, only two bands were located at the origin, and none migrated to the negative pole. Close comparison however, points up several similarities, particularly in the components that migrated to the anode. Band number two described by Crook et al, the largest and strongest in their work, appears to be in similar position to band number four in the present study. It was also the largest and strongest.

CHAPTER V

SUMMARY

An attempt was made to grow several species and strains of human leishmanias in large quantity and free from blood protein contamination. Because of the requirement of Leishmania for a factor or factors in blood, a dialysate culture technique was used in batch culture. The organisms were grown in a medium containing potassium chloride, sodium chloride, potassium dihydrogen phosphate and glucose. This was dialyzed against a mixture of lysed, post-dated human blood and beef extract, yeast extract, peptone and calcium chloride. Success was achieved with L. mexicana, L. tropica, L. donovani 2S and L. donovani CDC with yields reaching $\sim 10^7$ organisms per ml. Less successful results were obtained with two strains of L. brasiliensis where yields were no more than $\sim 10^4$ organisms per ml.

Cells from the four leishmanial organisms that were cultured in large numbers were harvested by centrifugation. Soluble antigens were prepared from each by freeze-thawing and sonication. The insoluble cellular debris was removed by centrifugation.

Five rabbits were immunized with each of these antigen preparations by subcutaneous injection of the material emulsified in Freund's incomplete adjuvant. Booster injections were given intravenously. The

antigens were then tested against homologous and heterologous antisera from the rabbits by immunoelectrophoresis and double diffusion.

Some of the rabbits showed detectable precipitins by day 14, and most responded by day 34 after the initial stimulation. The maximum response was with L. tropica in which one animal serum gave nine bands by immunoelectrophoresis. Minimum response was with L. mexicana where no more than six bands were detected at one time. When any given antigen preparation was compared with antiserum to a heterologous strain, from one to four bands were detected, with most showing two.

In gel diffusion studies, as many as four components could be detected with antigen and homologous antisera. By comparing one antiserum to all four antigen preparations, at least one component was detected that was common to all antigens tested. Some samples revealed as many as three shared components between two antigens.

Double diffusion studies indicated a close relationship between L. donovani and L. mexicana, but comparative immunoelectrophoresis demonstrated a closer identity of L. mexicana to L. tropica.

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