

The Effect of Chemotherapy on the Development
of Immunity to
Leishmania major



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(Name)
Deyene Petros

(Signature)

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Research Adviser

Hailu Kibret

External Examiner

Ghimja Fessahaye

by

Ghimja Fessahaye

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Leishmania major

by

Ghimja Fessahaye

Department of Biology

ADDIS ABABA UNIVERSITY

Approved by the Examining Board:

(Name)

Beyene Petros

(Signature)

Beyene Petros

Chairman, Department Graduate Committee

David Humber

David Humber

Research Adviser

Rolf Kiesslings

Rolf Kiesslings

External Examiner

Getachew Bolodia

Getachew Bolodia

Internal Examiner

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ABSTRACT

I

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I wish to express my indebtedness to my advisor, Dr. David P. Humber, with out whose unlimited assistance and indispensable advice, I would not have been able to complete this work.

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ABSTRACT

Successful treatment of cutaneous leishmaniasis has not yet been achieved due to toxicity and the need for parenteral administration of most antileishmanial drugs. One of the requirements of successful treatment is that after chemotherapy the patient should possess an adequate immune response to prevent re-infection on return to an endemic area. The aim of this thesis was to examine the effect of both parenteral and topically applied pentamidine dimethane sulphonate on cutaneous lesions caused by L. major, and its interaction with the immune response.

Two models have been established: L. major LV39 infection in Balb/c (inbred) and swiss albino (outbred) mice. L. major produced consistent lesions in these animals following subcutaneous inoculation of 10^5 promastigotes into the ear pinnae.

The effect of the treatment on the parasites was assessed by determining the mean lesion size plotted against time, and by biopsies and smears for detecting residual parasites. The interaction of the treatment with immune responses was assessed by measuring antibody titre, delayed type hypersensitivity responses (DTH) and challenge with live homologous parasites. Topical treatment cured Swiss albino mice before their controls which self-healed. In Balb/c mice eventhough the ulcer regressed they relapsed after termination of treatment, but the level of DTH was higher than their controls, which did not self-heal. Treatment increases the level of DTH response to L. major antigen, more in Swiss than in Balb/c and in those topically treated rather than systemically treated. Antibody titres increased during the development of the lesion, which then leveled off (in Balb/c) but decreased in Swiss albino. But this decreases in swiss albino did not correspond with clinical healing.

After about 12 weeks post treatment all mice were challenged, and no lesions produced in any group during the observation time of 12 weeks. Treatment, even immediately after infection, left the animals with immunity to subsequent challenge.

CHAPTER I

INTRODUCTION

INTRODUCTION

Clinical Aspects of Leishmaniasis

Leishmaniasis is a serious disease in endemic areas, and the World Health Organization has selected it as one of the six major tropical diseases. Leishmaniasis has been reported from about 85 countries and probably more than 100,000 new cases occur each year (McNair, 1961). It affects well over 10 million people world-wide.

CHAPTER I

INTRODUCTION

Leishmaniasis is a group of diseases that have very little in common except that they are caused by the protozoan parasite, *Leishmania*. There are now over 17 different species and subspecies of this genus. These parasites are transmitted to man from infected persons or animals by infected sandflies when taking a blood meal. The organisms of the genus *Leishmania* exist in two forms: the intracellular amastigote form, which lives in a parasitophorous vacuole of a mammalian host macrophage, and the flagellated promastigote which lives in the gut of infected sandflies.

The different species of *Leishmania* produce three different clinical pictures, with numerous variations (Fonseca, et al., 1954). The infection is now common in all continents and is usually acquired by contact with infected sandflies. The clinical picture is usually limited to the skin, but may be systemic (the leishmaniasis syndrome). Following natural inoculation with *Leishmania* parasites is summarized in Figure 1. The three major clinical types: cutaneous, mucocutaneous and visceral and their associated species are shown in Table 1 where it can be seen that a new division is required for the parasites normally visceral leishmaniasis, or wherever they remain in the skin. The exact mechanism controlling this tissue tropism of the parasite is unknown, but it has been suggested that temperature preference may be important (Fonseca, et al., 1954).

INTRODUCTION

Clinical Aspects of Leishmaniasis

Leishmaniasis is a serious disease in endemic areas, and the World Health Organization has selected it as one of the six major tropical diseases. Leishmaniasis has been reported from about 80 countries and probably some 400,000 new cases occur each year (Marinkelle, 1980), and it affects well over 10 million people world wide.

Leishmaniasis is a complex of diseases that have very little in common (Marinkelle, 1980) except for the morphology of the causative protozoa parasites, of the genus Leishmania. There are at least 13 different species and subspecies of this genus. These parasites are transmitted to man from infected persons or animals by infected sandflies when taking a blood meal. The organisms of the genus Leishmania exist in two forms: the intracellular amastigote form, which lives in a parasitophorous vacuole of a mammalian host macrophage, and a flagellated promastigote which lives in the gut of infected sandflies.

The different species of Leishmania produce three different disease patterns, with numerous variations (Faust, et al., 1974). The infection in man ranges from a mild, superficial and localized, spontaneously resolving lesion to systemic disease which is life threatening. The potential sequence of events (the leishmania spectrum), following natural inoculation with Leishmania parasites is summarized in Figure 1. The three major clinical types cutaneous, mucocutaneous and visceral and their associated species are shown in Table 1 where it can be seen that a major division is whether the parasites normally visceralize, or whether they remain in the skin. The exact mechanism controlling this tissue tropism of the parasite is unclear, but it has been suggested that temperature preferences may be important (Pearson, et al., 1983).

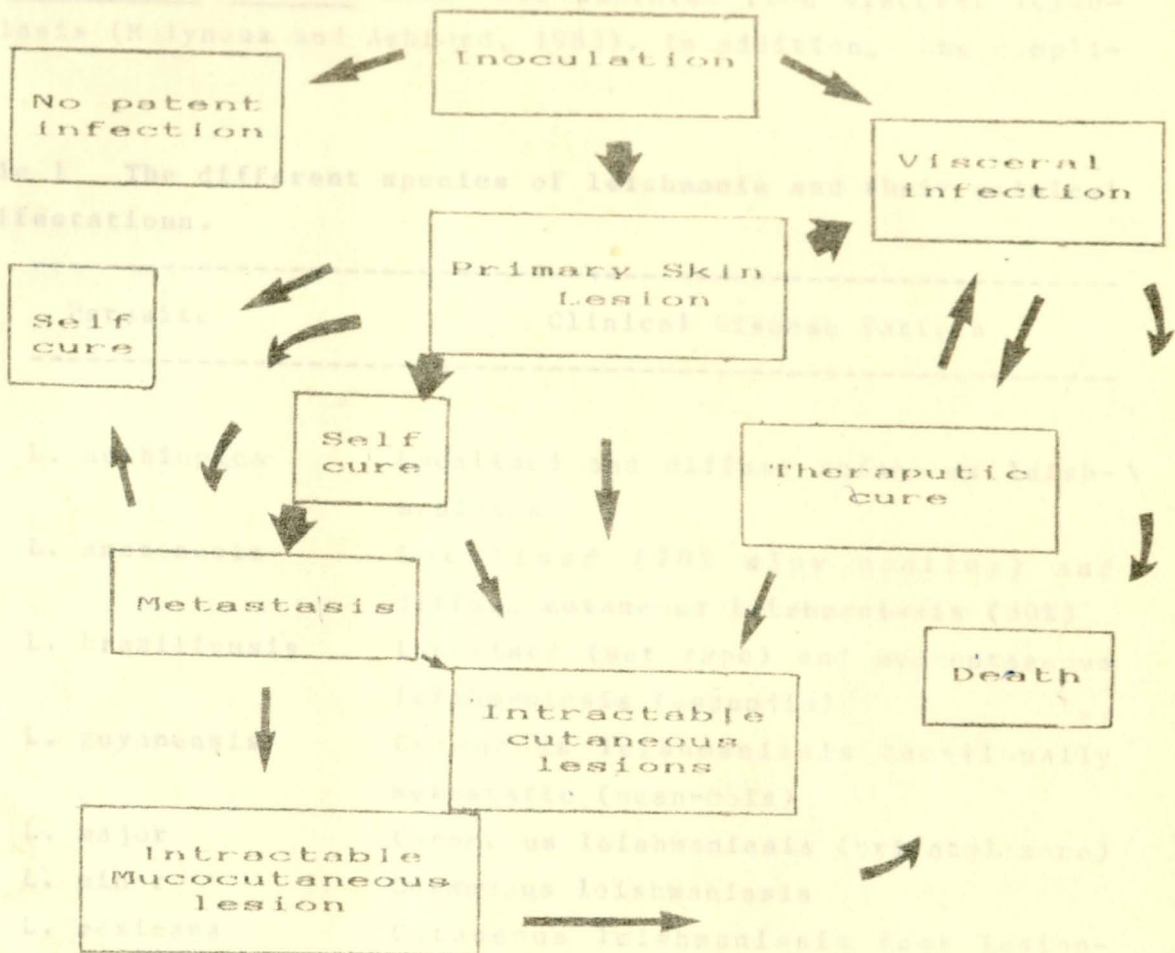


Fig.1; The potential sequence of events the (leishmania spectrum), following natural inoculation with leishmania parasites. (Modified from Molyneux and Ashford 1983).

L. panamensis: Cutaneous leishmaniasis (cutaneous)
L. peruviana: Diffuse cutaneous leishmaniasis
L. guyanensis: Visceral leishmaniasis (kala-azar)
L. donovani: Visceral leishmaniasis (kala-azar)
L. braziliensis: Diffuse cutaneous leishmaniasis (CL)
L. mexicana: Diffuse cutaneous leishmaniasis (CL)
L. major: Diffuse cutaneous leishmaniasis (CL)
L. tropica: Diffuse cutaneous leishmaniasis (CL)
L. infantum: Visceral leishmaniasis (kala-azar)
L. donovani: Visceral leishmaniasis (kala-azar)
L. braziliensis: Diffuse cutaneous leishmaniasis (CL)
L. mexicana: Diffuse cutaneous leishmaniasis (CL)
L. major: Diffuse cutaneous leishmaniasis (CL)
L. tropica: Diffuse cutaneous leishmaniasis (CL)
L. infantum: Visceral leishmaniasis (kala-azar)
L. donovani: Visceral leishmaniasis (kala-azar)

However, this difference is not clear cut and normally viscera-lizing strains can invade the skin and cutaneous strains, such as Leishmania tropica have been isolated from visceral leishmaniasis (Molyneux and Ashford, 1983). In addition, one compli-

Table 1 The different species of leishmania and their clinical manifestations.

Parasite	Clinical Disease Pattern
L. aethiopica	Localised and diffuse cutaneous leish- maniasis
L. amazonensis	Localised (70% slow healing) and diffuse cutaneous leishmaniasis (30%)
L. braziliensis	Localised (wet type) and mucocutaneous leishmaniasis (espundia)
L. guyanensis	Cutaneous leishmaniasis occasionally metastatic (pean-bois)
L. major	Cutaneous leishmaniasis (oriental sore)
L. minor	Cutaneous leishmaniasis
L. mexicana	Cutaneous leishmaniasis (ear lesion- Chiclero's ulcer)
L. panamensis	Localized and persistent cutaneous leishmaniasis (wet type)
L. peruviana	Cutaneous leishmaniasis (uta)
L. pifanoi	Diffuse cutaneous leishmaniasis
L. donovani	Visceral leishmaniasis (kala-azar) occasionally cutaneous (Post kala-azar dermal leishmanoid). Composed of a complex - infantum - donovani - chagas.

Compiled from various sources.

cation of visceral leishmaniasis is Post Kala-azar dermal leishmanoid (PKDL) in which large numbers of the visceralizing parasite, L. donovani are found in the skin. It is clear therefore that the tissue tropisms of various leishmanial species are not fully accounted for by temperature alone.

The experimental model that will be investigated in this study is cutaneous leishmaniasis in laboratory mice caused by the parasite L. major, a species which readily infects rodents and causes cutaneous leishmaniasis in the Old World. The remainder of this introduction will therefore be largely concerned with the clinical and immunological aspects of Old World Cutaneous leishmaniasis.

Human Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) in man, as in leprosy is a spectrum of diseases with clinical manifestations ranging from self-healing lesions to chronic disseminated lesions where both the host and the parasite factors control the pathogenesis of the infection (Scott, et al., 1983). Accordingly, it is classified into three different clinical patterns: self-healing, generalized non-healing (anergic), localized non-healing (ultra-hypersensitive or recidiva). The middle of the spectrum produces the self-healing forms of the disease, the two extremes: the anergic and recidiva are both non-self-healing (Bryceson, 1972).

The prototype of infections caused by Leishmania is the simple localized cutaneous lesion (LCL), classically known as "Oriental Sore" which is a self-healing disease. A nodule appears in the skin, some weeks after inoculation of the parasite by the sandfly which then ulcerates after a period varying from 2-8 months and is covered by a crust. It then starts to heal slowly, leaving in the end a characteristic depressed, mottled

scar (Bryceson, 1976). There are also varying amounts of humoral immunity and delayed hypersensitivity responses produced to leishmania antigen.

The lupoid form or recidiva leishmaniasis occupies one end of the spectrum. The lesion resembles oriental sore; but it never quite heals. Classically, there is a typical scar around whose borders small nodules recrudescence, ulcerate and heal incompletely so that the whole lesion spreads locally. Histologically, the lesion shows an intense cellular response in the form of tuberculoid granuloma, parasites are scanty or absent, and there are an unusual number of plasma cells. The condition is associated with marked delayed hypersensitivity to leishmanial antigens (Bray, et al., 1973).

Mucocutaneous leishmaniasis (MCL) of the New World is caused by L. braziliensis and is characterized by persistent disfiguring lesions that affect the mucous membranes. It is non-healing despite the presence of considerable humoral and cellular immunity throughout the infection. The disease is progressive, arises by metastatic spread, and is often resistant to conventional treatment. A form of mucocutaneous leishmaniasis is also found in the Old World and has been described in Ethiopia (Barnetson, et al., 1978) where it is relatively common. However, unlike the South American variety the mucous lesion normally arises by extension rather than metastatic spread, and is rarely aggressive and responds adequately to treatment. Mucocutaneous leishmaniasis has also been reported from the Sudan (Abdalla, et al., 1975), caused by L. donovani and also progressed slowly and responded well to treatment (Marinkelle, 1930).

At the other end of the spectrum is generalized non-healing or diffuse cutaneous leishmaniasis (DCL), which in the old world occurs only in Ethiopia and Mount Elgon in Kenya, although a

similar disease pattern also occurs in South America, notably in Venezuela. In Ethiopia, it is caused by L. aethiopica, and is associated with L. pifanoi in Venezuela. DCL is characterized by the presence of non-ulcerating nodules, rich in parasites, which do not heal spontaneously but are progressive and spread over large areas of the limbs, face, and occasionally trunk of the patient. Histologically, it is characterized by massive dermal infiltration with parasitized macrophages and an absence of lymphocytes. The leishmanin skin test is negative before treatment (Bryceson, 1970a). As far as is known, both in South America and in Ethiopia DCL is produced by the same strain of parasite that causes LCL lesions. Hence, the difference between the two types of clinical diseases is believed to be due to the modulation of host factors, rather than virulence of the strain involved (Bryceson, 1970b; Bray and Bryceson, 1969). However, it is clear that since DCL only occurs in a restricted number of leishmania species that failure of the hosts immune response is not entirely responsible for the development of this form of leishmaniasis.

Immune responses in Cutaneous Leishmaniasis

Cell Mediated Immunity - The nature of the mechanism controlling the immune response to cutaneous leishmaniasis is not fully understood, but according to most clinical and experimental evidence, CMI is of primary importance. Much of our knowledge regarding immunity to leishmania is based on animal studies and as such, have to be interpreted with caution. The two commonest animal models for leishmaniasis are L. enriettii in the guinea pig, and for Old World species (L. major, L. tropica, and L. donovani) in the mouse and hamster.

During the course of evolution the intracellular leishmania parasites have developed the ability to modify the hazardous

internal environment of the mononuclear macrophages into a suitable habitat. Although the mechanism by which the parasite tolerates the hostile environment of the macrophage is not known, there appears to be a fine balance between the host and the parasite which may last for many years. In most cases, the macrophages are able to control the parasite effectively, for example, a single cutaneous lesion of L. enriettii in the guinea pig, takes less than 10 weeks to heal. This healing is associated with the development of a variety of cell mediated immune mechanisms such as T cell activation, lymphokine production and macrophage activation (Bryceson, et al., 1970). Delayed type hypersensitivity responses to leishmanial antigens are also invariably increased during and after healing, although the exact relationship between immunity and DTH is not clear (Liew, 1986).

Humoral Immunity - The role of humoral immunity in the resolution of cutaneous leishmaniasis is not clearly defined, though anti-leishmanial antibodies will lyse leishmania promastigotes in vitro in the presence of complement (Pearson et al., 1983). In contrast, the high levels of circulating antibodies in visceral leishmaniasis and diffuse cutaneous leishmaniasis show no correlation with clinical improvement (Matossian and Robert, 1975). This lack of association between recovery from leishmanial infections and antibody production has also been demonstrated in a number of laboratory models (Preston, et al., 1978; Rezai, et al., 1980; Pearson et al., 1983). Thus, although there is no clear cut direct effect on the course of leishmanial infection it is possible that antibody may modulate the role of CMI in recovery from infection (Mauel and Behin, 1974; Poulter, 1980; Hale and Howard, 1981).

Impaired Immunity - The immunological response in the pathogenesis of the various clinical manifestations of leishmaniasis are mainly as a result of the hosts immune response; however,

the nature of the infecting parasite does play a role in the outcome of the infection (Bryceson, 1969,1975; Ridley, 1979; Belehu, Louis, Pugin and Miescher, 1980; Scott, Sacks and Sher, 1983). The main immunological features of cutaneous leishmaniasis are shown in Table 2. Thus, although development of cell mediated immunity is normally associated with healing, lesions such as Chiclero's ulcer in South America and leishmania recidiva in the Old World may fail to heal despite the development of delayed type hypersensitivity reactions. Conversely, leishmania infections may fail to self-heal due to impaired immunity, particularly cellular responses, as in the case of Diffuse Cutaneous Leishmaniasis where, despite an enormous parasite load the patients fail to develop any cell mediated immune reactions to leishmanial antigens. Although the underlying mechanism responsible for these quantitative differences in the response of these cells (Liew, 1985). However, it should be

Table 2 Principal immunological features of cutaneous leishmaniasis.

Parasite	Disease	DTH	Antibody	Parasite	Selfcure
L. aethiopica	LCL	+/-	+	+	+
	DCL	-	+/-	+++	-
L. braziliensis	LCL	+	+	+	+
	MCL	+	+	+/-	-
L. major	LCL	+	+	+	+++
L. mexicana	LCL	+	+/-	+	+
	DCL	-	+/-	+++	-
L. minor	LCL	+++	+/-	+/-	-
L. tropica	LCL	+	+/-	+	+

- No or Absent; + Present; +++ Rapid or Abundant; +/- Viable or weak (Modified from WHO, 1984).

for this anergic state is unknown, it is generally believed to be due to specific cell mediated immune deficiency in the host, possibly resulting from local lymphatic damage prior to infection or because of a genetically based defect in cell mediated immunity (Bryceson, 1970b). Experimentally, diffuse infections can be induced by prior immunosuppression (Lemma and Percy, 1973; Kadiivar and Soulsby, 1975) and as a result of genetically based defects in host immunity (James, et al., 1980; Howard, et al., 1981). In Balb/c mice the genetic defect in responses to leishmania appears to be mediated by the same T cells that are responsible for protection, either because of heterogeneity in this T cell population or because of quantitative differences in the numbers of these cells (Liew, 1986). However, it should be remembered that although experimental procedures will induce disseminated spread of an otherwise localized infection, that in general, dissemination under experimental conditions includes visceralization and is not restricted to the skin as in human DCL.

Treatment - Traditionally, cutaneous leishmaniasis has been treated with corrosive poultices prepared from plant juices such as Euphorbia and Ficus species, or blister beetles (Meloidae). later pastes of sulphuric acid and charcoal, and infra red radiation of the lesion, hot compresses, diathermy, scraping, curettaging, topical solid carbon dioxide and local infiltration with anti-leishmanial drugs were used (Reviewed by Molyneux and Ashford, 1983; Currie, 1983). In conventional clinical practice and experimental studies, many chemotherapeutic agents have been used (Table 3) although the efficacy of many of them is doubtful, some are toxic, and most of them need parenteral administration. In addition, standard regimens which do not require close patient supervision have not been developed.

sodium stibogluconate (Pentostam, Wellcome, U.K.) and meglumine antimonate (Glucantime, Specia, France). However, these drugs are potentially toxic and, at the high doses recommended for mucocutaneous leishmaniasis, they are invariably accompanied by serious side effects such as arthralgia, myalgia, anorexia etc. (Marsden, 1986).

In many parts of the world cases of antimony resistant leishmaniasis are treated with Amphotericin B (Fungizone, Squibb, U.S.A.). However, its use is again limited since it is seriously nephrotoxic, and since it is generally given intravenously patients have to be under close medical supervision. The other drug recommended for second line therapy in cases of antimony resistant leishmaniasis are the pentamidine derivatives of diamidine, pentamidine dimethane sulphonate (Lomidine, Specia, France) and pentamidine isethionate (Pentamidine, May and Baker, U.K.). However, these drugs also have serious toxicity problems and are both cardiotoxic and cause diabetes mellitus, although the induction of diabetes have been reported to be less marked with pentamidine isethionate (Belehu, 1982). Patients undergoing therapy must therefore have frequent clinical, electrocardiographic and biochemical monitoring.

Metronidazol (Flagyl, May and Baker, U.K.) used at a dose of 250mg orally has been reported to be successful against cutaneous leishmaniasis in Mexico (Long, 1973) although other studies in both the New and Old Worlds have been unable to demonstrate any effect (Walton, et al., 1974; Belehu, et al., 1978).

However, the majority of drugs used in the treatment of leishmaniasis are ineffective in the treatment of Ethiopian cutaneous leishmaniasis caused by L. aethiopica and the only drugs that are routinely effective against L. aethiopica are pentamidine compounds (Lomidine, Specia, France and Pentamidine, May and Baker, U.K.)

Table 3 Drugs used clinically and experimentally in the treatment of cutaneous leishmaniasis

DRUG	ROUTE	CLINICAL	EXPERIMENTAL
Aminoarsenophenol	il	++	NR
Amopyroguine	sc	NR	++
Amphotericin B	sc	NR	+++
Antimonial	iv	++	-
Antimonial	topical	NR	+
Anthiomaline	iv	++	NR
Chloroquine	sc	++	-
Benznidazole	sc	NR	++
Berberine sulphate	sc	NR	+++
Chloroquine	il	++	NR
Chloroquine	oral/sc	+	NR
Chlortetracycline	sc	NR	-
Clindamycin	sc	NR	++
Cycloguanil	sc	NR	-
Cycloguanil	im	+	NR
Dapsone	oral	NR	+
Dehydroemetine	oral	+	NR
Diformyl dapsone	sc	NR	-
Diminazine aceturate	sc	NR	+++
Emetine HCl	sc	NR	+++
Erythromycin	sc	NR	++
Griseofulvin	oral	-	NR
Hydroxynaphthoate	sc	NR	-
Macrocyclone	sc	NR	-
Mefloquine	sc	NR	++
Menoctone	sc	NR	+
Mepacrine methanesulphonate	sc	NR	++

Continued overleaf

DRUG	ROUTE	CLINICAL	EXPERIMENTAL
Metronidazole	oral	++	-
Moxipraguine	im	NR	++
Neomycine	oral	++	NR
Nifurtimox	oral	+	++
N-methylglucamine	im	++	++
Paromomycin	ip	NR	++
Paromycin sulphate	Topical	NR	+++
Pentamidine comp.	im	++	-
Primaquine	oral	+	+
Pyrimethamine	oral	++	-
Rifampicin	oral	++	+
Sodium stibogluconate	im	++	++
Solusurmin	oral	++	NR
Stibophen	im	+	++
Sulphadiazine	oral	NR	-
Sulphadoxine	oral	NR	-
Sulphaguinoxaline	oral	NR	+
Sulphamonomethoxine	oral	NR	-
Streptomycin	im	++	NR
Trimethoprim	sc	NR	++

im, intramuscular; ip, intraperitoneal; il, intralesional; sc, subcutaneous; ++, effective; +, slightly effective; -, not effective; NR, not reported.

Compiled from various sources.

Since most forms of cutaneous leishmaniasis are self-healing the use of pentavalent antimony compounds (which are poorly effective) and pentamidine which has serious side effects are not easily justified.

A number of novel methods of treatment have been investigated:

- a) Encapsulation of drugs into liposomes or red cell ghosts coupled to IgG are one method of delivering the drug directly to the infected macrophage and in this way the effective dose can be reduced by 80% (Berman and Aikawa, 1984; Blacks and Watson, 1977).
- b) Topical treatment is one possibility which might circumvent the disadvantages of systemic treatment and successful use of topically applied drugs such as pentamidine and paromomycin (PR, Humatin, Parke, Davis and Co.) has been reported (El-eh, et al., 1984; Humber, et al., 1983).
- c) A variety of other methods such as transfer factor (Bryceson, 1970b), localized heating (Neva, et al., 1984), surgical removal (Bryceson, 1970a) and curettage (Currie, 1983) have been investigated.

In terms of control of leishmaniasis, treatment plays an important role however, ideally, treatment should leave the patient with an adequate immune response in order to prevent re-infection following return to an endemic area. Little information is available regarding the effect of treatment on immune responses to leishmania, although it has been reported that protective immunity to reinfection only occurs following successful healing of a primary lesion (Bryceson, et al., 1970). However, Green, and colleagues (1983) have demonstrated that immunity may develop following the development of non-ulcerating nodules among human subjects vaccinated with live L. major. Since protective immunity follows infection and resolution of leishmaniasis, inoculation of live leishmania has been practiced in

protective immunity follows infection and resolution of leishmaniasis, inoculation of live leishmania has been practiced in the USSR and Israel for many years (cited by Marinkelle, 1980). This method of vaccination employs live non-attenuated strains of L. tropica to protect against infection; and the vaccination disease may be as severe as the naturally contracted infection. However, there is a cosmetic advantage in the fact that only one lesion is normally produced and it is in a cosmetically acceptable area.

MATERIALS AND METHODS

Since the drugs of choice in Ethiopian cutaneous leishmaniasis are pentamidine compounds, one of these drugs was chosen for this investigation. However, parenterally administered pentamidine is severely toxic and therefore a second treatment regimen, topical treatment, has been included for comparison. The topical treatment regimen chosen is one that has been used routinely at the All Africa Leprosy Rehabilitation and Training Center (ALERT) for LCL (Humber et al. 1983). Ideally, an animal model for L. aethiopica would have been chosen, however, since there is at present no suitable animal model for this parasite an alternative L. major in mice was chosen. In addition two strains of mice were used one of which, Balb/c, is permissive to L. major infection and the other, Swiss albino which produces a self-healing lesion similar to human LCL.

The aim of this study was to examine the effect of both parenteral and topically applied pentamidine on the course of leishmania infection and whether chemotherapy alters the development of immune responses to leishmania antigens.

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Leishmania Parasites: The species of leishmania used in this study, was L. major LV 39 (derived from a strain isolated from a wild rodent and obtained as a kind gift from the Max Plank Institute, Germany). The parasite was maintained by serial passage in Balb/c mice. Promastigotes were kept in culture for a maximum of 3 to 4 weeks in an attempt to prevent loss of infectivity. Mice were routinely inoculated subcutaneously into the ear margin with 10^5 Promastigotes. Promastigotes were normally obtained by culturing tissue obtained from the cervical lymph nodes draining these infections.

Preparation of inocula and inoculation of animals: Promastigotes used for inoculation were grown in RPMI 1640 culture medium (Flow Laboratories, U.K.) supplemented with 20% foetal calf serum with the addition of penicillin and streptomycin (200 units and 200ug per milliliter respectively). Prior to the preparation of the inocula, a growth curve was determined for L. major using triplicate bottles, each containing 10 ml of RPMI media, and incubated at 28°C. Samples were diluted with an equal volume of counting fluid (phosphate buffered saline, pH 7.2 containing 1% formaldehyde) to immobilize the parasites during counting using the hemocytometer. The promastigotes for infecting the animals were harvested from stationary phase cultures by centrifuging at approximately 800g for 15 minutes and resuspending in phosphate buffered saline prior to inoculation. The number of flagellates was estimated using a hemocytometer, following a 1:1 dilution of the suspension in counting fluid.

The ear pinnae of the animals were inoculated subcutaneously using a 30 gauge needle. Considerable care has to be taken with mouse ears since the pinnae are extremely thin.

Relationship between inoculum size and lesion development: A study was made of the influence of inoculum size on the nature and time course of the subsequent lesions in Balb/c and Swiss albino mice. A suspension of promastigotes harvested in the standard manner was diluted to yield inocula containing approximately 10^3 , 10^4 , 10^5 and 10^6 promastigotes per 0.05 ml. Each group of 4 (Balb/c) and 5 (Swiss albino) mice were examined at weekly intervals, and the extent of the skin lesions was assessed using a graded scale. Lesions were assessed clinically using a graded scale of +(1) to ++++(4). Large lesions with secondary infection involving most of the ear were graded as 4; ulcerated lesions involving approximately half of the ear as 3; small non-ulcerating lesions 2; localized induration 1 and no apparent lesion as 0.

Experimental animals: The mice used in this study were housed under conventional conditions in the Department of Biology animal house and fed animal pellets prepared by the Ethiopian Poultry and Dairy Enterprises, Addis Ababa. Mice were provided with water ad libitum. Two strains of mice were used; Outbred Swiss albino and inbred Balb/c both 6 to 7 weeks of age. Within a single experiment only one sex was used. For each strain there were 8 groups each containing either 10 mice (Balb/c) or 15 mice (Swiss albino). The groups were treated according to the following schedule:

Topically treated with pentamidine in 60% dimethyl sulphoxide (DMSO)

- Immediately post infection
- Two weeks post infection
- Three weeks post infection
- Control (treated with DMSO only)

Systemically treated with pentamidine

Immediately post infection

Two weeks post infection

Three weeks post infection

Control (untreated)

Treatment and Drugs and Clinical Assessment: The drug used during these experiments was powdered pentamidine dimethane sulphonate (Lomidine, generously donated by Specia, France). The topical preparation was prepared by dissolving 70mg of the drug per milliliter of 60% dimethyl sulphoxide (DMSO, Sigma). The parenteral preparation was 70mg per milliliter in sterile saline.

Pentamidine was applied topically by administering 0.05ml of the solution to each infected ear and was given parenterally by a weight adjusted intraperitoneal injection of 0.2ml daily (Balb/c mice) or 0.3ml (Swiss albino).

Histology: Tissue for histological examination was fixed in 10% buffered formalin, dehydrated, imbedded in paraffin wax and sectioned. The sections were stained with haematoxylin and eosin. Smears were prepared from lesions and other tissues, and stained with geimsa.

Delayed Hypersensitivity: The development of delayed type hypersensitivity was determined by the increase in foot pad thickness following the intradermal inoculation of 2×10^6 glutaraldehyde fixed L. major promastigotes into the right foot pad. Changes in foot pad thickness were measured at 24 hour intervals using a dial caliper (Schnelltaster, Germany) using the contralateral foot pad as a control.

Antibody Assay: Antibodies to leishmania antigens were assessed using a micro agglutination assay. Mice were tail bled every

two weeks upto 10 weeks after infection, and the serum separated and kept frozen at -20°C until required. Doubling dilutions (final volume of 50 μl) of the test antisera were prepared in flat bottomed 96 well microtitre plates (Flow Laboratories, U.K.) and to each well was added 50 μl of stationary phase promastigote suspension (2×10^6 per ml). The plates were incubated at 37°C for one hour. The degree of agglutination was assessed using an inverted microscope equipped with phase contrast. The last well with agglutinated clumps of promastigotes was taken as the end point.

Challenge: Six months after infection all mice were challenged with 10^5 stationary phase L. major promastigotes injected into the base of the tail. The animals were examined over a period of 3 months to assess the development of any lesion. Control animals were also inoculated.

Statistics: Means standard deviations and independant T tests were calculated using a Texas Instruments programmable calculator (TI 59).

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

L. major causes cutaneous leishmaniasis in man. In most cases it is confined to the skin, but in rare cases it metastasizes and invades the reticulo-endothelial cells of the viscera (El-On et al., 1984). In Balb/c mice it causes skin lesion as well as visceral infection and there is no self cure. Therefore during the present experiment the local clearance of the parasites in Balb/c mice is attributed to drug treatment only. On the other hand, L. major produces self-healing lesion in Swiss albino outbred mice which is one of the resistant strains and no visceralization was reported in these mice. Therefore the results must be interpreted with caution. In the present experiment the untreated controls healed after 20 weeks and the treated ones healed after 12 weeks, thus treatment accelerated healing.

The severity of the infection determines the ease of demonstration of drug action and it is affected by factors such as inoculum size and age of culture. Therefore experiments were carried out to investigate the lowest inoculum size (10^5) which is liable to produce apparent lesion in both strains of mice and a growth curve was determined.

Growth curve - The growth of L. major LV 39 in RPMI-1640 medium supplemented with 20% foetal calf serum was studied. Triplicate cultures of parasites were incubated at 28°C and counted daily for 12 days. The mean parasite growth was plotted against time (Figure 2). The growth was logarithmic. The stationary phase was reached between day 7 and 9, and the parasites started to die by day 10. During this experiment promastigotes for infecting the mice were harvested from the stationary phase of the growth, for promastigotes are said to be more virulent at this phase of growth (Sacks and Perkins, 1984)

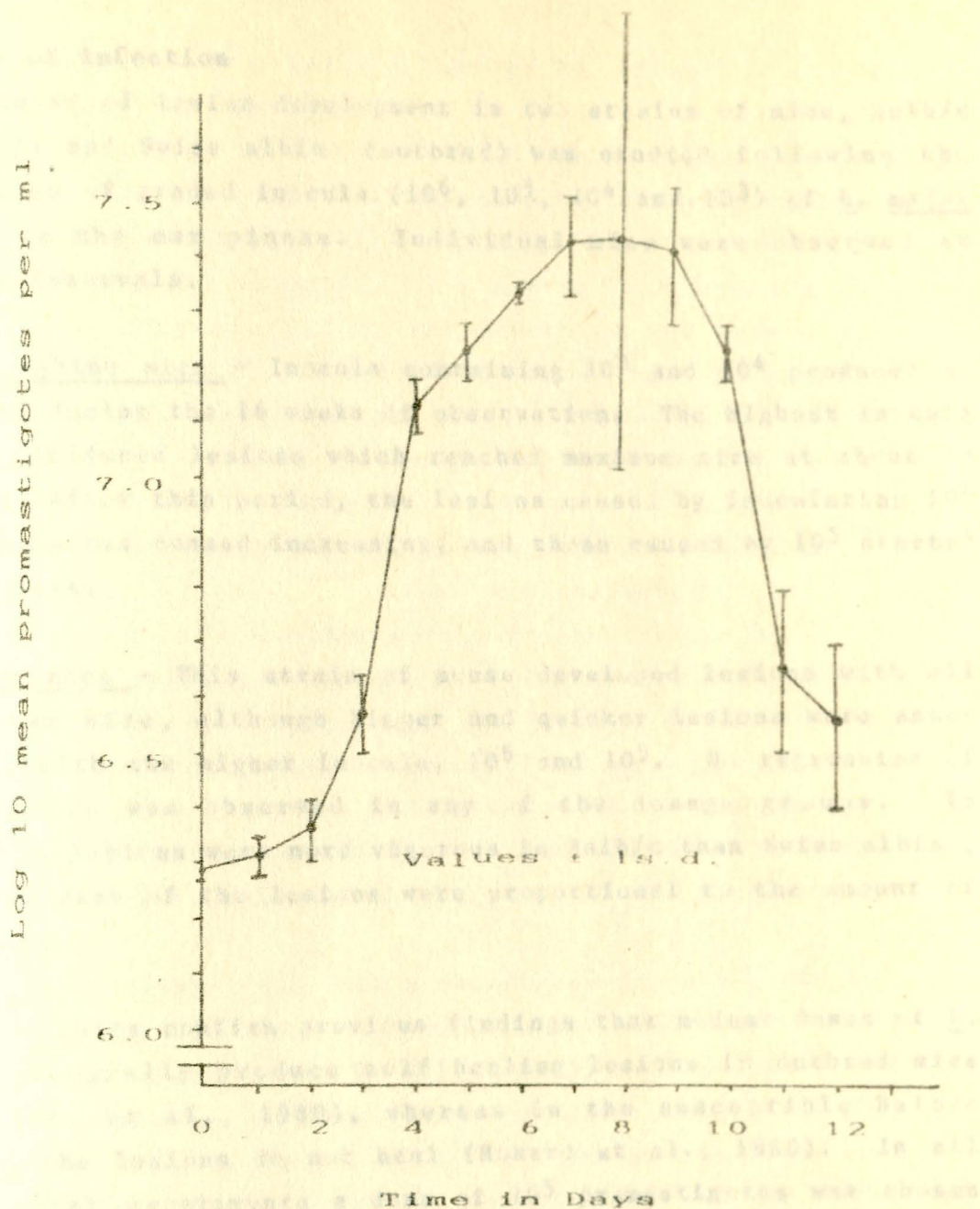


Fig.2: Growth curve of *L. major* LV39 at 28°C in RPMI-1640 media.

Course of infection

The course of lesion development in two strains of mice, Balb/c (inbred) and Swiss albino (outbred) was studied following the injection of graded inocula (10^6 , 10^5 , 10^4 and 10^3) of L. major LV 39 in the ear pinnae. Individual mice were observed at weekly intervals.

Swiss albino mice - Inocula containing 10^3 and 10^4 produced no lesions during the 16 weeks of observation. The highest inocula (10^6) produced lesions which reached maximum size at about 14 weeks. After this period, the lesions caused by inoculating 10^6 promastigotes ceased increasing, and those caused by 10^5 started to regress.

Balb/c mice - This strain of mouse developed lesions with all inoculum size, although bigger and quicker lesions were associated with the higher inocula, 10^6 and 10^5 . No regression of the lesion was observed in any of the dosage groups. In general, lesions were more vigorous in Balb/c than Swiss albino, and the size of the lesions were proportional to the amount of inoculum.

These results confirm previous findings that modest doses of L. major generally produce self healing lesions in outbred mice (Trotter, et al., 1980), whereas in the susceptible Balb/c strain the lesions do not heal (Howard et al., 1980). In all subsequent experiments a dose of 10^5 promastigotes was chosen since this was the lowest dose which consistently produced lesions in both strains of mice.

Effect of treatment on the development of the lesion- Drug activity was evaluated by determining the change in lesion size, and by assessing residual parasites in smears and sections taken from lesion and other sites. Improvement seen while under treatment in all animals was attributed to the treatment. The

dose selected was a level known to be effective clinically against cutaneous leishmaniasis.

After subcutaneous inoculation of 10^5 promastigotes of L. major LV 39 into the ear pinnae of Balb/c mice, a lesion started as a small nodule which gradually enlarged, ulcerated and generally became secondarily infected. The lesion progressively enlarged towards the periphery, and the margin of the ears started to erode. This condition persisted for more than 5 months and no self cure was observed in this mouse strain. On the other hand, Swiss albino inoculated similarly developed small nodules which gave rise to small lesions 2 to 3 mm in diameter and healed slowly after about 20 weeks. During this experiment both mouse strains were treated topically and systemically at different times post infection: Immediately treated after infection at 2 weeks and 3 weeks post infection. The lesion sizes was determined in each mouse weekly and the average score was computed using the scale described in material and methods. These scores are tabulated against time and Tables 4 and 5 show the effect of pentamidine methane sulphonate (Lomidine) given topically and systematically to Swiss albino and Balb/c mice respectively.

Topical treatment- Both mice strains were treated topically with 0.05 ml of 70 mg per ml of pentamidine dimethane sulphonate (Lomidine) incorporated in 60% dimethyl sulphoxide. DMSO has been used in many pharmaceutical ointments for increasing drug penetration (Review by Idson, 1975). Its median lethal dose in mice is approximately 20g/Kg body weight and is therefore virtually non-toxic to mice. The preparation was applied directly to the lesion daily for 7 weeks. Treatment was started at different times post infection to observe the effect of timing of onset of treatment on subsequent development of immunity. Controls were treated immediately with only 60% DMSO.

Table 4 Course of infection in Swiss Albino mice treated with pentamidine

Treatment		Weeks post-infection									
Group		1	2	3	4	5	6	7	8	9	10 20
Topical											
Immediate		-	++	++++	++++	+++	++	++	++	++	- -
2 weeks		-	-	++	+++	+++	++++	+++	++	+	+ -
3 weeks		-	-	-	+	++	+++	+++	++	++	- -
DMSO		-	-	+	+	++	++	++	++	++	++ -
Systemic											
Immediate		-	-	-	-	+	+	+	+	++ ++	-
2 weeks		-	-	-	-	-	+	+	+	+	-
3 weeks		-	-	-	-	-	-	+	++	++ -	-
Untreated		-	-	-	+	++	++	++	++	++ ++	-

Table 5 Course of infection in Balb/c mice treated with pentamidine

Treatment		Weeks post-infection									
Group		1	2	3	4	5	6	7	8	9	10 20
Topical											
Immediate		+	++	++++	++++	+++	++	++	+	+	+ M
2 weeks		-	-	+	+++	++++	++++	++	++	+	+ M
3 weeks		-	-	+	+	++	+++	+++	++	+	+ M
DMSO		-	-	+	+	++	++	++	++	++	++ ++
Systemic											
Immediate		-	+	+	+	++	+++	+++	+++	++ ++	M
2 weeks		-	-	-	+	+	+	+	++	++ ++	M
3 weeks		-	-	-	+	+	+	+	+	+	M
Untreated		-	-	-	+	++	+++	++	++	++ ++	++

Swiss albino mice treated immediately and at 2 weeks post infection developed lesions earlier than their controls and produced the biggest lesion by the third and sixth week respectively. Those treated after three weeks had lesions one week after the controls and ulceration was at its maximum at about the same time as the 2nd week treatment group but was less prominent. Ulceration was earlier, and vigour in the immediately treated mice than those treated later.

Treatment resulted in erosion of the ear pinnae particularly in the immediately treated mice. The controls (DMSO treated) produce indurated lesion after 2 weeks and small lesion developed after 4 weeks, then healed spontaneously after 20 weeks post infection with normal ears. After 12 weeks, all 3 treated groups were healed clinically and parasitologically as confirmed by smears and sections from the site of healed lesion and other sites (Table 6). The controls had parasites in the lesion and cervical lymph node which directly drain the lesion, but not in the spleen.

The development of lesions in Balb/c mice treated in the same manner, was accelerated in those immediately treated than the other treatment groups. The group treated after 2 weeks had more or less the same lesion development as that of the immediately treated, and those treated after 3 weeks developed lesions which were less vigorous than the above two groups, and persisted throughout the observation period. Ulceration reached its maximum after three weeks for the immediately treated and five weeks for those treated after 2 weeks and for those treated three weeks post infection after 6 weeks. The DMSO treated controls developed indurated lesions after 2 weeks and small lesions after 5 weeks which persisted up to the end of the observation period. No parasites were detected in smears or biopsy material in those immediately treated, but parasites were found in the spleens of mice treated 2 and 3 weeks after

Table 6 The presence and absence of parasites in biopsy material 12 weeks after infection

Treatment Group	Lesion		Lymph node		Spleen	
	Biopsy	Smear	Biopsy	Smear	Biopsy	Smear
Balb/c Topical Treatment						
Immediate	-	-	-	-	-	-
2 weeks	-	-	-	-	-	+
3 weeks	-	-	-	-	-	+
Control	+	+	-	+	-	+
Balb/c Systemic Treatment						
Immediate	-	-	-	-	-	-
2 weeks	-	+	-	-	-	-
3 weeks	-	-	+	-	-	+
Control	+	+	-	+	-	+
Swiss Albino Topical						
Immediate	-	-	-	-	-	-
2 weeks	-	-	-	-	-	-
3 weeks	-	-	-	-	-	-
Control	-	+	-	+	-	+
Swiss Albino Systemic Treatment						
Immediate	-	-	-	-	-	-
2 weeks	-	-	-	-	-	-
3 weeks	-	-	-	-	-	-
Control	+	+	-	+	-	+

infection. This may suggest that immediate treatment prevent visceralization of parasites in Balb/c mice.

In general in both strains of mice indurated lesions started with the onset of treatment before the controls. Ulceration was more prominent following topical treatment and ear erosion was more pronounced in those immediately treated than the other treatment groups.

Systemic treatment - To assess the influence of the route of administration on the efficacy of the drug, different groups of both mice strains were treated intraperitoneally with lomidine. A daily injection of 0.3 ml and 0.2 ml of 70 mg per ml/Kg body weight of pentamidine was given for 50 days to Swiss albino and Balb/c mice respectively. All treated Swiss albino produce lesion after their controls (Table 4). From these results it seems that systematic treatment retards lesion development. But among the treated groups, those immediately treated developed lesion earlier than the rest of the group. Besides healing earlier, generally the course of infection of the treated mice was not significantly different from the untreated controls. The ulcers were smaller than those topically treated and healed after about 12 weeks after infection, most with normal ears. At this time smears and biopsy materials show no parasites, but the control had parasites in smears taken from the lesion (Table 6). The untreated controls developed small lesion by the fifth week which self healed after about 20 weeks.

On the other hand, immediate treatment induce earlier induration in Balb/c mice. The other treated groups developed indurated lesions about the same time as their controls, and in the group treated 3 weeks after infection this induration persisted up to the 10th week and no ulceration was observed. The 2nd week treatment group developed small lesions after 8 weeks which persisted up to the 10th week. The immediately treated groups

had ulceration greater than the controls. After 12 weeks of infection the lesions in the treated groups regressed. Smears and biopsy materials showed no parasites in the immediately treated groups, but lesions of the 2nd week treatment group and lymph node and spleen of the 3rd week treatment group revealed parasites. It would seem therefore that topical treatment is superior to parenteral, in that no parasites were detected in lesions of the topically treated groups.

After 20 weeks all swiss albino (treated and untreated) were cured and had no disseminated lesion. A different picture emerged in Balb/c mice in that all produced metastatic lesions. Amastigotes were detected from smear of these lesion. The dissemination seemed to have a pattern in that all were occurred on the extremities. Most of the mice died after this condition. El-On et al. (1984), observed metastatic lesion on topically treated Balb/c mice after termination of paromomycin sulphate treatment, and considered this condition to be caused by the migration of unaffected parasites from the internal organs. The development of necrotic lesion on the extremities of L. enrittii infected guinea pigs following sodium stibogluconate treatment was also observed by Neal and Miles (1977) and they associated this condition with incomplete activity with the drug rather than acquired drug resistance, because transfers from these metastases confirmed that the sensitivity of the leishmania to glucantime was unaltered.

Interaction between Host Immune Response and Pentamidine Treatment

Humoral Response- The antibody titres to L. major during pentamidine treatment are shown in Figures 3 and 4. The titre of antibody in Swiss albino (treated or untreated control) was generally less than that of the Balb/c mice (Tables 7 and 8). There was an increase of antibody titre in both strains during the first 6 weeks of the lesion, which then leveled off in

Balb/c mice, but decreased in the Swiss albino. The decrease in antibody titre in the Swiss albino did not correspond to clinical healing, although Walton (1980) observed that clinical improvement and healing of lesions was accompanied by a diminution of antibody titre to amastigotes (Fluorescent antibody assay). In addition he found that those in which reversion to sero-negativity did not occur, a relapse occurred. In Balb/c mice the titre did not decrease (Figures 3 and 4) which may be due to the progression of the disease. Treatment seems to slightly, but not significantly, increase antibody production, since in all four groups, the treated groups were marginally higher than the controls.

Delayed type Hypersensitivity Responses (DTH)- The time curves of footpad enlargement following the injection of 2×10^6 glutaraldehyde fixed promastigotes 12 weeks post infection are shown in Figures 5, 6, 7 and 8. As can be seen from the shapes of the curves foot pad testing of these mice with L. major antigen shows a classical DTH pattern, begins at 24 hours, peaks at 48 hours and then decreases toward 72 hours. Figures 5 and 6 show the DTH time curve following the testing of Swiss albino mice treated topically and systematically respectively. Figures 7 and 8 show the DTH response of topically and systematically treated Balb/c mice to the same antigen respectively. It is apparent from these curves, that there is, as might be expected, greater DTH in Swiss than in Balb/c mice (Table 9). This could be due to visceralization of the parasite in Balb/c mice which causes relapse later on. According to Preston et al. (1978) elimination of DTH is often associated with progressive disease.

Log 2 Antibody Titre

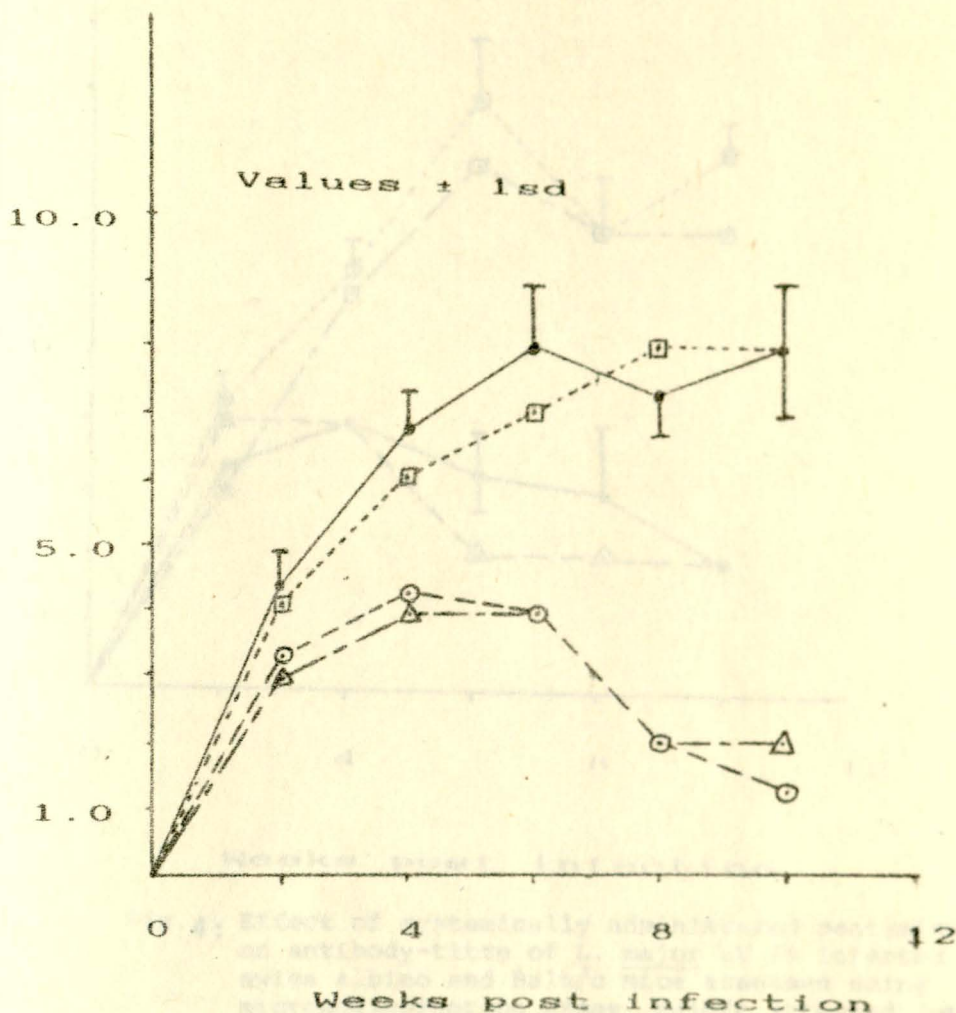


Fig.3: Effect of topically applied pentamidine on antibody-titre of *L. major* LV-39 infected swiss albino and Balb/c mice assessed using microagglutination assay. Balb/c (treated (—●—) and controls (—□—)); swiss albino (treated (—○—) and controls (—△—)).

Log 2 Antibody Titre

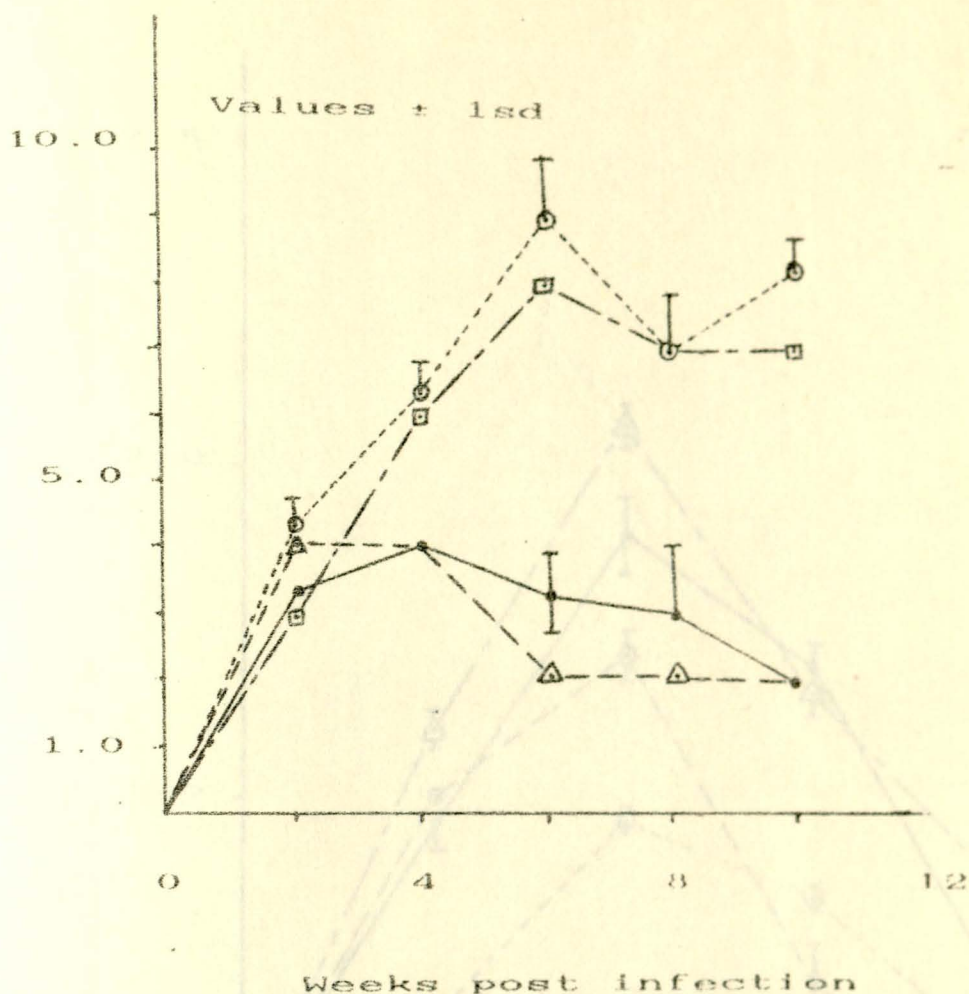


Fig. 4: Effect of systemically administered pentamidine on antibody-titre of *L. major* LV 39 infected swiss albino and Balb/c mice assessed using microagglutination assay. Balb/c (treated (●) and control (◻)); swiss albino (treated (▲) and control (△)).

Fig. 5: Effect of topical treatment with pentamidine on the skin response of swiss albino to *L. major* LV 39. Immediately post infection P.I. (●), 1 week P.I. (◻), 3 weeks P.I. (▲) and untreated controls (△).

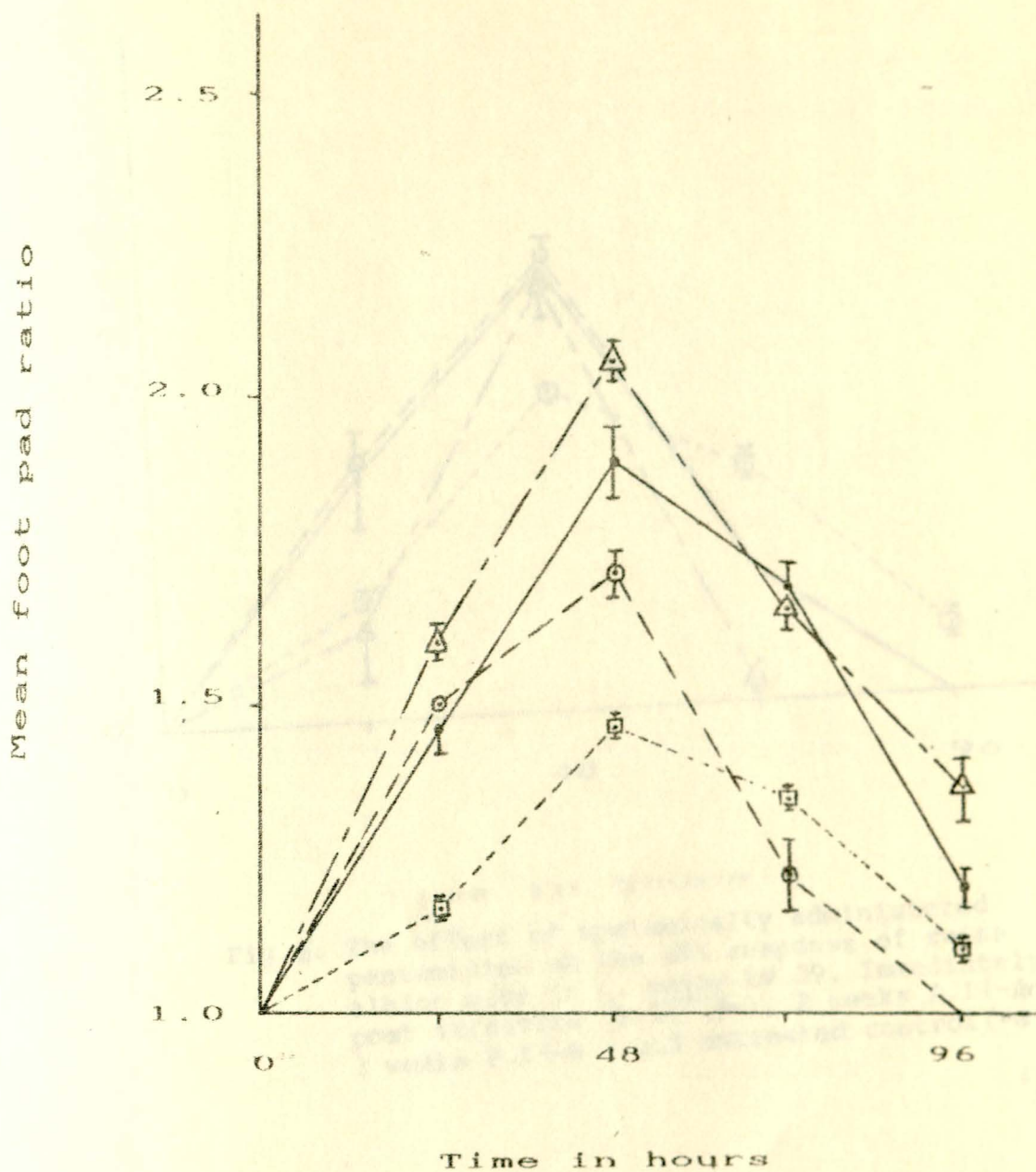


Fig. 5: The effect of topical treatment with pentamidine on the DTH response of swiss albino to *L. major* LV 39. Immediately post infection (P.I.) (-Δ-), 2 weeks P.I. (-→-), 3 weeks P.I. (-●-) and untreated controls (-□-).

Mean foot pad ratio

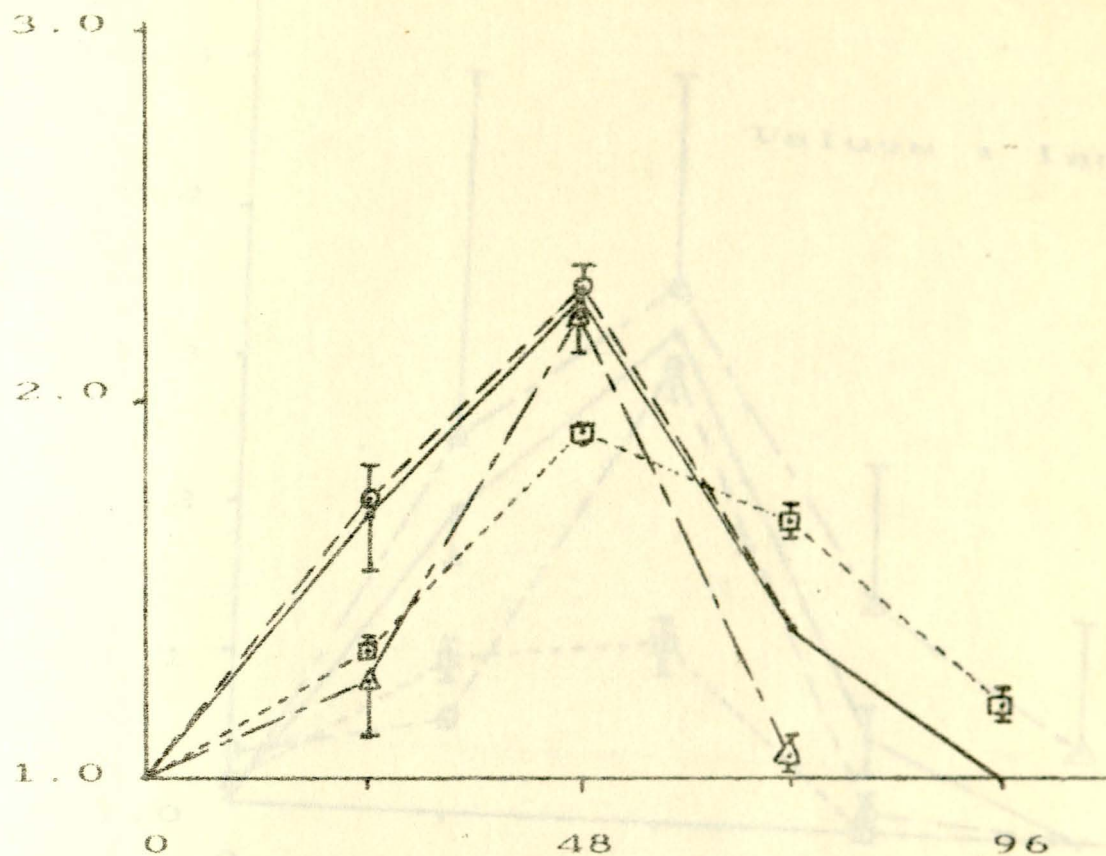


Fig. 6: The effect of systemically administered pentamidine on the DTH response of swiss albino mice to *L. major* LV 39. Immediately post infection (P.I.) (○-), 2 weeks P.I. (△-), 3 weeks P.I. (□-) and untreated control (○-).

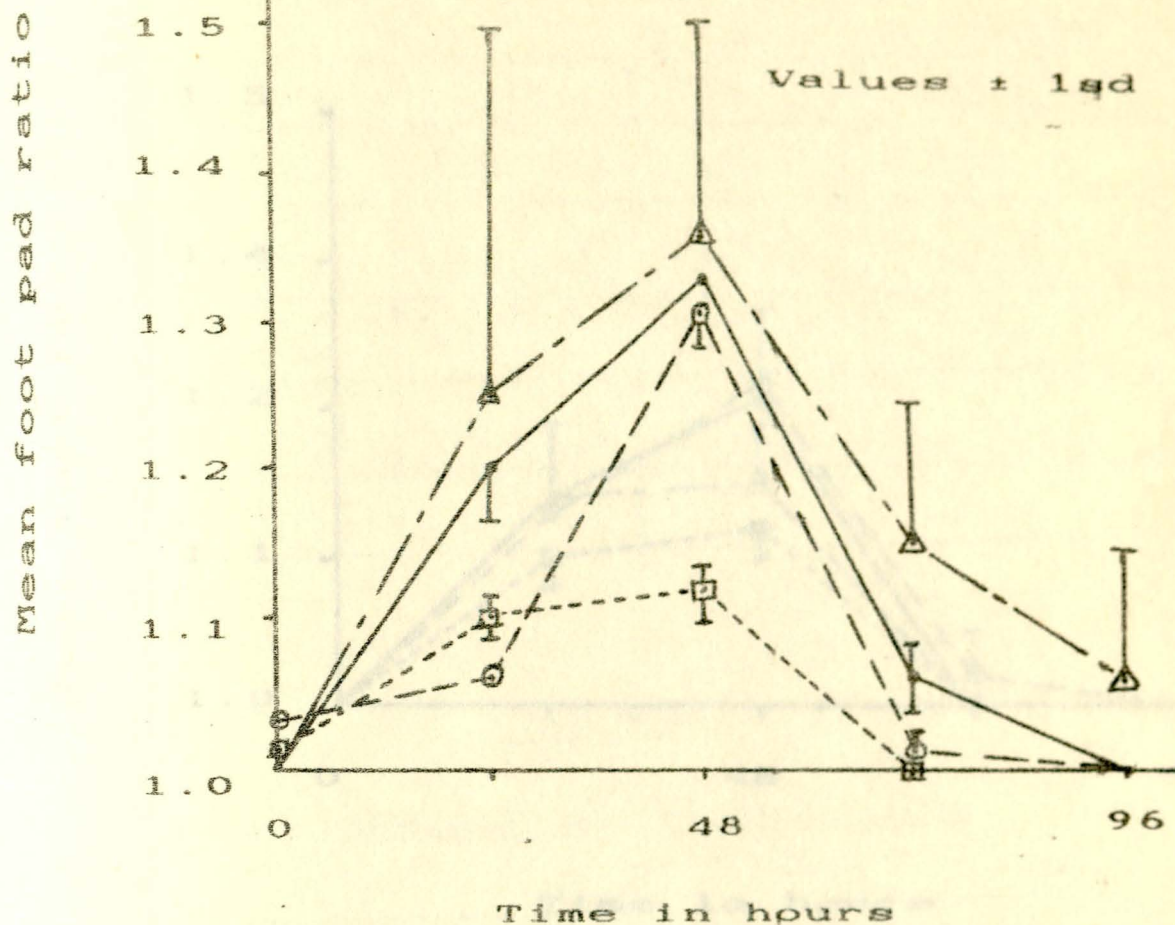


Fig.7: The effect of topical treatment with pentamidine on the DTH-response of Balb/c mice to *L. major* LV 39. Immediately post infection (P.I.) (-▲-), 2 weeks P.I. (-●-), 3 weeks P.I. (-○-) and untreated controls (-□-).

Mean foot pad ratio

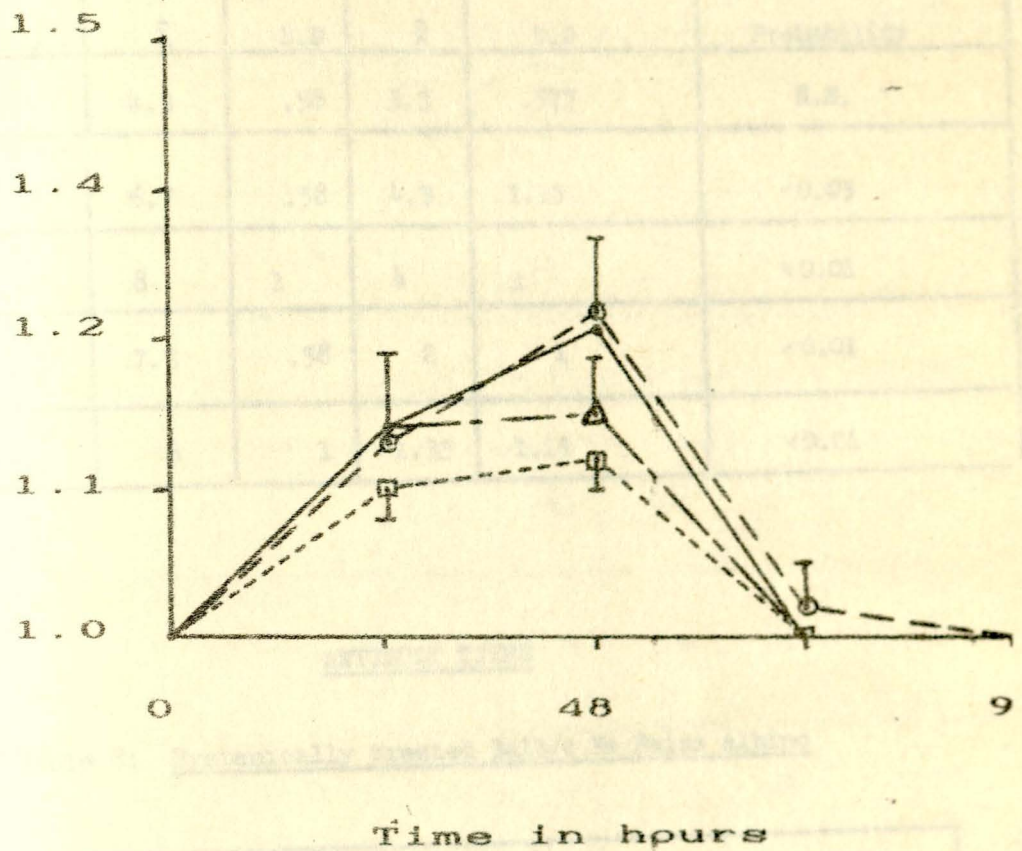


Fig. 8 : The effect of pentamidine given systemically on the DTH-response of Balb/c mice to *L. major* LV 39. Immediately post infection (P.I.) (-o-), 2 weeks P.I. (-+), 3 weeks P.I. (-A-) and untreated controls (-□-).

Table 7: Topically treated Balb/c mice versus swiss albino

Balb/c			Swiss albino		
Weeks P.I	$\bar{X}$	S.D	$\bar{X}$	S.D	Probability
2	4.3	.58	3.3	.577	N.S.
4	6.7	.58	4.3	1.15	<0.05
6	8	1	4	1	<0.01
8	7.3	.58	2	1	<0.01
10	8	1	1.33	1.15	<0.01

ANTIBODY TITRE

Table 8: Systemically treated Balb/c Vs Swiss albino

Balb/c			Swiss albino		
Weeks P.I	$\bar{X}$	S.D	$\bar{X}$	S.D	Probability
2	4.3	.58	3.3	.577	N.S
4	6.3	.577	4	1	< 0.05
6	9	1	3.3	.577	< 0.01
8	7	1	3	1	< 0.01
10	8.3	.58	2	1	< 0.01

N.S - Not significant

- Mean and standard deviation calculated from 3 pooled sera.

TABLE 9: DTH - RESPONSE, SWISS ALBINO VERSUS BALB/C AT 48 HRS.

	Treatment groups	N		$\bar{x}$		S.D		Probability
		Balb/c	Swiss	Balb/c	Swiss	Balb/c	Swiss	
T O P I C A L	Immediately P.I	4	4	1.36	2.07	.15	.03	<0.001
	2 weeks P.I	4	4	1.33	1.90	.04	.06	<0.001
	3 weeks P.I	4	4	1.31	1.72	.029	.04	<0.001
	Control	4	3	1.12	1.47	.02	.01	<0.001
S Y S T E M I C	Immediate P.I	4	3	1.22	1.64	.05	.05	<0.001
	2 weeks P.I	4	3	1.21	1.63	.06	.04	<0.001
	3 weeks P.I	4	4	1.18	1.65	.06	.04	<0.001

Table 10: DTH - RESPONSE AT 48 HRS. OF BALB/C MICE

	Treatment Groups	$\bar{X}$	S.D	N	Probability
S Y S T E M I C	Control	1.12	± 0.02	4	-
	3 Weeks P.I	1.18	± 0.06	4	*NS
	2 Weeks P.I	1.21	± 0.06	4	< 0.05
	Immediately P.I	1.22	± 0.05	4	< 0.01
T O P I C A L	3 Weeks P.I	1.31	± 0.03	4	< 0.001
	2 Weeks P.I	1.33	± 0.04	4	< 0.001
	Immediately P.I	1.36	± 0.15	4	< 0.05

* Not significant.

Table 11: DTH - RESPONSE AT 48 HRS. OF SWISS ALBINO

S Y S T E M I C T O P I C A L	Treatment Groups	N	$\bar{x}$	S.D	Probability
	Control	3	1.47	.01	-
	3 weeks P.I	4	1.65	.04	< 0.001
	2 weeks P.I	3	1.63	.04	< 0.01
	Immediately P.I	3	1.64	.05	< 0.01
	3 weeks P.L	4	1.72	.04	< 0.001
	2 weeks P.I	4	1.90	.06	< 0.001
	Immediately P.I	4	2.07	.03	< 0.001

** Significantly different
from control value

In both Balb/c and Swiss albino mice, treatment resulted in increased DTH when compared to controls (Tables 10,11). This could be related to the development of protective immunity. In both strains of mice greater responses were shown in topically treated rather than systemically treated groups. Immediate treatment induced greater DTH especially topical treatment and this may be related to the increased ulceration found following treatment. According to Hormaeche, et al. (1981) delayed (footpad) hypersensitivity testing is not always a reliable marker of natural or acquired resistance to infection, but probably parallels the development of CMI. It is a gross measurement of cellular reactivity involving a series of interacting T-cells and phagocytes (Liew, 1986) and the cell population which mediates it is Lyt 1⁺2⁻ cell line which is analogous to that of CMI (Howard, et al., 1982).

Challenge- Six months post infection all groups were challenged with 10⁵ L. major LV 39 promastigotes injected at the base of the tail, a site remote from the original point of inoculation. None of the mice, treated or untreated, showed any lesion over the observation period of 12 weeks. Although control animals never failed to produce lesions with 10⁵ promastigotes. At the time of challenge, treated Balb/c mice had disseminated lesions, but no lesion on the point of challenge. The reason why inoculated promastigotes did not develop while disseminate lesion were present, is not obvious although, Neal and Miles (1977) observed the same effect in sodium stibogluconate treated L. enriettii infected guinea pigs. That is, no lesion developed in treated or untreated controls when challenged after termination of treatment. In addition, Ercoli and Coelho (1967) found L. enriettii in the liver, spleen and kidney of guinea pigs following reinoculation after apparent successful treatment with glucantime. They suggest that this visceral localization was caused by the reinoculation rather than the primary infection. Normally guinea pigs infected with L. enriettii produce cuta-

neous lesion and they suggest the above condition may be due to a cutaneous, versus organ immunity, and may represent the inverse image of post Kala-azar dermal leishmanoid (PKDL) which usually appears after treatment of visceral leishmaniasis (Kala-azar).

CONCLUSION

The aim of this work was to examine the effect of both parenteral and topical pentamidine on the course of infection of L. major LV39 and whether chemotherapy alters the development of immune responses to leishmania antigens. Pentamidine was used during the experiment because the Ethiopian cutaneous leishmaniasis is sensitive only to pentamidine derivatives which are seriously toxic and need close medical attention during administration. Most forms of cutaneous leishmaniasis are self healing and the use of toxic parenteral drugs is unjustifiable, and topical treatment was used to overcome the disadvantage of systemic treatment and compared with parenteral administration.

L. major LV39 produced consistent lesions in Swiss albino and Balb/c mice. In Balb/c the lesion was progressive and non self-healing, and visceralizing. On the other hand Swiss albino develop small lesions which are self-healing after about 20 weeks.

The course of infection of the treated mice was different from that of untreated controls. The onset of lesion development was accelerated and ulceration was vigorous especially in topically treated mice. Treatment probably induced early development of immune response.

The earlier the treatment, the better the result, since even in the highly susceptible Balb/c mice, visceralization was hindered following immediate topical treatment. Topical treatment was more effective than intraperitoneal. In general, treatment cured all Swiss albino before their controls. On the other hand, the lesion of Balb/c regressed during treatment but relapsed after 20 weeks. This is analogous to DCL patients which relapse after successful treatment.

During parasitological examination more parasites were detected in smears than in sections. This finding is in relation with previous findings (Neal, 1964; Iskandar, 1978). The probable reason could be that the very thin section (cell line) taken decreased the chance of parasite detection.

Higher humoral responses and low DTH responses were observed in Balb/c mice than in Swiss albino mice. Green et al. (1983) observed high humoral responses accompanied by relatively low cell-mediated immunity in subjects vaccinated with live L. major.

Early during infection antibody titre increased in all mice, but later decreased in Swiss albino but not of the Balb/c mice. These changes are probably related to the progressive diseases in Balb/c mice. Treatment increased the DTH response in all treated mice being more prominent in Swiss than in Balb/c mice, and in topically treated than systemically treated. Moreover in most treated mice DTH is higher in immediately treated groups in parallel with regression of the lesion.

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DECLARATION

I, the undersigned declare that this dissertation is my own work and that all sources of material used for the thesis have been correctly acknowledged.

Name: Ghimja Fessahaye

Signature: 

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