A Comparison of Some Biological and Immunological properties of *Leishmania aethiopica* and *Leishmania major*

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by

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SUMMARY

This dissertation is concerned with a comparison between some of the biological and immunuological properties of two species of leishmania parasites that have been reported to occur in Ethiopia. Morphological and cultural assessment has shown that L.aethiopica is larger ($7.7u^3$) than L. major ($5.7u^3$), and has a lower optimal growth temperature (22^oC versus 28 - 30^oC).

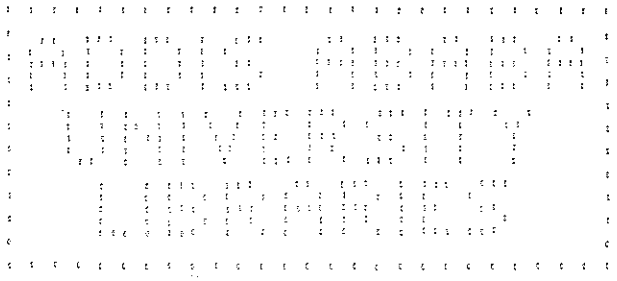
The use of in vitro promastigote culture to assess the effect of anti-ileishmanial drugs produced a good correlation with existing clinical experience. L. aethiopica responded poorly to the pentavalent antimony compound, pentostam (MIC 10ug/ml), but well to Lomidine (MIC 0.004ug/ml) and chlorpromazine (MIC 0.003ug/ml). In contrast L.major promastigotes responded similarly to all three drugs

L. aethiopica promastigotes did not produce clinical lesions in rabbits, guinea pigs, mice, outbred hamsters or Mastomys species. However, clinical lesions were produced in one of two inbred strains of hamsters inoculated. In contrast, of the species inoculated with L. major only swiss Albino and BALB/c mice developed demonstrable lesions.

Despite the close relationship between Old World leishmania species immunisation with L. aethiopica promastigotes (with or without adjuvant) did not induce cross-immunity to L. major promastigotes. This absence of immunity may have been due to a failure of DTH induction since foot-pad testing demonstrated that although DTH responses to homologus (L. aethiopica) antigens was produced, the level of cross reactive DTH to L.

Major antigens was not significantly different from unimmunised controls.

In contrsat to expectation, the interaction between mouse macrophages and the two species of leishmanial promastigotes shewed that L. aethiopica promastigotes are readily taken up. Therefore the failure of this species to establish a clinical infection must be due to either an effective immune response or to a post-phagocytic physiological incompatibility.



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CHAPTER I

INTRODUCTION

INTRODUCTION

General Features of Leishmaniasis.

Leishmaniasis is a collective term given to a protozoan disease cuased by members of the genus Leishmania and transmitted by sandflies of the genus Phlebotomus (Panosian and Wyler, 1983). The members of the genus Leishmania are responsible for causing a wide spectrum of disease (Table 1) that ranges from a simple cutaneous lesion (Localised Cutaneous Leishmaniasis) through extensive cutaneous and mucocutaneous involvement (Mucocutaneous and Diffuse cutaneous leishmaniasis) to a fatal visceralishing form Visceral Leishmaniasis. In the Old World four species of leishmania are known to be pathogenic for Man;

a) Leishmania major
b) Leishmania tropica
c) Leishmania aethiopica
d) Leishmania donovani

All Leishmania parasites have a digenetic life cycle (Figure 1), during which they alternate from a free living flagellated promastigote form in the gut of phlebotomid Sandflies to a non-flagellated intracellular amastigote form in the mammalian host (Marsden, 1979; Jaffe and Mchon, 1983). Although there are several different species of leishmania that infect man, they all share this type of life cycle in which an infected sandfly bites a suitable mammal, transmitting the leishmanial promastigotes directly from the proboscis or by regurgitating them during feeding.

TABLE - 1

Geographical Distribution of Leishmania Parasites.

Parasite	Clinical Disease Pattern	Geographical Location
L.aethiopica	Localised and Diffuse Cutaneous Leishmaniasis	Ethiopia and Mount Elgon (Kenya)
L.amazonesis	Localised and Diffuse Cutaneous Leishmaniasis	Amazon basin
L. braziliensis	Localised and Muco-Cutaneous Leishmaniasis	Brazil, Venezuela, Bolvia Guatemala, Mexico, Peru, Honduras Costa Rica, Panama,
L.donovani	Visceral Leishmaniasis	India, China, Mediterranean, Sudan, Southern USSR, North Africa, Brazil, Columbia, Guatemala, Argentina, Paraguay, El Salvador, Mexico, East & Equatorial Africa.
L. guyanensis	Cutaneous Leishmaniasis	Panama.
L. major	Cutaneous Leishmaniasis	North Africa, Middle East, India, Australia, Burma, China, Mediterranean, Southern USSR, Greece, Northern, Western, Southern & Eastern Africa.
L. minor	Cutaneous Leishmaniasis	Middle East, Mediterranean, Sudan, Egypt, Equatorial & West Africa, Pakistan, Sir Lanka.
L.mexicana	Cutaneous Leishmaniasis	Mexico, Honduras, Guatemala.
L. panamensis	Localised and Persistent Cutaneous Leishmaniasis	Pahang, Peru.
L. Peruviana	Cutaneous Leishmaniasis	Peru, Costa Rica, Panama.
L. pifanoi	Diffuse cutaneous Leishmaniasis	Venezuela, Carribean

Compiled from various sources.

FIGURE - 1

Life Cycle of Old World Cutaneous Leishmaniasis.

Leishmania major

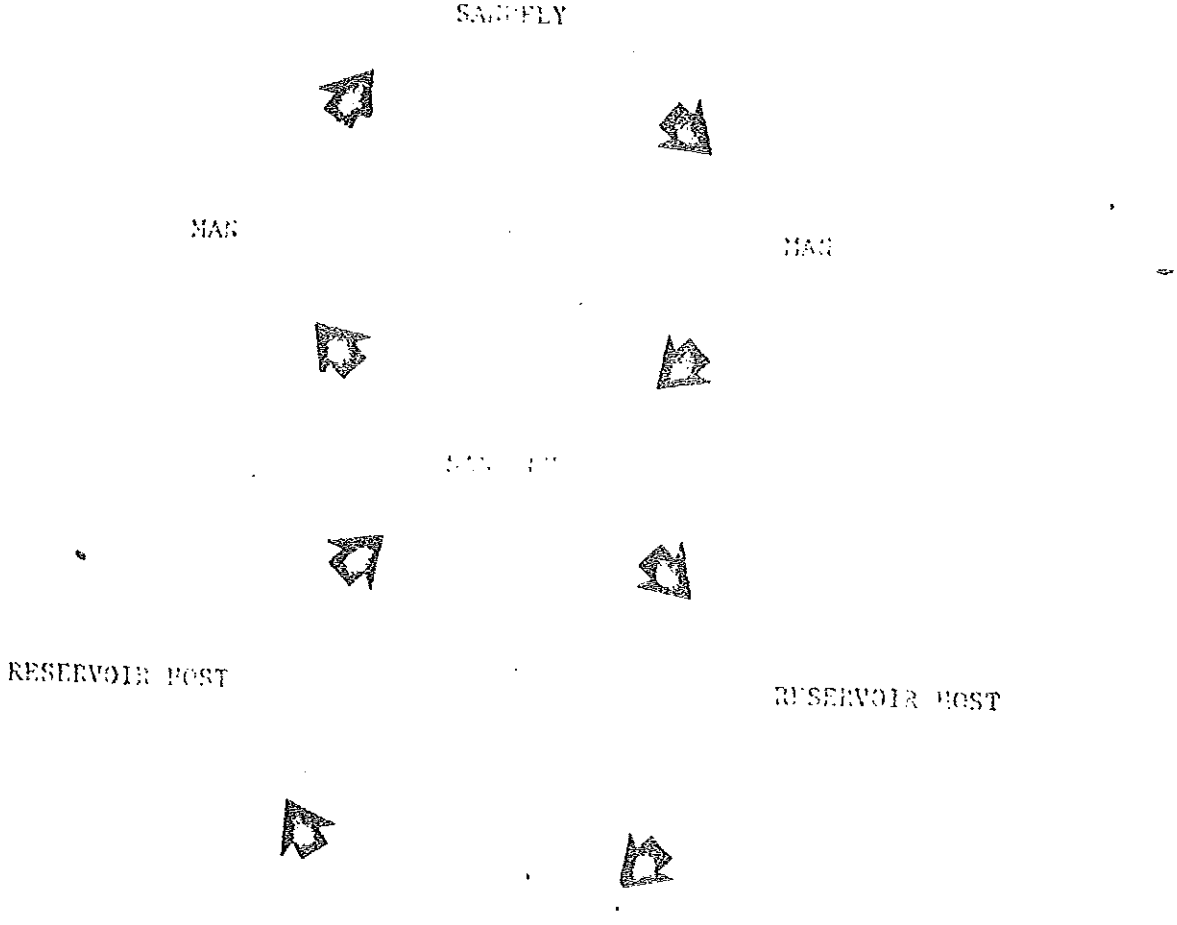
Wild Dogs (jackal), wolves, foxes, rodents, monkeys.

P. caucasicus
P. papatasii
P. mongolensis
P. clydei

Leishmania aethiopica

Heterohyrax brucei, Procavia habessinica.

P. longipes.
P. pedifer.



Once the leishmania parasites have entered the skin they are phagocytosed by macrophages in which they undergo a transformation to the non-flagellated amastigote form. These amastigotes divide approximately once every 24 hours and within a short time rupture the host macrophage liberating amastigotes which infect other macrophages. In both species of parasites that cause cutaneous or mucocutaneous leishmaniasis, the infected macrophages are restricted to the skin or occasionally to the lymph nodes draining the infected area (Snow, 1974). In contrast, Leishmania donovani, which causes visceral leishmaniasis rapidly metastasises to the visceral organs such as the liver and the spleen and may eventually be found circulating in the blood stream and in lymph nodes and bone marrow (Snow, 1974).

The cycle of infection is completed when leishmania-infected macrophages are ingested by another sandfly vector. The infected macrophage is digested in the midgut, releasing the amastigotes which then transform into the flagellated promastigote and multiply in the Sandfly gut (See Smyth, 1976). The promastigotes proliferate extensively until the foregut and proboscis contain very large number of promastigotes so that when the vector feeds, the parasites are once again transmitted to a mammalian host (Snow, 1974). One consequence of the insect vector is that in the case of cutaneous leishmaniasis lesions are restricted to areas exposed to sandfly bites (i.e. face, hands, arms and legs) and conversly a sandfly can only be infected by biting an infected lesion. In contrast visceral leishmaniasis is neither restricted in its distribution in the mammalian host (because it readily metastasises) nor does a sandfly necessarily have to bite an obvious lesion as the parasite undergoes haematogenic spread.

This dissertation is concerned with two species of leishmania which cause cutaneous leishmaniasis.

a) L. major is widely distributed throughout Europe, the Middle East and parts of Africa although only a single isolate has so far been reported from Ethiopia (Humber, Yemane-Berhan, Teklemariam, 1986).

b) L. aethiopica, in contrast, is almost entirely restricted to Ethiopia, except for a small foci around Mount Elgon in north-eastern Kenya (Belehu, 1981).

Cutaneous Leishmaniasis in Ethiopia..

Cutaneous leishmaniasis is widespread throughout the Ethiopian higlands (between approximately 1600m and 2650m) and is estimated to affect between 50 and 70, 000 people (Lemma, Foster, Preston, Bryceson, and Minter, 1969;Belehu, 1981). Two distinct clinical types of cutaneous leishmaniasis have been reported to occur in Ethiopia. The commonest form is Localised Cutaneous Leishmaniasis (LCL) which is generally restricted to the face, arms or legs and is eventually self-healing. This form of the disease may occur on, or spread to, mucous membranes and although termed mucocutaneous leishmaniasis (MCL) does not correspond to the metastatic form of MCL found in South America (Humber, et al, 1986). In contrast, Diffuse Cutaneous Leishmaniasis (DCL) is not self-healing, and the lesions are progressive and eventually spread over large areas of the

body. DCL is associated with immunological unresponsiveness to leishmanial antigens (Belehu and Humber, 1981).

The causative agent of cutaneous leishmaniasis in Ethiopia is principally L. aethiopica, although L. major has been isloated from a LCL lesion contracted in the Omo River Valley region of Ethiopia (Humber, et al, 1986). Thus, although DCL is entirely caused by L. aethiopica , Ethiopian LCL can be caused by both L. aethiopica and L. major. Originally L. major and L. aethiopica were considered to be sub-species of L.tropica (LeBlanc, Belhu and Peters, 1986). However, on clinical, serological and ecological criteria Bray and Lainson (1965) suggested that L. aethiopica should be separated from the other Old World cutaneous leishmaniasis. The classification of L. aethiopica as a separate species has recehtly been further justified by DNA-buoyant density analysis (Gardener, 1977). Analysis of 15 different isoenzyme patterns has also confirmed that L. aethiopica is not closely related to any other known species of Old World Leishmania (Le Blancq et al 1986).

It is believed that the different clinical manifestations of cutaneous leishmaniasis caused by L. aethiopica (LCL and DCL) are entirely the result of a defective host immunological response (Belehu and Humber, 1981), although differences in the frequency of plasma membrane-inserted proteins, and the presence of different antigens LCL and DCL isolates (Humber, personal communication) suggests that there may be slight differences between the strains of L. aethiopica causing LCL and DCl.

Leishmania species are essentially parasites of wild animals and only infect man zoonotically (Ayele,1981). No information is available

regarding the Ethiopian host species for L. major, although in otner parts of the world it has been reported to infect a wide variety of species especially rodents (Gemachu, 1981). L. aethiopica in contrast, has a very restricted reservoir host. The only recorded wild hosts are two species of hyrax, Procavia habessinica and Heterohyrax brucei, although 11 other species of hyrax occur in Ethiopia and may be potential reservairs (Humber, et, al, 1981).

L. aethiopica and L. major both cause cutaneous leishmaniasis; however, little published information is available about the biological and immunological features of L. aethiopica, probably due to its limited geographical distribution. In contrsat L. major which occurs throughout large areas of the Old World has received considerable attention. Since both of these species cause cutaneous leishmaniasis in Ethiopia, the aim of this dissertation was to examine and compare some of the biological and immunological features of these two pararsites.

CHAPTER II

MATERIALS AND METHODS

Leishmania Parasites.

Leishmania aethiopica Parasites were isolated at the Armauer Hansen Research Institute from patients with localised cutaneous leishmaniasis.Parasites were cultured from material taken from slit skin smears and inoculated into blood agar with a medium overlay. In order to ensure virulence, parasites were not maintained in culture for more than 3 weeks. All strains used isoenzyme typed at the London School of Tropical Medicine and Hygine, U.K. as corresponding to L. aethopica. within each experiment only one isolate of L. aethopica was used.

The L.major promastigotes were from a strain (LV 39) originally isolated from a wild rodent and was obtained as a kind gift from the max plank Institute, West Germany. The parasite was maintained by serial subcutaneous passage in a susceptible mouse strain, BALB/c, by inoculation into the subcutaneous tissue of the ears. Parasites were also only kept in-vitro for a maximum of 3 weeks when fresh parasites were isolated from infected mice by aseptically removing and homogenising the cervical lymph nodes in RPMI 1640 supplemented with human or foetal calf serum.

Parasite culture.

The promastigote stages of both L. aethiopica and L. major were grown in RPMI 1640 tissue culture medium (Flow Laboratories, U.K) supplemented with either 20 % foetal calf serum (Flow Laboratories, U.K) or 20 % human serum, and penicillin and streptomycin (200 units/ml and 200mg/ml

respectively). Serial passage of both species was performed very 3 or 4 days using an initial inoculum of approximately 5×10^6 promastigotes. Except where specified in the results section, all cultures were incubated at 25°C in 25ml glass bottles. All culture procedures were carried out in a static hood equipped with an ultra violet light.

Morphometric Measurements.

The length and width of leishmania promastigotes were determined using a calibrated micrometer eyepiece graticule using a x 100 oil immersion lens (Olympus Microscopes, Japan). In order to determine the volume of these parasites, the body was divided into three areas, two cone shaped anterior and posterior regions and a cylindrical central region (Edler, 1979). Thus the following formula was used to determine the volume:

$$\text{Vlume} = \underbrace{\frac{\Pi . d^2 . h}{4}}_{\text{Cylinder}} + 2. \underbrace{\frac{\Pi . d^2 . h}{12}}_{\text{Cones}}$$

Parasite smears for measurement were prepared by cooling aliquots of each species of promastigote to 0-4°C for 30 minutes and adding cold glutaraldehyde to a final concentration of 2%. The parasites were allowed to fix for 1 hour at 0-4°C at which time a smear was made on a clean microscope slide. After air drying the smears were stained with Wrights stain. The smear was covered with 10 drops of Wrights stain, left for 2 minutes, diluted with an equal volume of distilled water and left for a further 4 minutes. The stained slides were allowed to air dry after brief washing.

In order to determine the effect of fixation on parasite volume simila measurements were made on live parasites. Parasites were immobilis

by incubation at 0-4°C for 2 to 3 hours before measurement after which time movement had ceased or was very slow.

Promastigote Counting.

Promastigotes were counted using an improved Neubaure counting chamber with a x20 or x40 microscope objective. Smaples were diluted with and equal volume of counting fluid (Phosphate buffered saline, pH 7.2, containing 1% formaldehyde) to immobilise the parasites during counting.

Drug Sensitivity Testing.

The activity of 3 drugs was assessed on both species of leishmania parasites:

Lomidine - pentamidine methanosulphonate - Specia, France. (40mg/ml)
Pentostam - sodium stibogluconate - Welcome, U.K. (100mg/ml)
Chlorpromazine - Delmas perfusion, France (50mg/ml)

Dilutions of the drugs were made in RPMI 1640 with 20% foetal calf serum followed by sterilisation by membrane filtration through 0.22u Millipore filters (Millipore Inc., U.S.A.). Triplicate 5ml cultures of each species of leishmania were set up with a range of 8 dilutions of the original drugs were set up at the same time. The effect of each drug was assessed by counting at daily intervals.

Experimental Animals.

The following species and strains of animals were used in this study:

Animal Species or Strain	Source
Outbred Swiss Albino Mice	National Health Research Institute
Inbred BALB/c Mice	Addis Ababa University
Outbred Hartley Guinea Pigs	National Health Research Institute
Outbred Albino Rabbits	National Health Research Institute
Mastomys species	Dr. Colin Hetherington,
Outbred Hamster	National Institute of Medical Research,
Inbred Hamster	The Ridgway, Mill Hill,
Inbred Hamster	London, U.K.

With the exception of the experimental animals housed at the National Institute of Medical Research, U.K. (Mastomys species and Hamsters) all animals were maintained under conventional conditions in the Biology Department animal house. Animals were fed with laboratory animal food (Ethiopian Poultry and Dairy Enterprise, Addis Ababa) supplemented with fresh vegetables and given water ad libitum.

Sensitisation of Mice to L. aethiopica.

Stationary phase cultures of L. aethiopica were washed and resuspended in Phosphate buffered saline containing 1% glutaraldehyde and stored at 0-4°C until required. Before immunisation the fixed promastigotes were washed to remove the fixative and resuspended at 1 x 10^7 promastigotes/ml. Four groups of outbred Swiss Albino mice were immunised subcutaneously with 0.1 ml of 10^7 promastigotes per ml as follows:

a) L. aethiopica emulsified with an equal volume of Freunds Complete Adjuvant (Difco, U.S.A.)

b) L. aethiopica mixed with an equal volume of saline.

c) Freunds complete adjuvant emulsified with saline.

d) Untreated controls.

Animals in groups a to c were immunised on three occasions at weekly intervals. Three weeks after the final immunisation all animals were challenged with 1 x 10^6 L. major promastigotes in the rump just above the tail. Presence or absence of lesions was recorded at weekly intervals.

The development of delayed hypersensitivity to L. aethiopica following immunisation was assessed by changes in foot pad thickness following injection of 2 x 10^6 glutaraldehyde fixed L. aethiopica promastigotes into the right foot pad. The contralateral, uninjected, pad served as a control. Changes in foot pad thickness were measured daily using a dial

caliper (Schnelltaster, West Germany).

Preparation of Macrophage Cultures.

Peritoneal macrophages werecollected from the unstimulated peritoneum of BALB/c mice by injecting cold RPMI 1640 medium supplemented with 10% foetal calf serum into the exposed peritoneum. The aspirated macrophage suspension was then allowed to adhere to sterile coverslips overnight at 37°C in a 5% carbon dioxide in air atmosphere. The number of macrophages per coverslip was estimated by extrapolating from the number seen per x 100 high power field. Phagocytosis of leishmania promastigotes was assessed microscopically and the number of dead promastigotes assessed by methylene blue staining (Simposon, Roth and Loose, 1979).

CHAPTER III

RESULTS

Parasite morphology. The volumes of both live and glutaraldehyde-fixed promastigotes were determined as described in the materials and methods for L.aethiopica and L. major. The frequency distribution of the volumes are shown in Tables 2 and 3.

Table 2

Size Distribution of live and fixed L. aethiopica

Volume (u^3)	Frequency live	Frequency Fixed
<5.0	2	6
5.0 - 5.9	18	13
6.0 - 6.9	10	8
7.0 - 7.9	19	23
8.0 - 8.9	38	37
9.0 - 9.9	7	10
≥ 10.0	6	3
Mean Volume	7.70 +/- 1.55	7.69 +/- 1.48

Since there was no significant difference between the volumes of live and fixed parasites the data from these two groups were pooled for statistical comparison between L. aethiopica and L. major. The mean volume of L. aethiopica ($7.69u^3$) was significantly ($P<0.01$) larger than L. major ($5.66u^3$).

Table 3

Size Distribution of live and fixed L. major.

Volume (u^3)	Frequency Live	Frequency Fixed
$\leq$3.0	3	3
3.0 - 3.9	2	8
4.0 - 4.9	33	32
5.0 - 5.9	30	28
6.0 - 6.9	7	7
7.0 - 7.9	13	14
8.0 - 8.9	6	6
9.0 - 9.9	1	1
$\geq$10.0	1	1
Mean Volume	5.66 +/- 1.57	5.66 +/- 1.59

Optimum growth temperature. Promastigotes of L.aethiopica and L.major were grown in RPMI 1640 medium supplemented with 20% foetal calf serum as described in the materials and methods section. Triplicate cultures of both species of parasite were incubated at 22, 28, 30 and 37°C and counted daily for 14 days. The results are shown in Figures 2 and 3 (37°C data not shown) where it can be seen that L. major has a much higher temperature tolerance and grows well at temperatures between 28 and 30°C. In contrast the optimal temperature for L. aethiopica promastigotes was 22°C and growth was severely inhibited at higher temperatures.

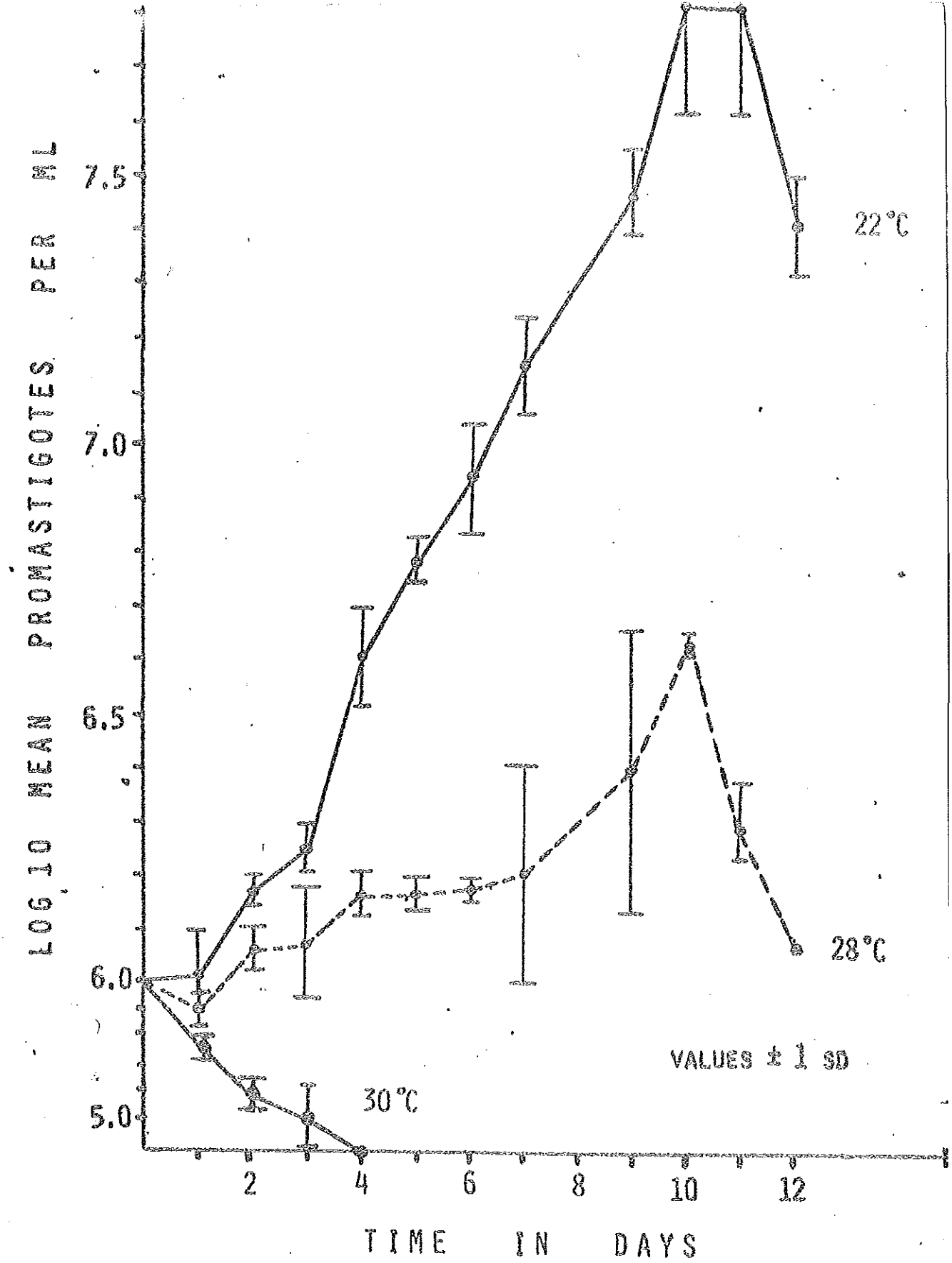


FIGURE 2 THE EFFECT OF TEMPERATURE ON THE GROWTH OF L. AETHIOPICA

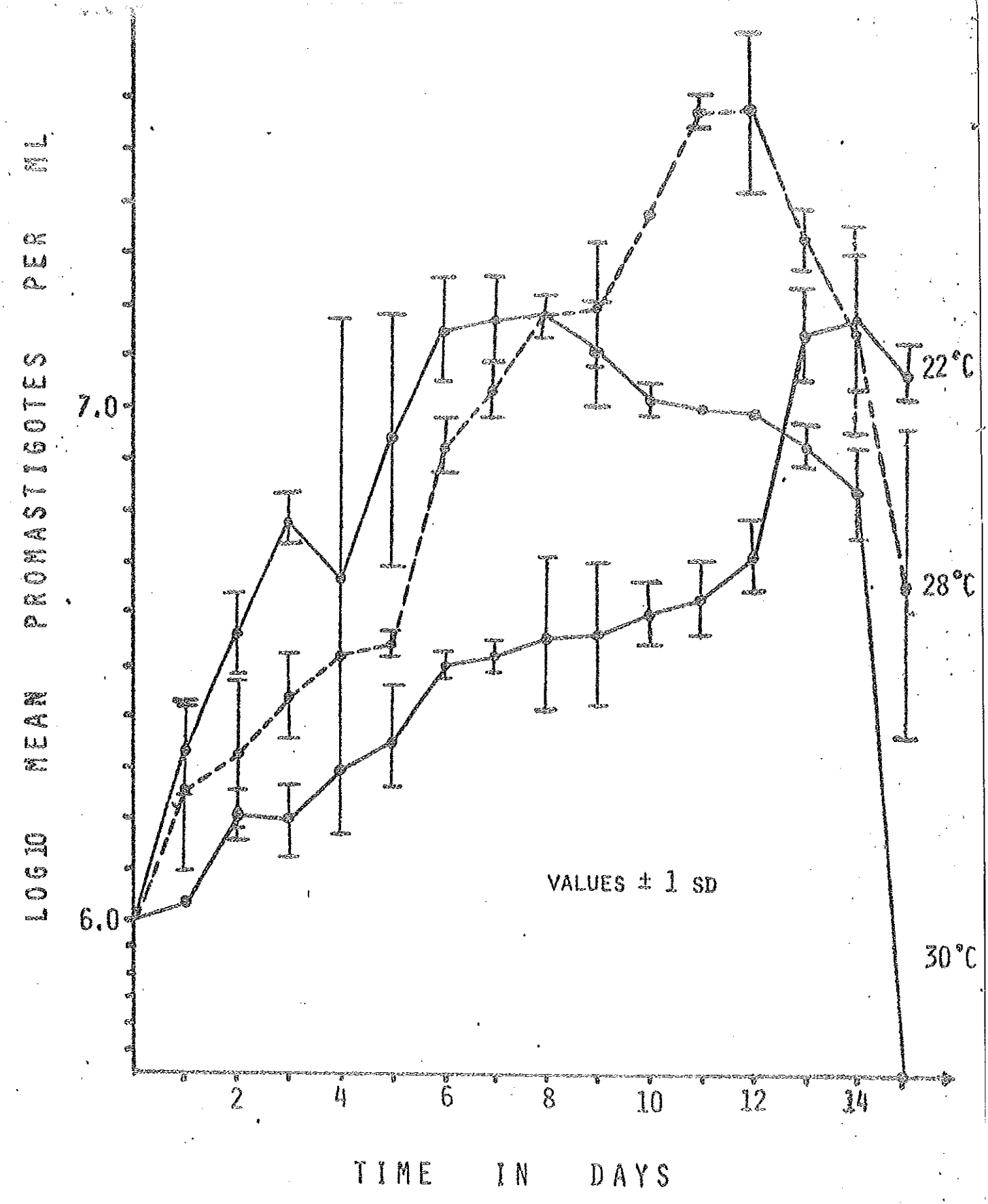


FIGURE 3 THE EFFECT OF TEMPERATURE ON THE GROWTH OF L. MAJOR

This is in contrast to L. major which grew slightly faster at 30°C than at 28°C, although the cultures reached a lower mean peak concentration and died off more rapidly at the higher temperature.

In both species of leishmania, higher temperatures caused the promastigotes to round up and become non-motile resembling the amastigote forms. In L. aethiopica this occurred at temperatures above 28°C and in L. major at temperatures above 30°C.

Drug Sensitivity. The response of both species of leishmania to the three drugs tested are shown in Tables 4 and 5.

Table 4

Effect of Lomidine, Pentostam and Chlorpromazine on the Growth and Survival of L. aethiopica

Drug	Minimum lytic Concentration	Minimum Inhibitory Concentration	Ineffective Concentration
Lomidine	4.0ug/ml	0.004ug/ml	0.0004ug/ml
Pentostam	100.0ug/ml	10.0ug/ml	1.0ug/ml
Chlorpromazine	Not Done	0.003ug/ml	0.0003ug/ml

Minimum Lytic Concentration is the concentration of drug which caused complete lysis of the parasites by day 2 of culture.

Minimum Inhibitory Concentration is the concentration of drug which caused a greater than 80% decrease in promastigote growth.

Ineffective Concentration is the concentration of drug that had no significant effect on parasite growth.

Table 5

Effect of Lomidine, Pentostam and Chlorpromazine on the Growth and Survival of L. Major.

Drug	Minimum lytic Concentration	Minimum Inhibitory Concentration	Ineffective Concentration
Lomidine	400.0ug/ml	0.4ug/ml	0.04ug/ml
Pentostam	10^4ug/ml	1.0ug/ml	0.1ug/ml
Chlorpromazine	250.0ug/ml	0.25ug/ml	0.025ug/ml

The results in these tables show that there were considerable differences between the sensitivities of the two leishmania species to the various drugs. Both L. aethiopica and L, major were affected least by the pentavalent antimony compound, pentostam although L. aethiopica need a ten times higher concentration before inhibition of growth was apparent. In contrast L. major required higher concentrations of both the diamidine Lomidine and chlorpromazine. The most effective drug for killing both species of leishmania promastigotes was chlorpromazine.

Growth of Leishmania in Laboratory Animals. There were large differences in the ability of the two species of leishmania promastigotes to infect different laboratory animals. L. major only caused distinct clinical lesions in the two strains of mice tested although small, transient swelling did occur in both rabbits and guinea pigs given 10^7 promastigotes. Since these lesions disappeared within 3 weeks no attempt was made to isolate parasites from the injection sites. In the mice there was no difference in the ability of the injected parasites to cause lesions at different sites. The presence of leishmania parasites in the suspected

L.major lesions in the mice was confirmed by isolation and culture. L. aethiopica did not infect any of the laboratory animals available in Ethiopia. However, L. aethiopica did grow in one strain of inbred hamster (HB) which was inoculated by Dr. Colin Hetherington of the National Institute of Medical Research, London. The presence of leishmania parasites in these lesions was confirmed by histology, culture and reinfection.

Immunity to Leishmania Infection and Development of DTH. Immunisation of Swiss Albino mice with L. aethiopica and Freunds complete adjuvant did not induce immunity to a subsequent challange with 10^5 live L. major promastigotes. Every animal (in all 4 groups) developed lesions within 3 weeks. No difference was apparent in the size or rate of lesion development between the animals in the different groups.

Development of DTH following immunisation or exposure to live L. aethiopica promastigotes was assessed using a coventional DTH foot-pad enlargement assay to leishmania antigens. The time curves of foot pad enlargement following the injection of 5×10^6 glutaraldehyde fixed promastigotes are shown in Figures 4, 5 and 6.

DTH responses to L. major antigens (Figure 4) shows the DTH time curve following the testing of Swiss Albino mice which had been challenged with live L. aethiopica promastigotes. Figures 5 and 6 show the DTH response to L. aethiopica antigens in mice and guinea pigs after immunisation with live L.aethiopica promastigotes. It is apparent from these curves, that the immunisation schedule employed, induced greater DTH in mice when compared to guinea pigs.

Table 6

Growth of Leishmania in Different Laboratory Animals

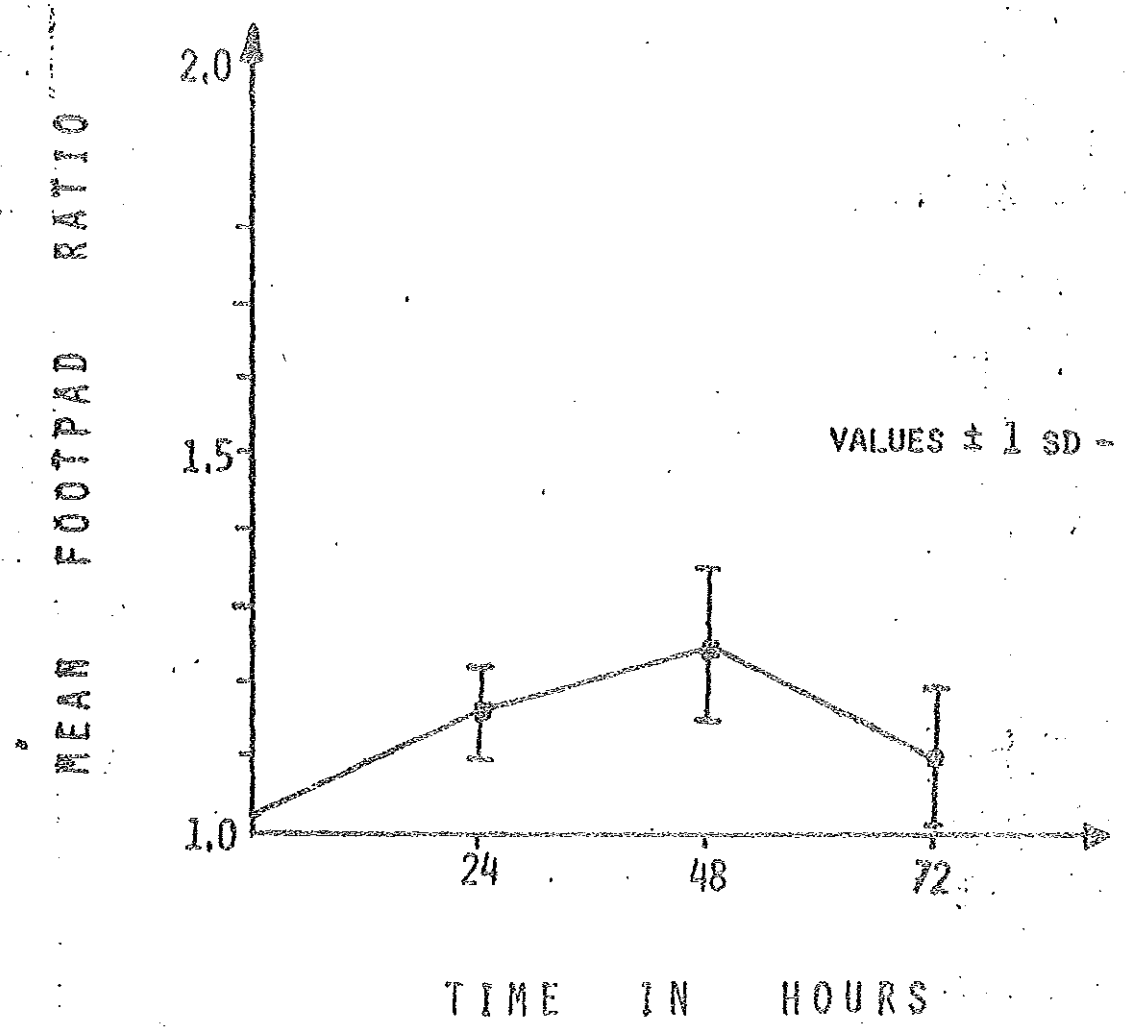
Animal	L.aethiopica		L. major	
	10^5	10^7	10^5	10^7
Swiss Albino Mice	No Lesions		Lesions on all Animals	
BALB/c mice	No Lesions		Lesions on all Animals	
Hartley Guinea Pigs	No Lesions		No Lesions	1/3 Lesions[1]
White Rabbits	No Lesions		No Lesions	1/1 Lesions[1]
Mastomys sp[1]	No Lesions		Not Done	
Hamsters (Outbred)[1]	No Lesions		Not Done	
HA Hamsters[1]	No Lesions		Not Done	
HB Hamsters[1]	5/5 with Lesions		Not Done	

[1] Transient lesions All Animals were inspected at regular intervals for 3 months following inoculation to assess the presence or absence of lesions.

[1] Inoculations and examinations done by Dr. Colin Hetherington at the National Institute of Medical Research, U.K.

Macrophage- Promastigote Interaction. The results of phagocytosis of both species of leishmania promastigotes are shown in Figure 7. It is apparent that L. aethiopica promastigotes are more effectively phagocytosed than those of L. major. After 60 minutes incubation almost twice as many L. aethiopica promastigotes had been phagocytosed by the peritoneal macrophages. In addition phgocytosis of L.major promastigotes was concentration dependant with over twice as many promastigotes being phagocytosed

at the 1:10 ratio compared to the 1:5 ratio. This effect was not seen with L. aethiopica promastigotes which were equally well phagocytosed at both 1:10 and 1:5 macrophage:Promastigote ratios. In all experiments the number of dead (methylene blue stained) promastigotes was too small to give any meaningful interpretation(generally less than 1-2%,data not shown).


MEAN FOOTPAD RATIO
2.0
1.5
1.0
VALUES ± 1 SD
24
48
72
TIME IN HOURS

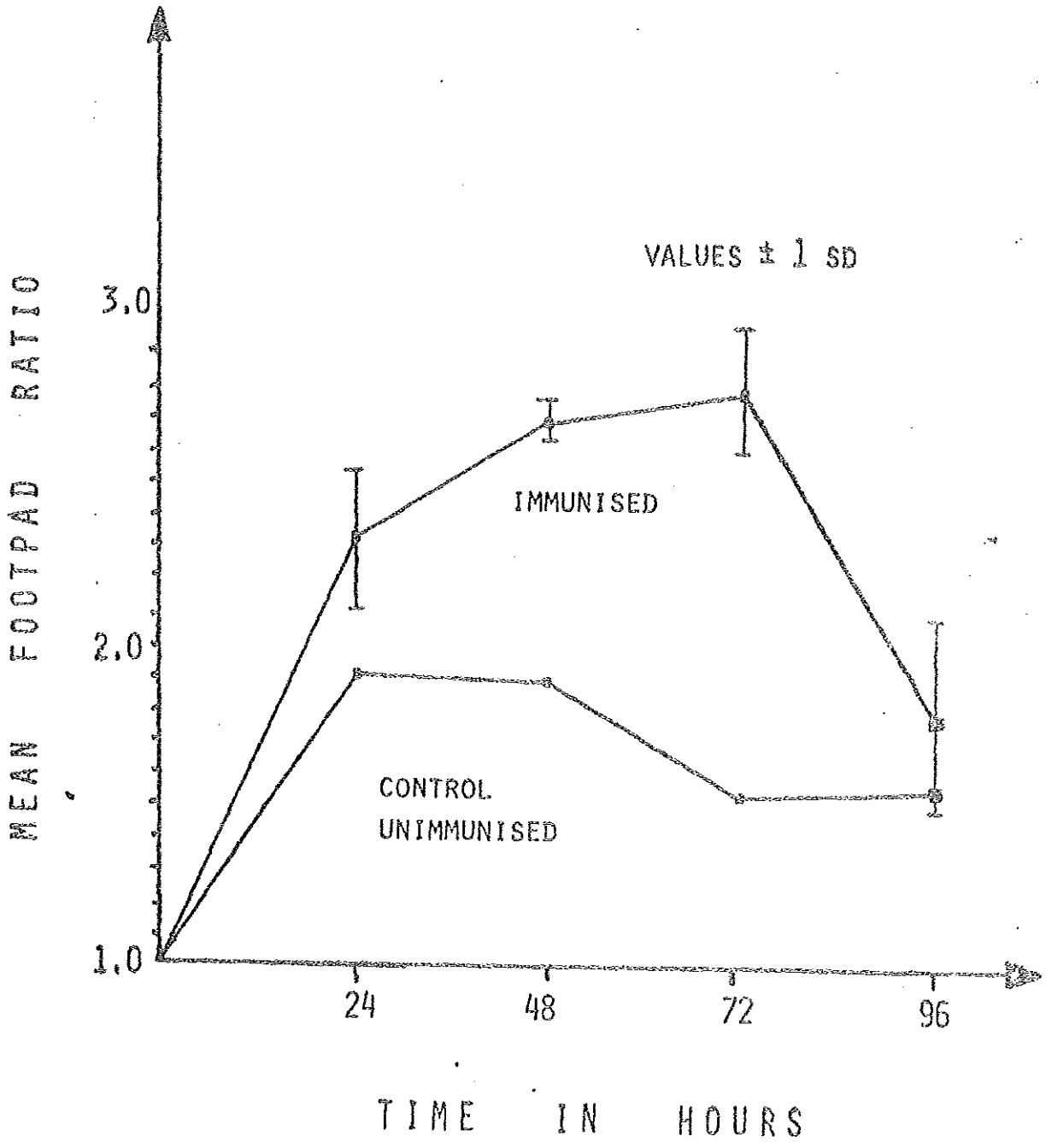

MEAN FOOTPAD RATIO
VALUES ± 1 SD
3.0
2.0
1.0
IMMUNISED
CONTROL
UNIMMUNISED
24
48
72
96
TIME IN HOURS

FIGURE 6 DTH FOOT-PAD RESPONSE OF GUINEA PIGS TO L. AETHIOPICA AFTER SENSITISATION TO L. AETHIOPICA

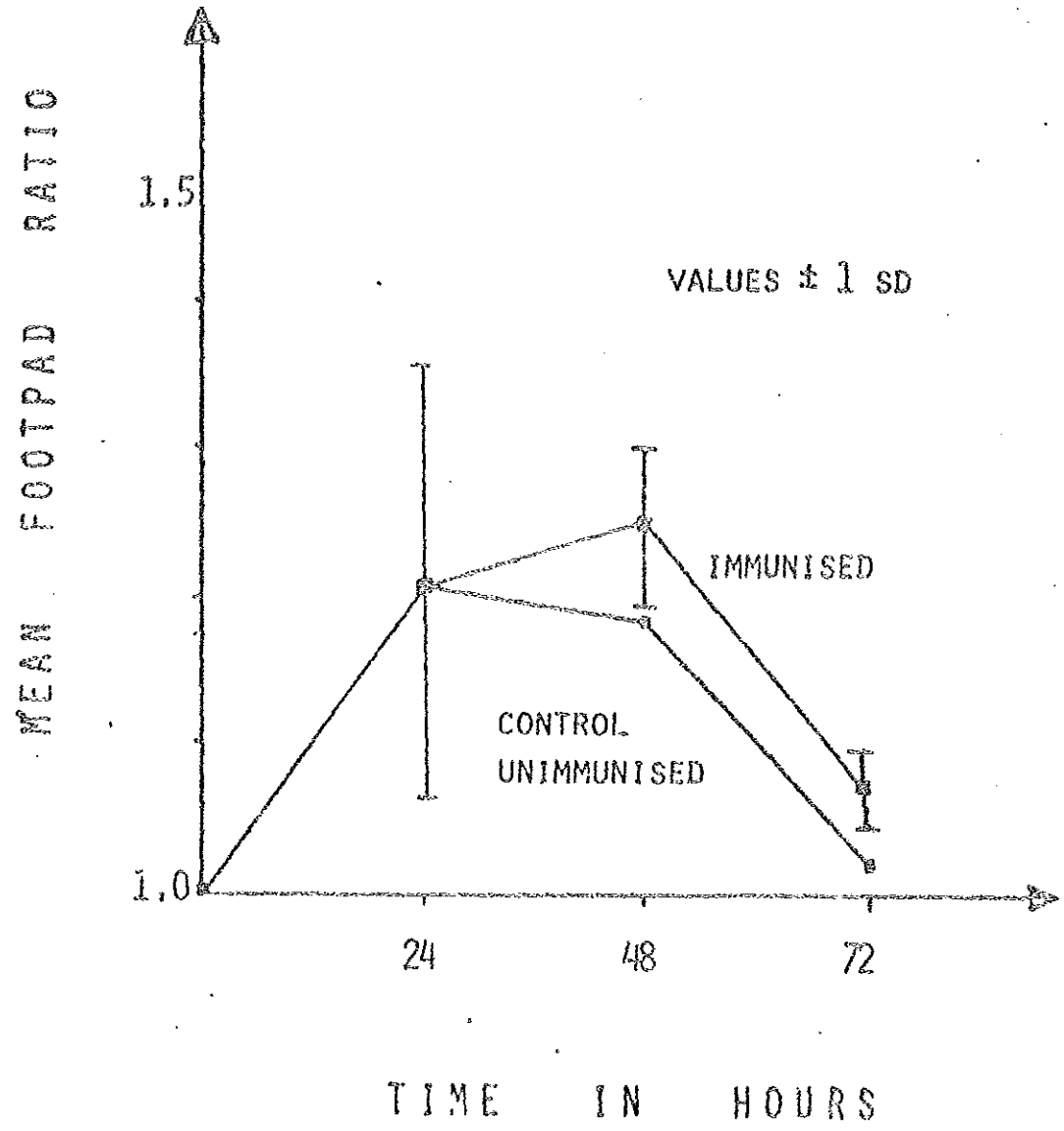
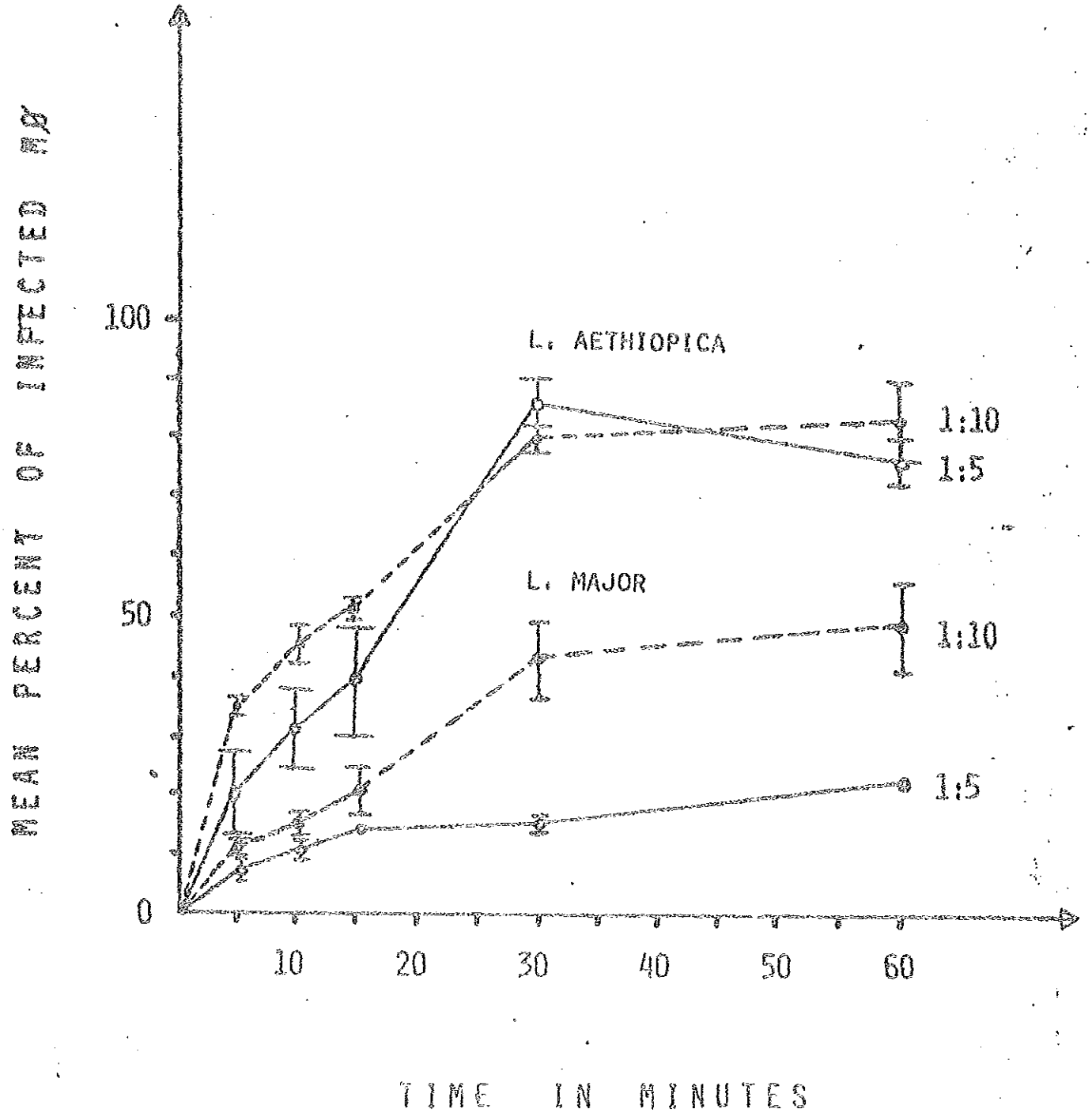


FIGURE 7 MICE MACROPHAGE - LEISHMANIA PROMASTIGOTE INTERACTION



CHAPTER IV

DISCUSSION

The results obtained in this experiment demonstrate that there is a considerable difference in volume between L. aethiopica and L. major, and that fixation with cold glutaraldehyde has no effect on parasite volume. L. aethiopica is almost 40% larger than L. major. However,,while these differences are statistically significant, some caution should be exercised in the interpretation of these data. Firstly, accurate measurment of promastigotes with the light microscope is difficult due to their small size. Secondly, although care was taken to ensure that parasites of both species were cultured under the same conditions, it was possible that the size differences may have been due to uncontrolled cultural factors. It is apparent from observations made in subsequent experiments that size, and morphology do change during culture. In particular, above optimum temperatures cause the parasites to resemble the amastigote, rather than the promastigote form. However, despite these reservations, it is doubtful whether cultural effects or measurement errors could acount for the large differences observed.

The principal cultural difference between L. aethiopica and L, major is in the former's relative sensitivity to temperatures above 22°C. This feature is in line with the epidemiological restriction of Ethiopian cutaneous leishmaniasis below the 23°C isotherm (Belehu, 1981). In common with other species of leishmanial, L. aethiopica also undergoes transformation to amastitgote-like form although this happens at a lower temperature than other species.From the growth data at the optimum temperature for both species it can be calculated that the cell cycle time for L. aethiopica is 36 hours compared to 56 hours for L. major.

Although leishmania promastigotes are not normally the stage on which chemotherapeutic agents act, difficulties in culturing amastigotes makes promastigote cultures a realistic alternative in drug screening. The results in these experiments show that anti-leishmanial agents can act at two separate levels. Prevention of growth (Minimum Inhibitory Concentration, MIC), and direct destruction of the parasite (Minimum Lytic Concentration, MLC) although from the practical point of view the MIC is probably of more relevance since the concentrations required for MLC are unlikely to be achieved in vivo.

It has been reported previously that conventional anti-leishmanial drugs, derived from antimony are ineffective in treating Ethiopian Cutaneous Leishmaniasis (Sarojini, et al, 1984). This clinical observation confirms the experimental findings in this thesis in that L. aethiopica promastigotes require a tenfold greater concentration of the pentavalent antimony compound, pentostam, to inhibit growth. In addition, the most effective drug in treating Ethiopian Cutaneous Leishmaniasis is the diamidine, Lomidine (Sarojini, Humber, Yemane-Berhan Fekete, Belehu and Warndorff, 1984) which in the in-vitro experiments described here was more efficient in inhibiting L. aethiopica promastigotes than L. major promastigotes.

The most effective drug in these experiments was chlorpromazine which inhibited growth in both species of promastigote at a much lower concentration than Lomidine and pentostam. Chlorpromazine was particular-

effective against L. aethiopica promastigotes (MIC of 0.003ug/ml), and has been also been repotred to be effective in the treatment of Diffuse Cutaneous Leishmaniasis (Hendriksen and Lende, 1983).

The apparent correlation between the in vitro results reported here and preivious clinical findings suggests that the use of promastigote cultures in screening for potential anti-leishmanial drugs is not only feasible but can yield useful information about drug activity.

The establishment of an animal model for Ethiopian Cutaneous Leishmaniasis would greatly facilitate investigations into the treatment and pathology of this disease. In particular, the in vivo screening of antileishmanial drugs, which at present can only be done on species of leishmania which readily grow in rodents, could be extended to L. aethiopica. The results presented here show that L. aethiopica promastigotes do not produce lesions in the majority of laboratory animals (Table 6), and in contrast to L. major, do not produce lesions in the highly permissive strains of mice, such as BALB/c (Bjovatn and Neva, 1979). However, L. aethiopica promastigotes did produce lesions in one inbred strain of hamsters (HB), although not in a second inbred strain, or in an outbred strain. It has been previously reported (Childs et al, 1984) that only recent isolates of L. aethiopical would grow in hamsters. This report, together with the data from this study, suggests that hamsters are indeeed permissive to L. aethiopica infections, but that the genetic background of the animal plays an important role in the outcome of the infection.

The reason for the failure of L. aethiopica and L. major to grow in

certain species is unclear, although it is likely to be due either to an effective immune response on the part of the host, or some physiological inability of the promastigotes to establish themselves in the host macrophages.

The data concerning cross- immunisation between L. aethiopica and L. major shows that immunisation with the former did not protect the mice against subsequent challenge with the latter. This finding is in agreement with previous investigations which have shown that closely related leishmania species do not necessarily induce protective immunity to each other. Thus L. major has been reported to protect against L. minor but not vice versa (Levine, 1973).

Although immunisation with L. aethiopica was unable to protect against challenge with L. major it was able to induce DTH responses in both mice and guinea pigs, even without the presence of Freunds complete adjuvant, to homologous antigen (i.e. L. aethiopica). Interestingly guinea pigs appeared to develop much smaller DTH responses to the homologous antigen than mice. In the heterologous promastigotes of L. major (following sensitisation with L. aethiopica) were much lower, and not significantly different from the response of control unsensitised mice.

Phagocytosis, or uptake, of leishmania promastigotes is of primary importance in establishing infection. The results of these experiments show that although L. aethiopica is incapable of causing clinical lesions in mice, it is readily taken up by virgin (unstimulated) peritoneal macrophages. Thus the failure of L. aethiopica

to establish an infection is not due to its inability to establish itself in the mouse macrophage. However, the much more rapid phagocytosis of L. aethiopica promastigotes might result in parasite overload within the macrophage and subsequent rapid triggering of anti-leishmanial mechanisms. Additional, longer term cultures of mouse macrophages with phagocytosed promastigotes would reveal whether these cells do indeed have an increased capacity to kill L. aethiopica parasites.

In conclusion this preliminary investigation into the biological and immunological features of L.aethiopica and L. major has been that there are considerable differences between these two species that warrant further investigation.

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