USE OF LIVE ATTENUATED *LEISHMANIA MAJOR* PARASITES AS A CANDIDATE VACCINE AGAINST *LEISHMANIA MAJOR* INFECTIONS IN BALB/c MICE.

BY

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Immunology of Kenyatta University.

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DECLARATION

I, Janet Achieng' Onyalo, hereby declare that this is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted after examination with our approval as University Supervisors.

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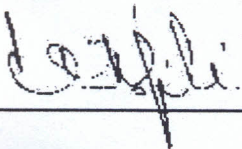

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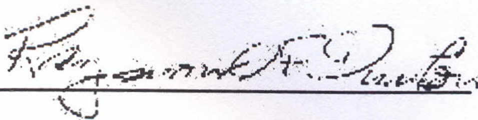

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DEDICATION

I dedicate this thesis to my late husband, Mr. Caleb Ogutu who until the time of his death was instrumental in my pursuing the Msc. Degree course and for ably providing financial support to enable me complete my coursework

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GLOSSARY OF ABBREVIATIONS

ABTS	Azinodi[3-ethyl- benzthiazoline sulphate].
AIDS	Acquired Immune Deficiency Syndrome.
ALM	Automated *Leishmania major*.
ANOVA	Analysis of Variance.
BCG	Bacille Calmette Guerin.
C3	Third complement component.
Con A	Concavalin A.
CR 1/3	Complement component type 1 and 3.
CMI	Cell Mediated Immunity.
DCL	Diffuse cutaneous leishmaniasis.
ELISA	Enzyme linked immunosorbent assay.
GP 63	63 Kilodalton glycoprotein.
HI	Humoral Immunity.
HIV	Human Immunodeficiency Virus.
IFN-γ	Interferon gamma.
I.M.	Intramuscular.
I.P.	Intraperitoneal.
IPR	Institute of Primate Research.
I.V.	Intravenous.
KEMRI	Kenya Medical Research Institute.
LHFP	Left Hind Footpad.
Log	Logarithmic.
LPG	Lipophosphoglycan.

M2	Major surface antigen type 2 of unknown fraction.
MCL	Mucocutaneous leishmaniasis.
mRNA	Messenger ribonucleic acid.
NNN	Novy Nicolle McNeal medium.
NaOH	Sodium Hydroxide.
NaCl	Sodium Chloride.
OD	Optical Density.
PBS	Phosphate Buffered Saline.
PBST	5% Tween bovine albumin in phosphate buffered saline.
PKDL	Post kala-azar dermal leishmaniasis.
PNA	Peanut Agglutinin.
RHFP	Right Hind Footpad.
S.C.	Subcutaneous.
TNF-α	Tumour Necrosis Factor -alpha.
WHO	World Health Organization.

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ABSTRACT

Several studies have demonstrated that *Leishmania* and other protozoan parasites lose their infectivity and virulence when serially cultured *in vitro* for a long time. Serially cultured *L. major* parasites were used in this study as live attenuated vaccine candidate and its ability to induce protective immunity against cutaneous leishmaniasis in BALB/c mice was examined.

Four experimental groups of 30 BALB/c mice each were used. The first group was immunized with live attenuated metacyclic promastigotes, the second one was immunized with heat killed whole parasites, third group was given soluble antigens derived from *L. major* metacyclic promastigotes while the last group was not immunized and served as the controls. The immunization was administered intravenously, four times at an interval of 7 days.

Blood from all the animals was obtained through tail snipping and was used for the Enzyme-linked immunosorbent assay (ELISA) test to detect production of antibodies. Five animals from each group were kept separate from the others for 14 weeks, they were tested for delayed hypersensitivity test (DTH) reaction and then sacrificed. Their splenocytes were then used for lymphocyte proliferation assay to detect retention of immunological memory.

The remaining immunized and control mice were challenged (infected) with 10^6 culture derived metacyclic *L. major* promastigotes on the left hind footpads (LHFP) while the right hind footpads (RHFP) were left as contralateral controls. After every 7 days both (RHFP) and (LHFP) were measured and the difference between the two footpads of each mice was recorded as the lesion size. Fourteen weeks post-infection,

all the mice were sacrificed. Impression smears and cultures of spleen and liver were prepared in order to determine the level of metastasis and visceral infections.

Results obtained from the study showed that live attenuated parasites induced the best protection against the disease (ANOVA 1, P=0.000) compared to the other vaccines used. All the immunized mice produced significant level of antibodies, exhibited evidence for retention of immunological memory with T cell stimulation although non produced DTH response. While live attenuated parasites did not cause lesion, mice immunized with them exhibited minimal lesion development upon challenge with virulent *L. major* parasites therefore it is possible to use them as a candidate vaccine against cutaneous leishmaniasis.

`CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION.

Leishmania is a genus of flagellated protozoan parasites that cause a wide range of human diseases collectively known as leishmaniases. The parasites are in the subkingdom;- *Protozoa*; phylum:- *Sarcomastigophora*; Order:- *Kinestoplastida* (Vickerman, 1976); Genus:- *Leishmania* (Ross, 1903). The parasites are transmitted by female sandflies (Order:-Diptera, Family:-Psychodidae) of genus *Phlebotomus* in the old world and *Lutzomyia* in the new world (the Americas) (Kar, 1995).

Leishmaniases are worldwide in distribution, accounting for 20 million new cases per year with 1.5 persons at risk (Ho *et al.,* 1994). In Kenya several cases of leishmaniases have been reported. These include visceral leishmaniasis caused by *L. donovani* (Fendall, 1952; McKinnon; 1962; Mebrahtu *et al.*, 1987; Perkins *et al.*, 1988) in Baringo, Kitui, Tharaka Mithi and Machakos.

Treatment of leishmaniases generally involves the use of high doses of pentavalent antimonial compounds, sodium stibogluconate (Pentostam®) and meglumine antimonate (Glucantine®), (Modabber, 1989). However, the cost, duration of administration, toxic nature of antimonial compounds and the tendency for the disease to relapse after an initial successive regime of chemotherapy underscore the need for an effective, safe and a cheap vaccine to be used as an integrated approach to control leishmaniases.

Previous studies with *Leishmania tropica* and *Leishmania donovani* showed that prolonged *in vitro* cultivation of promastigotes reduced the infectivity and the virulence of these parasites (Ebert, 1979; Katakura and Kobayashi, 1985). The efficacy of such promastigotes (attenuated by prolonged *in vitro* cultivation) to induce protective immunity in the host against

the *Leishmania* infections had not been examined. It was for this reason that this study to investigate the possibility of serially subcultured promastigotes of *Leishmania major* to provoke adaptive immunity was designed.

Lesions caused by *L. major* parasites can be easily monitored over a period of time and this is why they were given preference. Moreover, the lesions caused by these parasites heal spontaneously in humans making it a safer vaccine candidate compared to its counterparts in the old world. BALB/c mice are a suitable model for this study because they are highly susceptible to *L. major* infections and they mimic human infections (Howard, 1986).

It is already known that there exists cross-reactivity of surface antigens between various species of *Leishmania* parasites (Howard *et al*., 1982) and cross protection by *L. donovani* antigens against *L. major* infections has been demonstrated (Gicheru *et al*., 1997). Success of this study may therefore offer an opportunity to plan a strategic control of *Leishmania* parasites through immunization using either live attenuated whole parasites or its antigens.

1.2 LITERATURE REVIEW

1.2.1 Aetiology and clinical presentations of various forms of the leishmaniases.

The leishmaniases are a wide a spectrum of clinical presentations of diseases caused by flagellated protozoan parasites of the genus *Leishmania*. The disease can be broadly categorized epidemiologically as either anthroponotic with humans as the only or main source of infection or zoonotic, with domestic animals or wild animals serving as the important source of infection (WHO, 1990). Leishmaniases are transmitted by over seventy species of adult female sand flies of the genus *Phlebotomus (*Old World) and genus *Lutzomyia* (New World) through a blood meal (Kar, 1995).

Clinically, several forms of infection are recognized in humans ranging from the simple cutaneous ulcers to the disfiguring or sometimes fatal mucocutaneous form in *L. brazilensis* or to visceral infections, kala-azar, which entails a high mortality rate if not treated (WHO, 1987). The three main forms of leishmaniasis are cutaneous (Oriental sore), kala-azar (visceral), and mucocutaneous (espundia) leishmaniasis (WHO, 1984).

1.2.1.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is caused by a wide variety of *Leishmania* species namely; *Leishmania major (L. major), L. tropica* and *L. aethiopica* for Old World forms (OWCL) (Gramiccia *et al*., 1987) while in the New World the disease is caused by *L. panamensis, L. brazilensis, L. guyanensis, L. peruviana, L. mexicana, L. amazonensis* and *L. venezuelensis* (WHO, 1989). Cutaneous leishmanisis caused by *L. major* produces wet skin ulcers, which leave unsightly scars on healing. New World cutaneous leishmaniasis tends to

be more severe and chronic than Old World cutaneous leishmaniasis (WHO, 1989). Cutaneous leishmaniasis caused by either *L. major* or *L. tropica* is normally uncomplicated and their lesions heal spontaneously within the range of nine months to two years although unlike *L. major*, *L. tropica* parasites cause dry ulcers (WHO, 1987).

Infections by *L. aethiopica* in Kenya and Ethiopia cause diffuse cutaneous leishmaniasis (DCL) while in the New World DCL is caused by *L. pifanoi* and *L. amazonensis* (WHO, 1984). Diffuse cutaneous leishmaniasis causes widespread disseminated thickening of the skin in plaques, papules or multiple nodules sometimes resembling lepromatous leprosy. There is neither ulceration nor mucosal involvement, the disease does not heal spontaneously and tends to relapse after treatment (WHO, 1987).

1.2.1.2 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL) initially causes lesions that are similar to those of ordinary CL which may heal, but later, may metastasize in the oral nasal and pharyngeal mucosae causing disfiguring leprosy-like tissue destruction and skin granulomas (WHO, 1987). *Leishmania brazilensis, L. panamensis* and *L. guyanensis* in the New World cause mucocutaneous leishmaniasis. In the Old World this disease is caused by *L. donovani sensu lato* in adult males in Sudan (WHO, 1987).

1.2.1.3 Visceral leishmaniasis

Visceral leishmaniasis is a very severe systemic disease which is almost fatal if left untreated (Modabber, 1993). Old World visceral leishmaniasis is caused by parasites of *L. donovani* complex, which may be either sporadic or endemic. This disease is caused by *L. chagasi* in the New World. Common symptoms are fever, malaise, and weight loss. Pathological changes consist of hyperplasia of the reticulo-endothelial cells in most internal organs causing enlargement of the spleen, liver and in the lymph nodes. Splenomegaly with or without hepatomegaly, wasting and anaemia are the most common clinical signs (WHO, 1990).

In Kenya and some parts of India, following treatment, *L. donovani* migrates from the viscera to cause a form of cutaneous leishmaniasis known as post kala-azar dermal leishmaniasis (PKDL) (Muigai *et al.,* 1991). This disease may occur six months to several years after apparent cure of visceral leishmaniasis. Hypopigmented or erythromatous macules on any part of the body or redness of the face occur and may later become papilla or nodular especially on the face. Chronic lesions consist of multiple papilla or nodular infiltration of the skin, usually without ulceration and so far no PKDL has been reported in the New World (WHO, 1984).

1.2 2 Leishmaniases in Kenya

Leishmaniases in Kenya produce almost a full spectrum of clinical manifestations attributed to the disease namely visceral (kala-azar), cutaneous, and mucocutaneous leishmaniasis.

1.2.2.1 Visceral leishmaniasis (kala-azar) in Kenya

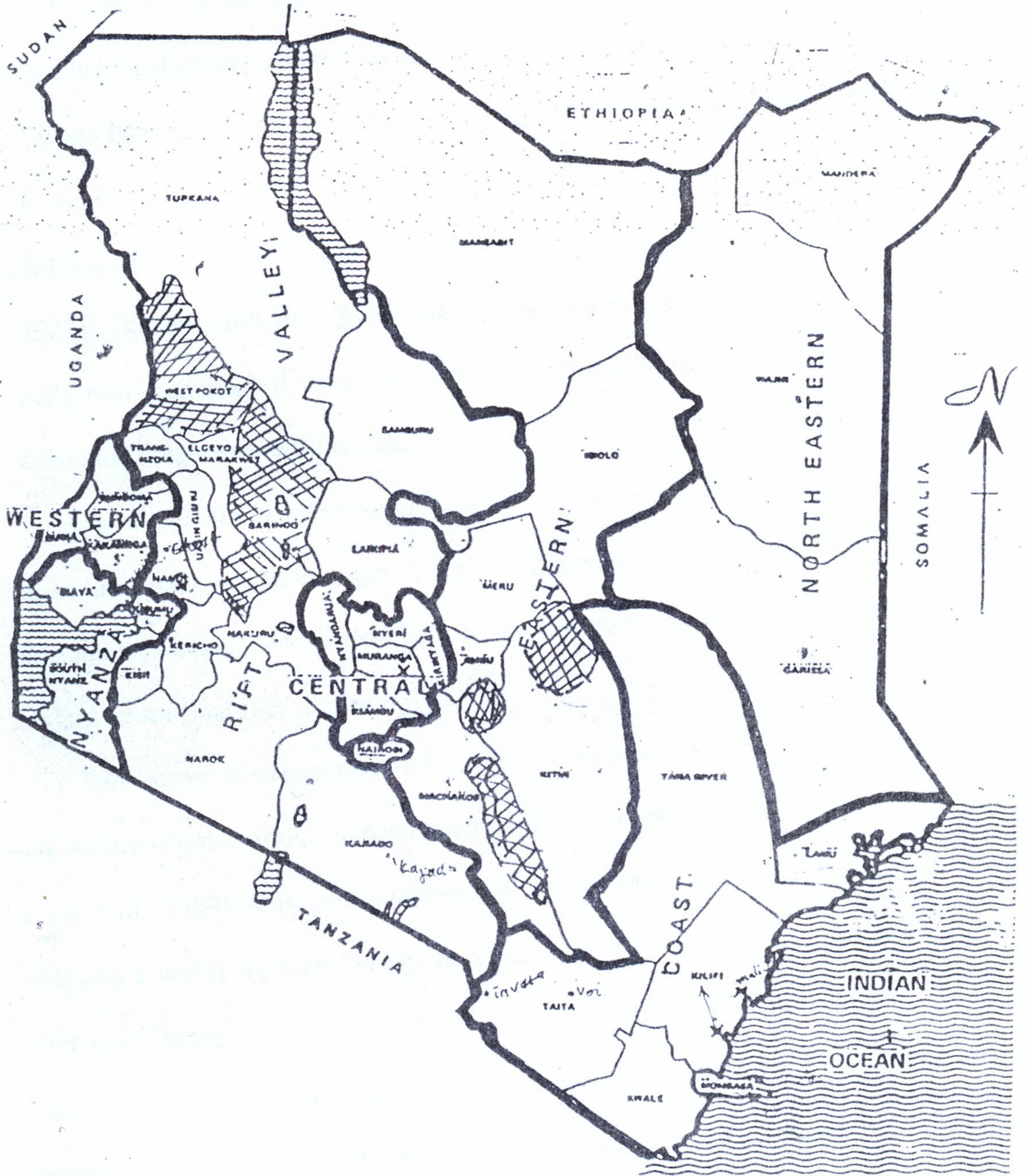
Kala-azar is believed to have been introduced into the country as early as 1940 during the second World War possibly by the soldiers who camped north of Lake Turkana in South West of Ethiopia (Mutinga *et al.,* 1975). After the war, sporadic cases started showing up in three localities of Saricho on the Uaso Nyiro River, between Kitui, Tana River and Machakos districts. Kala azar was first reported in Machakos district between 1942 and 1950 (Fendall, 1952) and in 1952/ 1953 there was a major epidemic affecting about 3,000 people in Tseikuru, in Kitui district. Although control measures including treatment were taken, the disease did not disappear completely. By 1980, other reports of increasing number of patients were confirmed (Mbugua and Siongok, 1981). These foci have since remained endemic for kala-azar with sporadic epidemics. From the time kala-azar was established to be endemic in Kenya, the disease has continued to show a rapid spread. Although the disease was previously known to be limited in sparsely populated areas whose inhabitants are nomads, the sporadic cases reported in many areas of the country has shown some changes in its trend of spread (Mutinga, 1988). *Phlebotomus martini* is the natural vector for *L. donovani* in Kenya (Perkins *et al.*, 1988).

Baringo and West Pokot Districts were established as endemic for kala-azar in 1955 and 1956 respectively and it was suggested that probably the introduction of the disease in these areas followed cattle trade routes (McKinnon, 1962; Mebrahtu *et al.*, 1987). The population in these two areas is comprised of pastoralists who are very mobile with their flocks in search of better pastures. In these foci, the disease manifests in different ways: namely; asymptomatic, sub clinical which is self healing and clinically manifest disease and half of the reported visceral leishmanial patients are aged between 5-15 years old of which 66% are males (Schaefer *et al.,* 1994).

No wild or domestic reservoir host has been found in these foci except for the isolated case when *L. donovani* parasites were obtained from two domestic dogs (Mutinga *et al.,* 1980) and this has led to workers to believe that visceral leishmaniasis is anthroponotic in Kenya (Githure, 1989; Robert *et al.*, 1997).

The distribution of visceral leishmaniasis in Kenya is shown in Figure 1.

Figure 1: The distribution of visceral leishmaniasis in Kenya. Shaded areas are the foci. (Adapted from Lawyer *et al.*, 1989).



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1.2.2.2 Cutaneous leishmaniasis in Kenya

In 1964, the first case of cutaneous leishmaniasis was reported in Baringo (Kungu *et al.,* 1972). While capturing sandflies from animal burrows in Baringo district, Beach was bitten on the hand several times by a female sand fly *P. duboscqi* that formed lesions. The sand fly was later found to be infected with *L. major* parasites (Beach *et al.,* 1984). Following the discovery, a survey was conducted among the human population and *L. major* was isolated from lesion on the face and arm of an 8 year old girl in Marigat location (Muigai *et al.,* 1987). There could have been other unreported cases of the disease since *L. major* normally heal spontaneously without treatment or it could have been mistaken for cutaneous manifestation of visceral leishmaniasis.

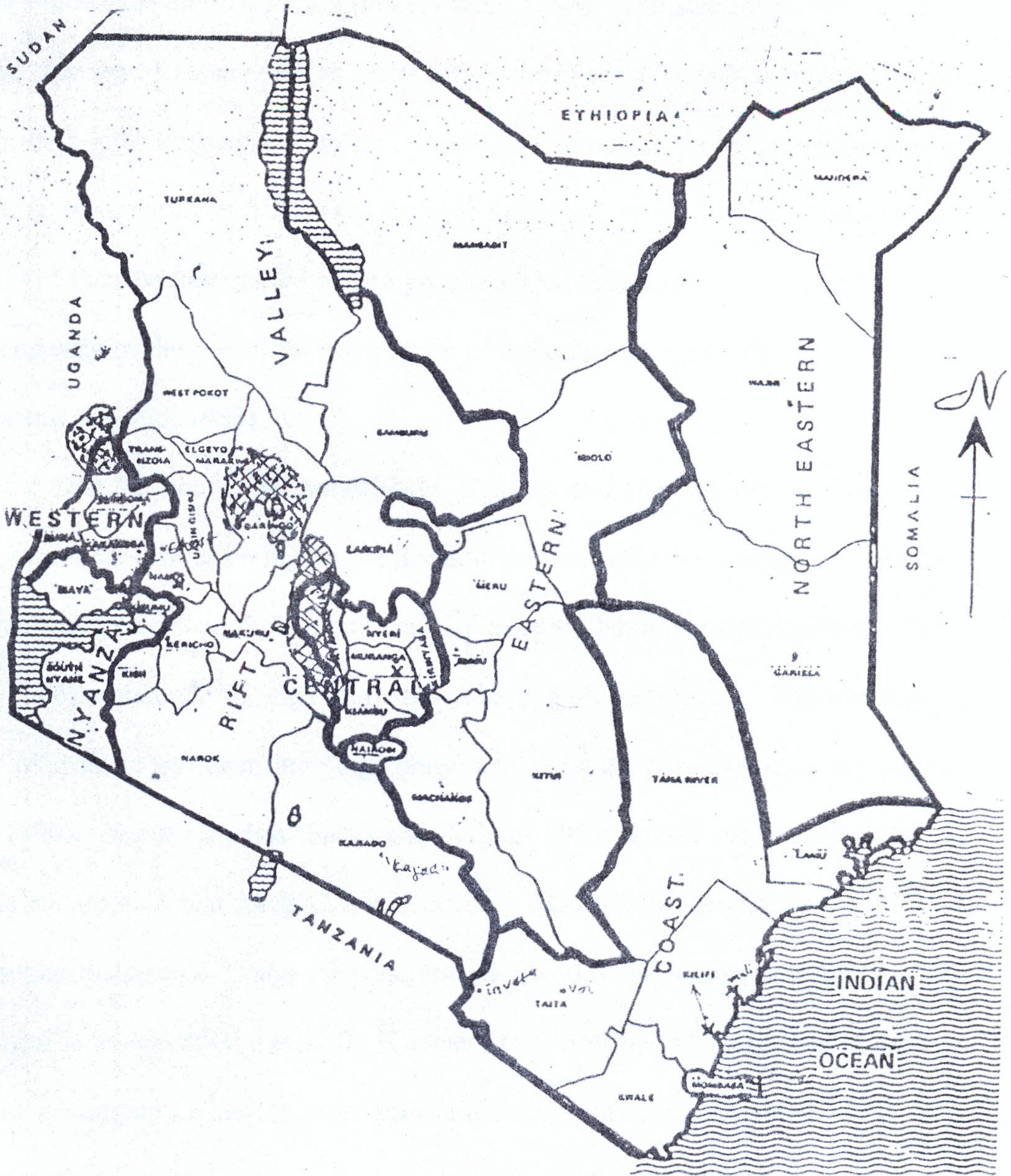
Leishmania major infection in Kenya is zoonotic with a wide variety of natural hosts being wild animals, which are little affected by the presence of the parasites (Lainson, 1982). Man is almost always an accidental host, when he intrudes into the natural habitat of the wild hosts and vector sand flies (Lainson, 1982).

The disease is transmitted by *P. duboscqi* (Beach *et al.,* 1984) and the parasites have been isolated from various rodents such as *Tatera robusta, Xerus rutilus* (Heisch, 1957; Heisch *et al.,* 1959), *Mastomys natalensis, Taterillus emini, Aethomys kaiseri* (Githure *et al.,* 1984) and a vervet monkey *Cercopithecus aethiops* (Binhazim *et al.,* 1987). The disease is endemic in Baringo and West Pokot Districts, Rift Valley Province (Githure *et al.,* 1984; Muigai et al., 1987). Baringo district is a dual focus of both visceral and cutaneous leishmaniasis and a human case of a mixed *L. donovani* and *L. major* infections has been reported (Mebrahtu *et al.,* 1991).

Like *L. major*, infection with *L. aethiopica* is zoonotic and was found to be sporadic among the communities living in the vicinity of two mountainous regions of Mt. Elgon Bungoma District (Sang and Siongkok, 1983) and Aberdare range in Nyandarua district (Githure, 1988). *Phlebotomus pedifer, P. longipes* and *P. elongensis* have been implicated as the vectors of *L. aethiopica* in this area (Mutinga and Odhiambo, 1986; Sang *et al.*, 1983). The rock and tree hyraxes (*Procavia johnstoni* and *Dendrohyrax arboreus*) and the giant rat *Cricetomys gambianus* are the reservoir hosts (Kungu *et al.*, 1972; Mutinga *et al.*, 1975).

Infections with *L. tropica* in indigenous Kenyan was reported fairly recently from Narok, Nakuru, Laikipia, Nyandarua and Samburu Districts (Mebrahtu *et al.*, 1988; Lawyer *et al.*, 1991). *Phlebotomus guggisbergi* was incriminated as the vector and was found to be prevalent in these areas (Johnson *et al.*, 1999). The distribution of cutaneous leishmaniasis in Kenya is shown in Figure 2.

Figure 2: The distribution of cutaneous leishmaniasis in Kenya. Shaded areas are the foci. (Adapted from Lawyer *et al.*, 1989).



1.2.3 Development, transmission and general pathology of *Leishmania* parasite infections.

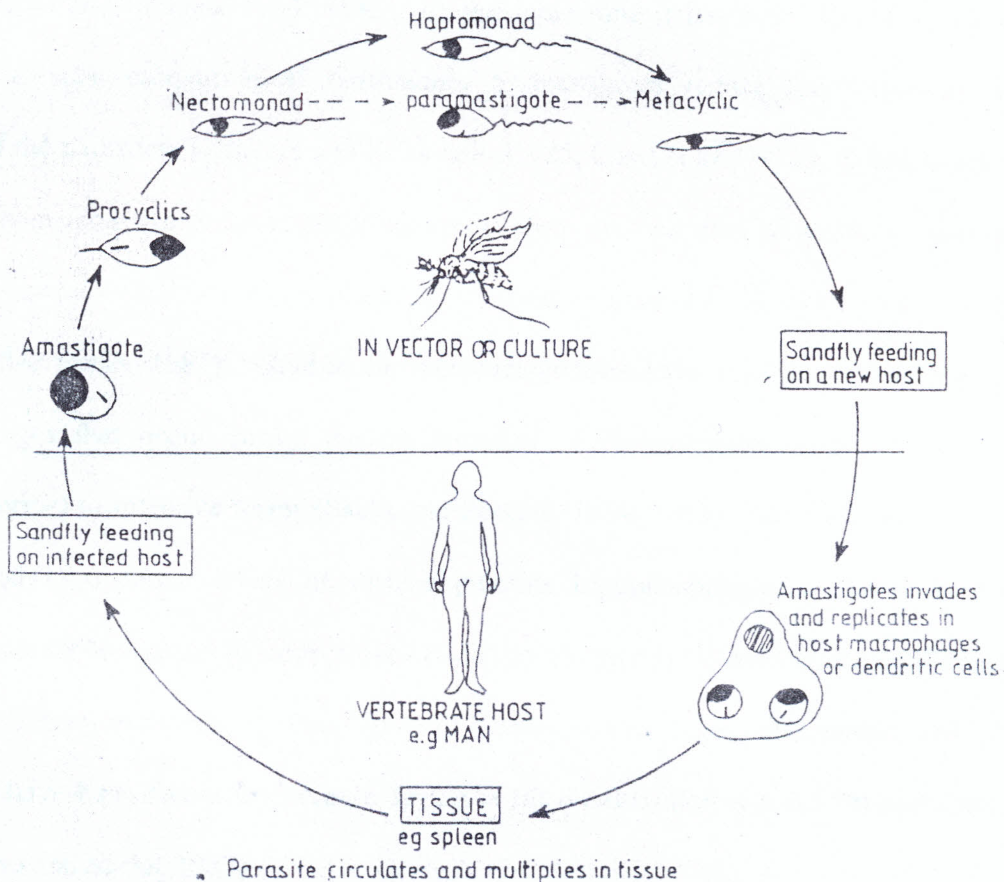
Leishmania parasites are transmitted by the female sand flies during a blood meal since they require blood for their ovulation process (Kar, 1995). The susceptibility of a sand fly to a particular species of *Leishmania* is genetically controlled (Killick-Kendrick 1985). If the vertebrate host is infected with *Leishmania* amastigotes liberated from the macrophages and/or intracellular amastigotes may be taken up with the blood meal. Further development of the parasites will then be determined by the genetic physical and physiological suitability and sufficient longevity of the vector for completion of the extrinsic cycle (Killick-Kendrick, 1985; Molyneux and Ashford, 1983).

During the first 72 hours, the amastigotes elongate and the rudimentary flagellum grows resulting in a long slender highly motile dividing promastigote (nectomonads). By the 5th day later, they transform to a short broad actively dividing haptomonads (Lainson 1982; Warburg *et al.,* 1986) which can be seen in the thoracic midgut and foregut. The two stages are collectively referred to as logarithm (log) phase and the parasites known as procyclics (Lawyer *et al.,* 1990). *In vitro* studies (Sacks and Perkins, 1985; Sacks, 1987) demonstrated that these procyclics are avirulent for BALB/c infection. The infective promastigotes known as metacyclics appear between 4-7 days after bloodmeal infection coinciding with the time at which another meal is sought by the sand fly (Lawyer *et al.,* 1990). The metacyclics migrate to the foregut and mouthparts where they are transmitted through bites. The metacyclics are believed to be the end-point of the life cycle in the sand fly, and are required for initiation of an infection in a vertebrate host (Killick-Kendrick, 1986) which takes place during the next blood meal. While the development of promastigotes from non-infective to infective form

(metacyclogenesis) (Sacks and Perkins, 1984) is not accompanied by any obvious morphological change, *L. major* metacyclics can be distinguished and even purified because they are not agglutinated by the lectin peanut agglutinin (PNA) at concentrations which agglutinate all the noninfective (procyclic) promastigotes (Sacks and Perkins.1985). Reduction in the level of Lipophosphoglycan (LPG) a 9Kda glycoconjugate molecule and glycoprtease kilodalton 63 (gp 63) have been observed during metacyclogenesis (Wilson *et al.*, 1989; Sacks *et al.*, 1990; Kimsey *et al.*, 1993). These two surface proteins have been implicated in parasite attachment and uptake into host macrophages and virulence of *Leishmania* parasites (Etges *et al.*, 1986; Russell and Wilhem 1986).

Once introduced into the mammalian host, the promastigotes enter the macrophages, or dendritic cells where they transform into non-motile amastigote forms, within an acid phagolysosomal compartment (Locksley and Louis, 1992; Moll *et al.*, 1995). Inside the macrophages, the amastigotes divide, finally rupturing the host cells after which they enter the macrophages of reticulo-endothelial organs of the skin where they continue to divide leading to various forms of clinical presentation (Moll *et al.*, 1995). Some parasites infect circulating mononuclear monocytes in the peripheral blood and are picked up in these or with skin macrophages during another sand fly bite (Peters and Gillies, 1985). The amastigotes then differentiate into promastigotes within the sand fly (Lawyer *et al.*, 1991). Figure 3 shows the life cycle of the *Leishmania* parasite.

Figure 3: Life cycle of *Leishmania* parasite.



1.2.4 Effect of prolonged *in vitro* cultivation on *Leishmania* parasites.

Studies have shown that it is possible to grow *Leishmania* parasites *in vitro* using culture medium (Sacks and Perkins, 1984; 1985). At the same time it has been demonstrated that prolonged *in vitro* cultivation of *Leishmania* promastigotes reduce the infectivity and virulence of the parasites (Katakura and Kobayashi, 1985, Ebert *et al.*, 1979). It has also been observed that promastigotes from log phase (procyclics) are avirulent to normal susceptible BALB/c but as they reach stationary phase, their virulence progressively increase (Sacks and Perkins, 1984; Sacks, 1987). Studies on metacyclogenesis have illustrated that there are several changes that occur during the development of promastigotes from non-infective (procyclic forms) to infective forms (Sacks and Perkins, 1984, 1985). Most of these changes are attributed to different levels of surface proteins Lipophosphoglycan (LPG) a 9Kda glycoconjugate molecule and glycoprotease kilodalton 63 (gp 63) (Wilson *et al.*, 1989; Sacks *et al.*, 1990; Kimsey *et al.*, 1993). Both gp 63 of *Leishmania mexicana amazonensis* and LPG of *L. major* have been found to decrease during *in vitro* cultivation (Chaudhuri and Chang, 1988; da Silva and Sacks, 1987).

It is possible that efficiency of metacyclogenesis decreases with subsequent culture and the loss of virulence for the parasites with frequent culture could be attributed to drastic decline in metacyclogenesis potential overtime (daSilva and Sacks, 1987). The decrease in infectivity/ virulence could therefore be a reflection of the faster growth rate of the infective subpopulation that might predominate after prolonged cultivation *in vitro* (Handman *et al*, 1983).

1.2.5 Invasion of macrophages by *Leishmania* and evasion of host innate mechanisms.

Leishmania parasites are obligate intracellular organisms in mononuclear cells namely; monocytes, histocytes and macrophages which are phagocytic cells. To establish an infection, the metacyclic promastigotes deposited in the skin must find or be found by a cell of the mononuclear phagocyte series (Moll *et al.*, 1995). It is known that successful parasitization of macrophages is aided by chemical components in the sand fly saliva (Anjili, 1995; Theodos *et al.*, 1991). LPG and gp 63 are major *Leishmania major* surface molecules; which have been implicated in parasite attachment and uptake into host macrophages (Etges et al., 1986; Russell and Wilhem 1986). Although the exact details of the parasites interaction with various macrophage surface molecules remain unclear, good evidence implicates the macrophages integrins CR1 and CR3 in the binding of both gp 63 and LPG (Talamas-Rohana *et al.,* 1990; Russel and Wright, 1989).

Although a natural infection takes place in the presence of serum, unlike the procyclics, the metacyclic promastigotes readily resist the lethal activity of complement components and are engulfed by the macrophages (Puentes *et al.*, 1988; Franke *et al.*, 1985). This has been attributed to the elongation and thickening of LPG on metacyclics (Saraiva *et al.*, 1995; Kimsey et al., 1993). LPG plays a vital role in interaction of promastigotes with murine and human macrophages by acting as the C3 acceptor of *L. major* (Davies *et al.*, 1990) and there is evidence that thickening of the LPG activates complement efficiently, resulting in extensive deposition of C3b (Sacks, 1989) which prevents channel formation and hinders lysis by the C5-9 membrane attack complex (MAC) (Puentes *et al.*, 1991; 1988; Sacks, 1989; Franke *et al.*, 1985). The parasite is efficiently opsonised with C3 cleavage fragments; which have been shown to enhance the uptake and the subsequent intracellular

survival of the parasite in the host macrophages (Mosser and Edelson, 1987; da Silva *et al.*, 1989). Within the macrophages, although protected from the lytic effects of serum and killing by polymorphonuclear leucocyte, the amastigotes are in an environment, which is potentially harmful to them. However, they are capable of surviving and multiplying in this environment. Some of the mechanisms put forward to explain the apparent ineffectiveness of the microbial activities of the macrophage against *Leishmania* amastigotes are: (i) LPG acts as a cell surface barrier preventing hydrolases from attacking the parasites, and that the highly anionic nature of LPG may protect the parasite against enzymatic attack in lysosomes (Turco, 1988). A lysosomal enzyme that has been shown to be clearly inhibited by LPG is B-galactosidase (El-On *et al.*, 1980). It is also thought that LPG may protect the parasites within phagolysosomes of host macrophages by inhibiting the action of protein kinase C, the enzyme believed to be responsible for activating the normally dormant oxidative killing events in phagocytic cells (Turco, 1988). (ii) The anionic LPG acts as a free radical and hydrogen peroxide scavenger (Hughes, 1988). Amastigotes and not promastigotes show a high activity of enzymes able to neutrolize or eliminate oxidative metabolites. These enzymes are glutathione peroxidase, superoxide dismutase and catalase (Murray, 1981; Pearson *et al.*, 1983). (iii) ATP-ase in the proton pump of *Leishmania* amastigotes plasma membrane drives transport of glucose and amino acids while maintaining a neutral pH within the parasite inside the acidified phagoglysosome (Blackwell *et al.*, 1986). (iv) The protease activity of gp63 with an optimum at pH 4, inactivates proteolytic macrophage enzymes and thus protects parasite proteins from phagolysosomal degradation (Kweider *et al.*, 1987). (v) The acid phosphate on the surface of *L. donovani* is capable of blocking the production of superoxide (0_{-2}) and hydrogen peroxide (H_2O_2) by phagocytes (Bogdan *et al.*, 1990; Chan *et al.*, 1989;

Buchmuller-Rouiller and Mauel, 1987). (vi) Amastigotes suppress the expression of macrophages major histocompatibility complex (MHC) class II gene products and interleukin 1 (IL-1) secretion, which impairs cell induction of protective parasite-specific T cell immunity (Reiner and Locksley, 1995). LPG has been further implicated as an important virulence factor (Kimsey *et al.*, 1993; da Silva *et al.*, 1989).

It is however, believed that there are innate properties of subsets of macrophages that determines their ability to support or resist the intracellular replication of amastigotes (Wikel, 1984). Innate resistance is controlled by a single autosomal gene designated *Lsh* and the expression of genetic control is through macrophages with the resistant phenotype displaying a greater response of lymphokines (Bradley and Kirkley, 1977).

1.2.6 Control of Leishmaniases.

The diversity of the epidemiological patterns of various forms of the disease makes it impossible to control leishmaniases with a single approach and require an integrated approach. Some of the strategies tried so far include vector and animal reservoir control, chemotherapy and vaccination.

1.2.6.1 Vector and Animal reservoir control.

Control of leishmaniasis through control of vectors and animal reservoirs is complicated by the fact that many species of sandfly are potential vectors and some 100 species of animals could act as reservoir hosts (WHO, 1990). Effective control of sand flies requires clear understanding of their bionomics (Koirala *et al.*, 1998). The use of insecticide

is believed to have helped reduce transmission of *L. donovani* in India, *L. tropica* in many parts of the Middle East and *L. brazilensis* in Brazil (WHO, 1987) although resistance to the insecticides has been reported in North Eastern India (WHO, 1990). Total eradication of sandfly vectors through chemical control has been difficult due to the nature of their breeding and resting sites such as termite hills, rock crevices and caves (Kaul et al., 1978). Recent studies on the use of impregnated bednets proved to be a potential control measure against leishmanial infections (Kroeger *et al.,* 1999).

Physical destruction of animal burrows helped to interrupt transmission of *L. major* in parts of Southern USSR and control of leishmaniases by controlling reservoir hosts has been recommended for zoonotic visceral and cutaneous leishmaniasis (WHO, 1987).

However, control of animal and vector reservoirs cannot be used as a single tool against leishmaniases since not all species of animal host have been identified. To control leishmaniases there is an urgent need for integrated approach, which could be achieved through the combination of chemotherapy and vaccination.

1.2.6.2 Chemotherapy of leishmaniases.

The current drugs of choice for all forms of leishmaniases are pentavalent antimonials (SB5) in the form of sodium stibogluconate or meglumine antimonate (Olliaro and Brycesson, 1993). Unfortunately, treatment failure in kala-azar (Mebrahtu *et al.,* 1989), mucosal leishmaniasis (Franke *et al.*, 1994) and forms of cutaneous leishmaniasis (Costa *et al.*, 1986) are becoming a common problem in many endemic areas, occurring in 5-70% of patients. Furthermore, none of the drugs used in the treatment of leishmaniases are deprived of significant toxicity and all must be administered in large doses over a large period of time.

The drugs on the other hand are quite costly and are associated with relapses (Moddaber, 1989). This leaves vaccination as one of the best option in the struggle for the control of leishmaniases.

1.2.7 Acquired immunity to cutaneous leishmaniasis.

1.2.7.1 The role of antibodies.

Upon deposition of the promastigotes by the sand fly into the host skin, before being taken up by the macrophages, they are exposed to the serum components that consist of the complement system and the antibodies. Although cell mediated immunity (CMI) rather than humoral immunity (HI) has been implicated to play a major role in the protection against cutaneous leishmaniasis (Liew, 1989; Locksley and Louis, 1992), anti-leishmanial antibodies have been shown *in vitro* to lyse promastigotes in the presence of complement (Pearson and Steigbigel, 1980) and to enhance phagocytosis (Herman, 1980; Mosser and Edelson, 1987). Serum IgE concentration and the expression of low affinity receptor IgE (FC ε Rii/CD23) or IgG have been shown to increase *in vivo* or after immune challenge with *Leishmania* antigens (LazamaDavilla and Gallager, 1995). Furthermore, antibody mediated protection against murine cutaneous leishmaniasis has also been demonstrated with monoclonal antibodies that are directed against leishmanial surface antigens (Andersons *et al.*, 1983).

1.2.7.2 Macrophage mediated killing of *Leishmania.*

Previous studies have shown that infected macrophages are capable of either killing *Leishmania* parasites directly or require being stimulated in order to eliminate the parasites (Molyneux and Ashford, 1983). The infected macrophages are activated to generate toxic oxygen metabolites namely:- superoxide dismutase (SOD), singlet oxygen(O) and hydrogen peroxide (H_2O_2) which significantly kill and inhibit the growth of the intracellular parasites (Murray and Carteli, 1983). On the other hand the infected macrophages can present antigens in the context of class II MHC antigen together with IL-1 to T- cells which leads to T- cell activation (Liew, 1989).

1.2.7.3 Role of T-cell mediated immunity.

Various studies have demonstrated the pivot role of T-cells in acquired immunity to *Leishmania* parasites and that immunity to leishmaniasis is achieved through macrophage activation by cytokines from specifically stimulated T-cells (Locksley and Louis, 1992; Hoerauf *et al.,* 1996). Experimental infections of mice with *Leishmania* have shown that the T- cells subset stimulated depends on the genetic background of the animal, which in turn leads to two different patterns of the disease (Howard, 1982). During active infection with *L. major* parasites, susceptible mice such as BALB /c inbred strains have been shown to mount CD 4+ T- helper type 2 (Th 2) cell response. This is characterized by high IL- 4, IL-5, IL- 10 and low IFN- y production, which is correlated with non-healing (exacerbation) of the disease (Scott *et al.,* 1988, Heinzel *et al.*, 1989). In active human cutaneous leishmaniasis, *in situ* expression if IL-10 and IL-12 have been observed (Mebly *et al.,* 1996). Resistant strains of mice like C57/46 mount CD 4+ Th 1 response on infection, characterized by high IFN-y

production and low IL- 4, IL- 5 production which are correlated with healing of the disease (Titus *et al.*, 1989). Thus the CD 4+ parasite- specific T-cell subset may be beneficial or detrimental and has an influence on the course of infection. The CD 8+ T- cells have also been implicated in the elimination of the *Leishmania* infected cells (Maasho *et al,* 1998). Additionally, healing of cutaneous leishmaniasis has been linked to activities of T cells that mediate specific delayed type hypersensitivity (DTH) (Convit *et al.,* 1989). However, protection of murine cutaneous leishmaniasis can be conferred by cells which do not mediate parasite specific DTH (Howard *et al.*, 1982; Liew, 1989, Muller and Louis, 1989). Kimsey *et al.,* (1993) observed an emerging complex picture with cytokine secretion in leishmaniases in mice in which certain T cell cytokines either conferred protection or exacerbated the disease in *L. major* infections.

Another cytokine, interleukin-12 produced by monocytes and B-cells stimulate production of interferon-gamma (IFN-γ) by T cells and natural killer (NK) cells (Liew, 1989). Natural killer cells have been shown to play an important role in the elimination of *Leishmania* infected cells (Maasho *et al.*, 1998). Interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-alpha (TNF-α) generated by the activated CD 4+ Th 1 (Liew, 1989) are macrophage activating factors which enhance intracellular killing of the parasites by the macrophages leading to self healing (Mc Gurn *et al.,* 1990). Significant amounts of TNF-α and IL-12 have been demonstrated to play an important role in the development of protective immune response against cutaneous leishmaniasis (Liew 1989, Titus *et al.,* 1991). Scott *et al* (1996) showed that IL-12 rather than IL-4 was the critical cytokine in the determination of susceptibilty in experimental *L. major* infections. Failure of susceptible

mice to cure infections is normally due to selective induction of T suppressor cells and low response to lymphokines (Howard *et al.*, 1982).

1.2.8 Vaccine development in the leishmaniases and related problems.

To date there is no potent vaccine against any human parasitic diseases including leishmaniasis. Although leishmanization (deliberate inoculation of virulent *Leishmania* parasites at a site where no disfiguring lesions can develop) appeared protective in USSR (Modabber, 1989), Israel (Greenblatt, 1988) and Iran (Dowlatti *et al.*, 1996) it became unpopular. This was because not every one who received the inoculation healed within a year and could still require treatment (WHO, 1995).

1.2.8.1 Preparation of experimental antigens.

Experimentally a number of antigen preparations have been used to immunize inbred mice against murine leishmaniases with varied degrees of success. *Leishmania tropica* parasites attenuated by gamma irradiation (Howard *et al.*, 1982) and later heat killed (Howard *et al.*, 1984), soluble extracts of *L. major* promastigotes (Scott *et al.*, 1987) have been used to induce protective immunity in BALB/c mice against cutaneous leishmaniasis. Although studies on killed *Leishmania* with or without Bacille-Calmette Guerin (BCG) provoked a good adaptive protection against murine cutaneous leishmaniasis, results in human volunteers were discouraging (Convit *et al.*, 1987).

Significant progress has been made with protein antigens namely 63 kilodalton glycoprotein (gp 63) (Medonca *et al.*, 1991), 46 kilodalton glycoprotein (gp46/M2) (Handman *et al.*, 1995) and promastigote surface antigen- 2 (PSA-2) (Handman *et al.*, 1995; Kemp *et al.*,

1998) which were able to induce Th1 mediated protection against murine cutaneous leishmaniasis. A study on gene encoding *Leishmania* surface protease gp 63 cloned in BCG has also been shown to induce a protective immune response in mice (Connell *et al.*, 1993).

Meanwhile a lot of attention is currently focused on developing transmission blocking vaccines. It has been shown that when sand flies are fed on mice immunized with subcellular and sand fly gut antigens (Anjili *et al.*, 1997) and purified LPG (Tonui, 1997), the growth of *L. major* in the gut of these sand flies is inhibited. However, killed or sub unit vaccines are known to produce short term immunity thus requires frequent boosting. This makes a vaccine expensive in terms of production and administration (Modabber, 1993). Live vaccines may have advantage over killed or sub units in that the parasites mimic the natural behaviour thus eliciting both primary and secondary immune responses as in the case of a natural infection.

1.2.8.2 Live attenuated parasite vaccines.

Vaccination with live *L. major* has proven to yield effective immunization in humans (Dowlatti *et al.,* 1996; Greenblatt *et al.*, 1988) but has been discontinued due to problems associated with virulence of the vaccines. Recent studies that try to circumvent this problem are mainly focused on generating live vaccines by knocking out a gene encoding a particular protein either by gamma irradiation or use of chemical inhibitors. Studies on chemical compounds conferring mutations in *Plasmodium falciparum* dihydrofolate reductase (Plowe *et al.*, 1995) going on in an attempt to combat chloroquine resistant malaria and in toxoplasmosis (Aymedir and Bilaloglu, 1996) has been applied in a vaccine trial against cutaneous leishmaniasis as well. Dihydrofolate reductase mutant (dhfr-ts- null mutant) *L. major* induced a protective immunity in mice (Titus *et al.,* 1995) and the results obtained

from lipophosphoglycan (LPG) deficient *L. major* (Kimsey *et al.,* 1993) demonstrated that genetically constructed or gene-knockout live parasites may be used as safe live vaccines. Studies with chemically mutagenized *L. major* (McGurn *et al.,* 1990) and gamma irradiated *L. major* (Rivier *et al.*, 1993) parasites although induced protective immunity, may be risky if not all parasites are inactivated. Recombinant vaccinia viruses expressing GP46/ M-2 (MacMahan- Pratt *et al.*, 1993) and genes for gp 63 incorporated into live vector (Xu *et al.*, 1995) demonstrated significant immune response although no delayed type hypersensitivity (DTH) was produced to live parasites in the latter study.

However, despite the encouraging results with the live attenuated vaccines, the cost involved in their production still calls for a vaccine which will not only be safe but also cost effective. The success of a vaccine does not only depend on the identification of productive antigen(s) but also on the adoption of a suitable vaccine protocol. A vaccine should be able to produce long term protection, safe, inexpensive, and easily administered or stored. (Moddaber, 1993). It is for this reason that *L. major* promastigotes that have been attenuated by serial cultivation were designed for this study.

1.2.9 Rationale of the study.

To date, there is no potent vaccine against any form of leishmaniases and chemotherapy has proven inadequate in the control of leishmaniases (WHO, 1995). Cutaneous leishmaniasis is a zoonotic disease (except *L. tropica* and *L. donovani* in Kenya) with humans occasionally acting as secondary reservoirs while some peridomestic animals such as *Rattus rattus* are reservoir hosts and this may result in the spread and persistence of the disease (WHO, 1995). Cases of leishmaniases have increased due to non-immune

individuals travelling to endemic areas (Olliaro and Brycesson, 1993). Moreover, leishmaniases have emerged as serious opportunistic infection among HIV/ AIDS in developed and developing countries (Orago *et al.*, 1993).

Loss of infectivity or virulence of *Leishmania* promastigotes has been shown to occur after prolonged *in vitro* cultivation (Katakura and Kaboyashi, 1985), and that the parasites may become non-infective after extensive subcultivation (Ebert *et al.,* 1979). This protocol therefore portends to establish the efficacy of live *L. major* parasite attenuated by 116 times serial subculture (NLB-144, 116^0) as a candidate live vaccine against cutaneous leishmaniasis. Preliminary studies using these avirulent parasites have shown that they do not induce lesions in BALB/c mice (Anjili, personal communication).

BALB/c, an inbred strain of mice are the most suitable models for vaccine because they can easily be infected in the laboratory and infection monitored over a period of time (Playfair *et al.*, 1990). They are also highly susceptible to *L. major* infections and produce a disease which mimic that of human. The ease with which *L. major* parasites can be cultured and attenuated makes it an ideal model for this investigation.

Experiments on heat-killed or soluble promastigotes have shown that repeated intravenous immunization induce a strong protective immunity (Howard *et al.*, 1982; Scott *et al.*, 1987). Intravenous (iv) or intraperitoneal (ip) routes induce protective T- cells while intramascular (im) or subcutaneous (sc) routes induce disease exacerbation (Scott *et al.*, 1987). If the method is successful, this will offer an opportunity to plan strategic control of *Leishmania* infections.

1.2.10 Hypothesis.

Leishmania major promastigotes maintained serially in culture *in vitro* without passage through the vector or reservoir host can induce protective immune response in BALB/c mice against the homologous virulent strain due to loss of infectivity.

1.3 OBJECTIVES OF THE STUDY

1.3.1 General objective.

To assess the potential of whole live attenuated *L. major* parasite as a candidate vaccine against cutaneous leishmaniasis in BALB/c mice.

1.3.2 Specific objectives.

1.3.2.1 To determine humoral responses against *Leishmania* derived antigens.

1.3.2.2. To estimate cell mediated responses against *Leishmania* derived antigens.

1.3.2.3 To quantitate the parasiticidal activities of peritoneal macrophages from previously immunized mice.

1.3.2.4 To measure lesion size in immunized mice that were challenged with virulent *L. major* parasites.

1.3.2.5 To determine metastasis in the immunized mice that were challenged with virulent *L. major* parasites.

1.3.2.6 To determine persistence of the live attenuated *L. major* parasites at the site of inoculation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Source of mice.

Weanling BALB/c mice (6 to 8 week-old) were obtained from animal house, KEMRI, Nairobi and were maintained in the KEMRI animal house under standard hygienic conditions. The mice were fed on nutrition standard food pellets ((Unga Limited, Kenya) and given clean tap water *ad libitum*. Wood shavings that were used as bedding were changed from the cages twice a week.

2.2 *Leishmania* promastigote cultures and preparation of live attenuated antigens

Virulent *L. major* parasite (strain IDUB/KE/83=NLB 144) was originally isolated from a female *P. duboscqi*, collected near Marigat, Baringo District (Beach *et al.,* 1984) and has since been maintained in BALB/c mice. Parasites used in this study were obtained from aspirates of infected footpad lesions of BALB/c mice and cultured in NNN/ Schneider's *Drosophila* insect medium supplemented with 20% foetal bovine serum (FBS), 250U/ml penicillin, 250μg/ml streptomycin and 500g/ml 5-Flourocytocine arobinoside at 25^{0}C. The parasites were grown and harvested at metacyclic promastigote stage.

2.3 Preparation of *Leishmania* antigens.

2.3.1 Live attenuated parasites.

Live attenuated parasites were originally obtained from the virulent *L. major* parasites strain (NLB 144) and then cloned. These parasites were attenuated by serial *in vitro* subculturing of metacyclic promastigotes upto stationary phase 116 times.

2.3.2 Preparation of heat killed antigens.

Stationary phase promastigotes of virulent *L. major* parasites were suspended in PBS, placed at 60^0C in a water bath for 10 minutes to provide the heat killed whole parasite antigens which were freshly made and administered at each time of immunization.

2.3.3 Preparation of soluble *Leishmania* antigens.

Virulent *L. major* promastigotes from stationary phase were washed three times at 4°C with sterile phosphate buffered saline (PBS). The frozen parasites from liquid nitrogen were suspended in PBS and then subjected to several cycles of freeze -thawing between -196^0C and 37^0C to lyse the cells. The lysate was centrifuged at 10,000 rpm (385g) for 15 minutes at 4°C . The supernatant was removed and made isotonic with 1.5 sodium chloride (NaCl). Protein in the supernatant was estimated calorimentrically using Lowry technique (Lowry *et al.*, 1951) and then stored at -20^0C. This provided the soluble *Leishmania* antigen.

2.3.4 Protein estimation.

Albumin stock containing 200µg/ml of bovine serum was serially diluted in PBS and aliquoted in separate test tubes. These were used as protein standards against the unknown. The soluble *Leishmania* antigen was then diluted in the ratio of 1:20 and 1:5 respectively in 0.5N sodium hydroxide (NaoH). A test tube containing 1ml of PBS without any protein was used as a control (blank). Solution of 2% Di-sodium carbonate (Na_2Co_3), 1% copper sulphate ($CuSO_4$) and 2.7% Potassium sodium Tartrate (PST) were added to all the tubes, vortexed and allowed to stand at room temperature for 15 minutes. Phenol reagent of 0.5ml was added to each tube, vortexed again and let to stand at room temperature for a further 45 minutes.

Aliquot from each tube was put into a cuvet and using a spectrophotometer (DU 650, Beckman, USA) at 675nm, their absorbance value was read. The absorbance value of the standard against the corresponding protein concentration was plotted. Finally, using the absorbance values obtained, the protein of content of the protein in the soluble antigen was read form the graph.

2.4 Immunization procedure

Four groups each containing thirty weanling BALB/c mice (six to eight weeks old) matched by age and sex were immunized intravenously through the tail vein.

The first group of mice was immunized with 1 x 10^6 live attenuated metacyclic parasites. They were boosted with the same dose of antigen at seven days interval for four weeks. The second group was immunized with 1 x 10^6 heat killed whole parasites and were boosted with the same antigen dose at seven days interval for four weeks. The third group was immunized with 5.15μg/ml soluble antigen (amount of protein estimated from 10^6 promastigote) and were also boosted with the same dose of antigen at seven days interval for four weeks. Lastly the control group was injected with sterile PBS and every time the experimental animals were boosted, they were also given the same dose of PBS.

2.5 Determination of humoral immune responses against *L. major*-derived antigens.

Twenty (20) mice from each group were bled one week after the third booster by tail snipping method as previously described (Tonui, 1998). Briefly, two haematocrit tubes full of blood were collected from each mouse and blotted onto two opposite spots of a filter paper. Filter papers were dried and stored at 4^0C until. Spots containing serum were cut out and put in an elution tube containing 5% Tween 20 bovine albumin in saline containing phosphate 0.05% Tween 20 (PBST) as a diluent. Enzyme linked immunosorbent assay (ELISA) as described by Voller *et al.*, (1976) was used to assay the antibody level in the sera. Briefly, U-well polyvinyl chloride microtiter plates were coated overnight at 4^0C with 100µl of 100µg soluble *L. major* antigen. The plates were washed three times with boiled casein at 37^0C for 1 hour, washed again three times and coated with 100µl of animal serum at a dilution of 1:50 in PBST. Plates were then incubated for 1 hour at 37^0C and then washed three times in PBST. Rabbit anti-mouse Immunoglobin G (IgG) peroxidase conjugate was added at a recommended working dilution of 1:2000 and incubated for 1 hour at 37^0C. The plates were washed and 100µl of 2.2'-azinodi [3-ethyl-benzthiozoline sulfate] (ABTS) peroxidase substrate was added. The plates were incubated for 30 minutes in the dark at room temperature. Finally the reaction was stopped by adding 25µl of 1N HCL and the optical density was read using an ELISA reader with a 492nm filter.

2.6 Estimation of cell mediated response towards *L. major* derived antigens.

2.6.1 Lymphocyte proliferation assays.

Lymphocytes were obtained from mice spleens according to the method of Shephard *et al.*, (1983). Briefly, the spleen from previously immunized animals was aseptically crushed on an autoclaved wire mesh using a syringe plunger. The cells were washed out of the wire mesh into a test tube containing RPMI growth media and then overlaid in Ficoll isopaque to separate the cells. The tubes were centrifuged at 2500 rpm (24g) for 30 minutes at 4^0C. The ring of cells which comprised of the lymphocytes was harvested, put in a sterile test tube then centrifuged at 1500 rpm (8.65g) for 10 minutes. The cells were then cultured in 96 well trays at a concentration of $3x10^6$ viable cells per ml (cell viability was determined by the trypan blue exclusion test) and stimulated with *L. major* soluble antigens at various dilutions. The culture plates were incubated at 35^0C in 5%$C0_2$ 95% air. After 72 hours, the cultures were pulsed with 0.5µI [3H] thymidine for 18 hours after which lymphocytes were harvested onto glass fibre filters using an automated harvester and counted by the liquid scintillation counter (LS 1801, BECKMAN, U.S.A.)

2.6.2 Determination of delayed type hypersensitivity (DTH).

Both immunized and control mice were injected in the left hind footpad with 25µl of the soluble antigen (Nolan and Farrell, 1987). The induration was expressed as the difference between the thickness measured before and 48 hours after eliciting the injection. The measurements were made using a vernier calliper.

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2.7 Macrophage activation assays.

To determine the parasiticidal efficiency of peritoneal macrophages of immunized mice, these cells were infected with virulent *L. major* parasites. This was done as described by Mbati *et al.* (1994). Briefly, three weeks after the third booster, peritoneal macrophages from both immunized and naive mice were collected by lavage of the unstimulated peritoneal cavity with RPMI 1640 medium buffered with 25mM HEPES. After three washes in PBS, cells were plated on culture flasks containing buffered RPMI 1640 medium, supplemented with 10% FBS plus penicillin/streptomycin and incubated *in vitro* at 37°C in 5% CO_2 95% air. After two hours of incubation, non-adhering cells were removed by rinsing with cold medium while the adhering cells were cultured for 40 hours to allow for adequate spreading.

After three rinses and renewal of the RPMI 1640 medium- FBS, the cultured cells were exposed to virulent *L. major* promastigotes and incubated at 37°C in 5% CO_2 95% air. After periods of infection of 4, 24 and 48 hours, the macrophage monolayers were washed, fixed for 5 minutes in absolute ethanol and stained for 30 minutes with 10% Giemsa. The level of infection in these cultures was calculated by counting macrophages in random microscopic fields in two separate culture dishes and expressing the number of infected macrophages as a percentage of the total number of macrophages counted.

2.8 Challenge procedure.

In order to assess the effectiveness of the vaccines, after the third booster, all the immunized and control animals were infected with 10^6 virulent promastigotes on the LHFD. Thereafter lesion development was monitored by measuring both infected and uninfected footpads once a week and the extent of parasite metastasis in all the animals was determined 14 weeks post challenge.

2.8.1 Lesion measurement.

After the third booster, sizes of the left and right hind footpads were measured and recorded. Following the challenge infection, both infected and uninfected food pads were measured once a week for 14 weeks using a vernier calliper and the difference between the thickness of the two footpads was reported as the lesion size.

2.8.2 Determination of parasite load in the liver and spleen biopsies.

At 14 weeks post-infection, all mice were sacrificed, liver and spleen cultures were obtained to determine extent of metastasis and visceralization. Impression smears were also made from liver and spleen, fixed in absolute methanol and stained for 30 minutes in 10% Giemsa then observed under the microscope. The number of amastigotes per 1000 organ cell nuclei were counted and the number of parasites (x) in organs was derived from the formular

X= LDU/1000 x wt (mg) x 2x 10^5 (Mbati *et al.*, 1994)

2.9 Determination of persistence of the live attenuated parasites

Some 10^6 stationary phase promastigotes of live attenuated *L. major* and virulent parasites were inoculated into two different groups of BALB/c mice on their LHFP. The presence of these parasites was monitored by obtaining aspirates from the infected site 6 hrs, 12 hrs and 24 hrs post-infection. Some of the material from the aspirates was cultured while the rest was used in preparing slide smears which were then stained in 10% Giemsa as previously described. Slides were then observed under the light microscope at x100 magnificationfor the determination of the presence of promastigotes or amastigotes. The same procedure was repeated 7 and 30 days post- infection respectively.

CHAPTER 3: RESULTS

3.1 Humoral responses against *Leishmania*-derived antigens.

Immunized mice in this investigation produced substantial amount of antibodies on repeated intravenous injection with various antigens derived from the metacyclic *L. major* promastigotes. Table 1 below presents the mean of antibody titre produced by 20 mice from each group after the fourth immunization with PBS as the diluent of the antigens.

There was a significant difference in antibody titre produced by the various antigens (ANOVA I, df=79, F=7123.53, P= 0.006). The live attenuated parasites induced the highest production of antibody titres followed by soluble antigens whereas the heat killed whole parasites induced the lowest antibody titre.

Table 1: Mean antibody response against *L. major* derived antigens after the fourth immunization

IMMUNIZATION ANTIGENS	MEAN OD ±SE
Live attenuated parasites	0.283±0.004
Heat killed whole parasites	0.198+ 0.085
Soluble antigens	0.223±1.040
PBS (Controls)	0.000±0.000

OD- Optical Density

SE- Standard Error of mean

3.2 Cell-mediated responses against *Leishmania* derived antigens in immunized mice.

Cellular immunity against the various *Leishmania* antigens was assessed in the vaccinated animals by skin testing using soluble antigen derived from *L. major* and by measuring the capacity of spleen cells from immunized mice to proliferate on stimulation with the antigen preparation. All the immunized mice exhibited minimal delayed type hypersensitivity (DTH) after 24 hrs and 48 hrs before challenge as shown in Table 2 below:

Table 2: Mean nodule size of 5 BALB/c mice from each group at different hours post treatment with virulent *L. major* derived soluble antigens

	NODULE SIZES AFTER SPECIFIED TIME ± SE	
IMMUNIZATION ANTIGENS	24 Hours	48 Hours
Live attenuated parasites	0.130± 0.08	0.100± 0.09
Heat-killed parasites	0.120±0.01	0.000±0.00
Soluble antigens	0.100±0.09	0.000±00
PBS (Controls)	0.000±0.00	0.000±00

All the vaccinated mice exhibited varied T-cell responses, an indication that immunological memory persisted for at least 12 months after the final immunization. Animals vaccinated with soluble antigen revealed the highest T-cell proliferation (Stimulation index =4.8) followed by those vaccinated with heat killed whole parasite antigen (SI=3.8) whereas the mice immunized with live attenuated parasites induced the least (SI=3.0). Figure 4 below illustrates the proliferative response of splenocytes (of 5 mice from each group) after stimulation with *L. major* derived soluble antigen.

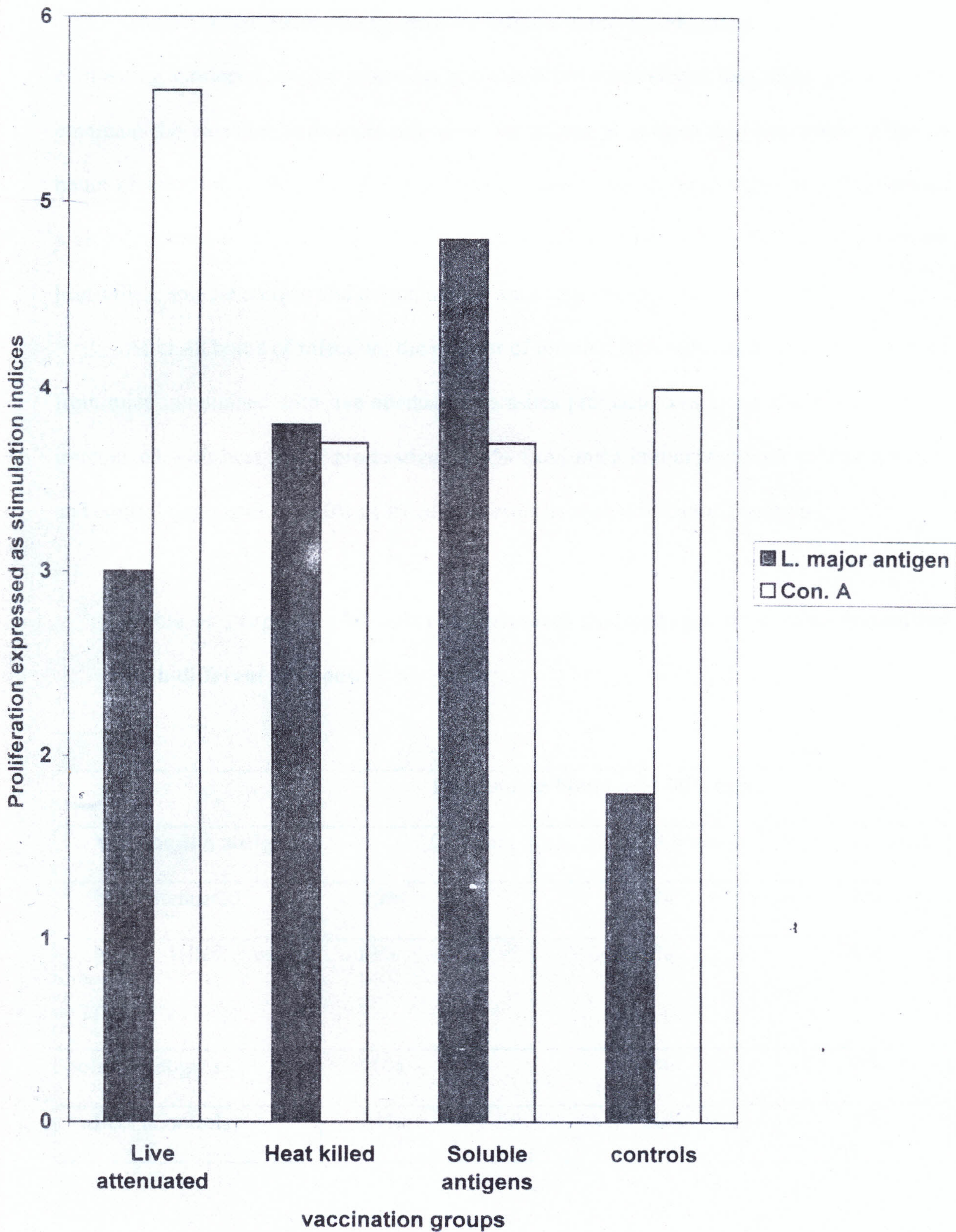


Fig. 4:Recall proliferative response of splenocytes of mice stimulated with L. major antigen and Con A.
Proliferation expressed as stimulation indices
6
5
4
3
2
1
0
Live attenuated
Heat killed
Soluble antigens
controls
vaccination groups
L. major antigen
Con. A

3.3 Parasiticidal activities of peritoneal macrophages obtained from the immunized mice.

The peritoneal macrophages from the various immunization groups were all capable of eliminating virulent *L. major* promastigotes which were introduced into them, but ability to eliminate the parasites varied depending on the nature of antigen that was used. After 24 hours of infection, only 10% of the resident peritoneal macrophages from mice immunized with live attenuated parasites were still parasitized compared to 13%, 10% and 24% from the heat-killed, soluble antigen and unimmunized mice respectively.

After 48 hours of infection, the number of infected cells reduced drastically with those from mice immunized with live attenuated parasites promastigotes being 2%, 8% from mice immunized with heat killed promastigotes, 4% from mice immunized with soluble antigens and control group still had 20% of its cells infected as shown in Table 3 below:-

Table 3: Proportion of infected peritoneal macrophages from mice immunized with different antigens.

	Duration in hours post infection		
Vaccination antigens	24 hours	48 hours	72 hours
Live attenuated	10%	2%	0%
Heat killed whole parasites	13%	8%	0%
Soluble antigens	10%	8%	0%
PBS (Controls)	24%	20%	6%

3.4 Lesion development of mice post-inoculation with virulent parasites.

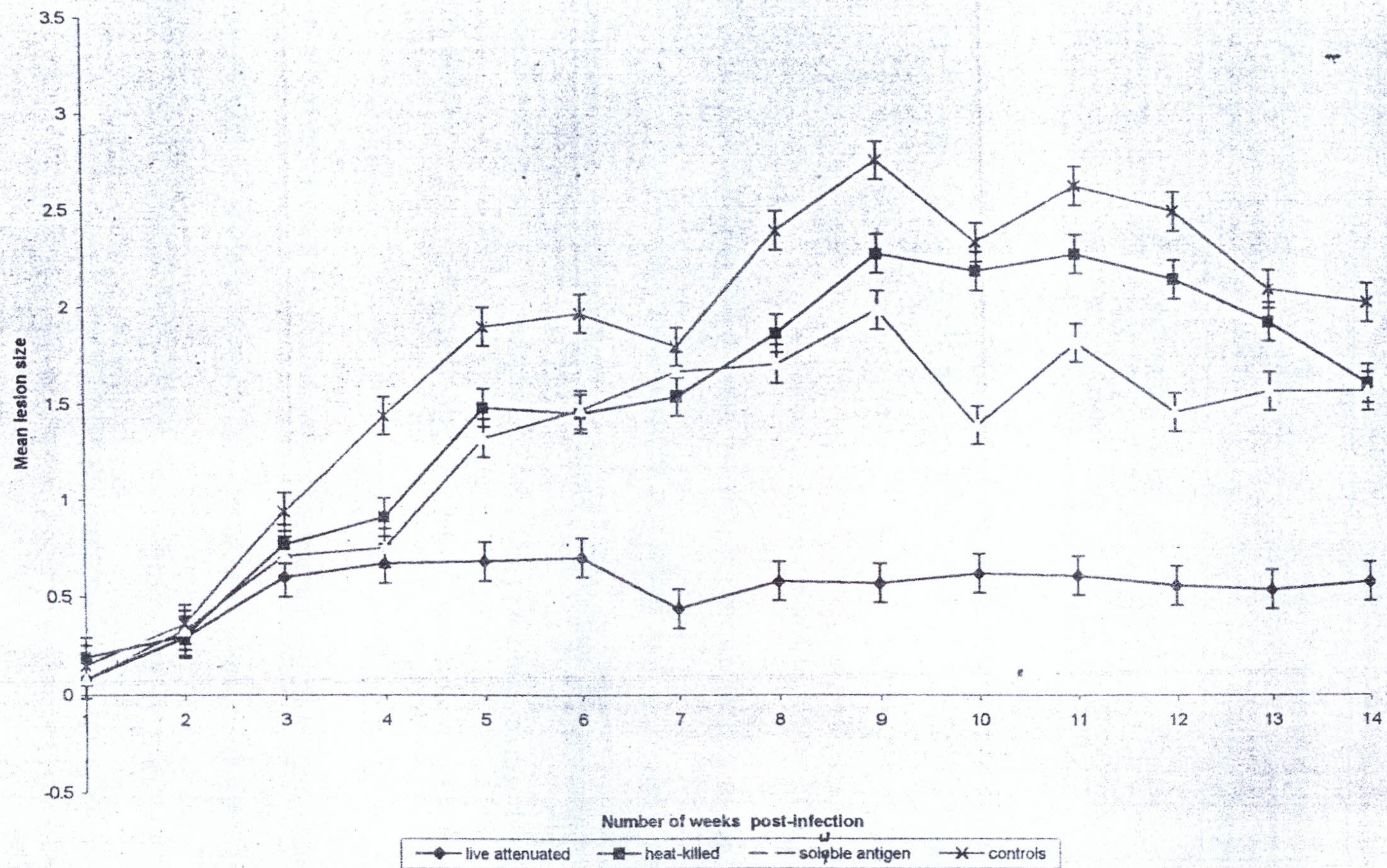
By the end the second week, post-inoculation with virulent *L. major* parasites, all mice had measurable nodules of which by the fifth week, had progressed in size. Figure 5 below represents the mean lesion size of 30 mice from each group over a period of 14 weeks post the challenge.

Throughout the study, mice immunized with live attenuated parasites exhibited only minor footpad swelling and failed to develop into progressive infections with a maximum mean lesion size of 0.7 mm recorded in the 5th week. The difference in the lesion development in this group compared to the rest started appearing as early as the 4th week (ANOVA 1, P=0.000) and remained consistent throughout the study period.

Animals immunized with soluble antigens and heat-killed whole parasite antigen although had different mean lesion sizes throughout the study period, the disease progression did not vary statistically (ANOVA 1, P=0.673) as early as the 4th week and as late as the 12th week see Appendix I. Compared to the controls, generally the lesion development in these two groups was slower (ANOVA 1, P=0.011, P=0.00), for mice immunized with soluble antigens and heat killed whole parasite antigen respectively).

Unimmunized mice, (controls) had quite prominent lesions and by the 5th week post-infection most of the lesions had ulcerated. By the end of the 7th week, necrosis had started taking place in most mice and by the 14th week 3\15 animals from this group had lost their footpads. Interestingly, 5\15 mice immunized with soluble antigens developed more severe lesions compared to the controls.

Fig 5: Mean lesion size of BALB/c previously vaccinated with various antigens and challenged with virulent *L. major*



3.5 Parasitaemia levels in the liver and spleen biopsies.

No amastigotes were seen on the slides except on one slide of control group. Cultures from liver and spleen biopsies were positive in the rest of the experimental groups except in the group immunized with live attenuated parasites. Table 4 below shows the mean weight of the biopsies from 10 BALB/c mice from each group.

From the mice immunized with heat killed parasites, 2/10 of spleen and 1/10 liver cultures were positive compared to 4/10 of spleen, 1/10 of liver cultures from mice immunized with soluble antigens. From the control mice, 6/10 culture flasks had promastigotes while 1/10 liver flasks had promastigotes see Appendix II. Liver and spleen sizes from various vaccination group did not show a big difference in their mean weights (ANOVA 1, P=0.113, for liver and P=0.684 for spleen).

Table 4: Mean weights (in grams) of liver and spleen of BALB/c at 14 weeks post-infection.

	Biopsied organs	
Vaccination groups	**Liver**	**Spleen**
Live attenuated parasites	1.08±0.046	0.43±0.024
Heat killed whole parasite antigen	1.18±0.079	0.45±0.02
Soluble antigens	1.19±0.09	0.46±0.042
Controls	1.33±0.056	0.48±0.03

3.6 Persistence of the live attenuated parasites at the site of inoculation in BALB/c mice.

Live attenuated parasites inoculated in mice were eliminated before 48 hours and the amastigotes could not be traced from the impression smears. Cultures from the infected sites after 2 days, 7 days all turned out negative. While those infected with virulent *L. major* parasites all had parasites. Aspirates obtained from the footpads 2 months post-infection with live attenuated parasites were similarly negative.

CHAPTER 4: DISCUSSION

4.1 Humoral Immune response to *Leishmania*-derived antigens.

All the immunized mice responded to the soluble antigen preparations by showing high absorbance density. Animals vaccinated with live attenuated parasites produced the highest antibody titre (ANOVA 1, P=0.00) compared to the other vaccinated groups. Animals that were immunized with soluble antigens produced better humoral response than the group of heat killed whole parasite antigen (ANOVA 1, P=0.00). This difference in antibody titre may be attributed to the nature of the antigens produced. It may be that live-attenuated parasites retained normal surface antigens found on the surface of virulent *L. major* parasites and are capable of eliciting antibody production as seen during normal infection. In addition, due to the fact that they are poorly phagocytosed as observed by previous workers (Ebert, 1979; Giannini *et al.*, 1981) provided them a longer time to be outside the cells which gave them a longer period to provoke B-cells. On the other hand, the process of heat-killing parasites may denature proteins leading to distortion of surface antigens. This may explain why the humoral response in mice immunized with heat-killed whole parasites was poor. Similarly, freeze-thawing was shown to destroy surface antigens on promastigotes (Titus *et al.*, 1984) and could have been the case with the soluble antigens used in this study.

4.2 Cell mediated immunity to *Leishmania* derived-antigens.

Although healing of cutaneous leishmaniasis has been linked in both human and murine leishmaniasis to the activities of T-cells that mediate parasites specific DTH, none of the immunization antigen used in this study mediated a DTH response. Protection in murine cutaneous leishmaniasis can be conferred by cells that do not mediate parasite specific DTH. Several studies have confirmed this. A study by Liew, (1989) showed that it is possible to generate protective T-cells by immunizing BALB/c mice intravenously with heavily irradiated *L. major* promastigotes. He reported that these protective cells did not express DTH but instead suppressed it. In addition, McGurn *et al.* (1990) also realized the same results while using mutated clone of *L. major* and further noticed that the protective T-cells when adaptively transferred to a naive animal and then challenged with virulent *L. major* parasite, the T-cells mediated strong DTH reactions. Kimsey *et al* (1993), generated protective CD 4+ T-cells by using LPG deficient *L. major* parasites which also failed to elicit DTH reactions. Although earlier studies demonstrated good correlation between susceptibility and resistance to clinical and experimental leishmaniasis (Howard *et al.*, 1980). Studies suggest that DTH could be detrimental to the host. Mice resistant to *L. major* infections after intravenous immunization are DTH negative, while mice exhibiting exacerbated disease after sub-cutaneous immunization show strong DTH reaction (Dhaliwal *et al.,* 1985; McGurn et al., 1990). Gicheru *et al.,* 1998 (data unpublished), while working with vervet monkeys, demonstrated that, despite showing strong DTH reaction, following a vaccination with Autoclaved *L. major* (ALM) plus rhIL-12, the animals were not highly protected. These results imply that DTH may not be a good correlation of protection in cutaneous leishmaniasis.

4.3 Lymphocyte proliferation assay

The CD 4+ T-cells generated in mice after intravenous. immunization by various vaccine antigens proliferated *in vitro* in response to soluble antigen preparations from *L. major* promastigotes. The proliferative response of the T-cells was an evidence of immunological memory that persisted in these vaccination groups for the last 12 months post-sensitization.

The group immunized with soluble antigens induced higher proliferation followed by that of heat-killed whole parasite antigens and lastly was the live attenuated group. There was no much difference in the response produced by the two groups immunized with heat killed whole parasite antigen and live attenuated parasites. The poor T-cell induction in the latter group could either be because they were poorly phagocytosed by the macrophages as had been earlier demonstrated (Giannini *et al.*, 1981; Ebert, 1979). Poor phagocytosis may be attributed to the reduced level of LPG always associated with long term cultivation of *Leishmania* parasites. Alternatively, the phagocytosed parasites might have been digested fast and most of them not transforming into amastigote forms within the cells. Investigations carried out earlier (Murray, 1981; Hernandez *et al.*, 1983) revealed that although there was poor phagocytosis of an avirulent clone of *Leishmania* parasites, once engulfed they were destroyed quite fast. Transformation of the parasites into the amastigote form leads to the expression of parasite specific antigens by the infected macrophages which effectively trigger protective CD 4+ T-cells.

Heat killing of parasites denatures proteins thus distorting the surface antigens and also as found by Makwali (1997) heat-killed *L. major* promastigotes release

immunosuppressive antigens. This may explain why there was poor stimulation of the cells in the animals immunized with heat killed whole parasites antigen. On the other hand, it is not clearly understood whether the T-cells provoked by the soluble antigens were all the protective CD 4+ T-cells. As mentioned earlier in this text the process of freeze-thawing induced exercabative T-cells in mice (Titus *et al.,* 1984). Previous studies in mice also confirm that, depending on the type of antigen presented, there are two subsets of CD 4+ T-cells that can either be stimulated; CD 4+ Th 1 or Th 2 (Howard et al., 1986). The Th 1 response is characterized by high IFN-γ production and is correlated with healing of the infection while Th 2 response is correlated with disease exacerbation (Scott *et al*., 1988; Heinzel *et al.,* 1989). Therefore, there was a possibility that most of the T-cells that proliferated from the mice immunized with soluble antigens were CD 4+ Th 2 cells.

4.4 Parasiticidal activities of the peritoneal macrophages from immunized mice towards virulent *L. major.*

There was poor survival of parasites in the macrophages from the immunized animals compared to those from the controls. This demonstrated that the macrophages had a more enhanced parasiticidal activity than the controls and this may be attributed to the prio-sensitization of the macrophages during immunization. Furthermore, earlier studies have shown that macrophages from immunized mice produced greatest amount of oxygen singlet and hydrogen peroxide which is neccesary for intrcellular killing of the parasites (Murray *et al*., 1980; Ayesta *et al.,* 1985; Frommel *et al.,* 1988).

4.5 Parasiteamia in spleen and liver biopsies.

There was no difference in the weight of the liver (ANOVAI, df=36, P=0.113) and the spleen (ANOVA 1, df= 36, P=0.686) from the various immunization groups. As it was observed from the proliferation assay, all the protected animals retained immunological memory of *Leishmania* derived antigens and earlier studies confirmed that specific T cells are capable of preventing dissemination of the parasites (Hill, 1988) and thus circumvent mechanisms that permit metastasis (Howard, 1986). This could further explain why no amastigotes were seen on the smears. Otherwise, the presence of parasites in the cultures of the liver and spleen from the other immunized and control animals except those given live attenuated parasites is an indication that this antigen was more immunogenic than its counterparts thus induced more *Leishmania* specific protective CD 4+ T-cells. These cells therefore either eliminated the parasites at the site of inoculation or suppressed the mechanism of metastasis.

4.6 Lesion development.

All the immunized animals exhibited considerable variation in protection against cutaneous leshmaniasis between the varied groups. Mice immunized with live attenuated parasites failed to develop progressive infections and only minor footpad swelling was noticed thus showing a big difference with the other experimental animals. The protection must have been brought about by the activities of the macrophage activating lymphokines stimulated by Th 1, a subset of CD 4+ T-cell. The poor protection observed in the animals immunized with soluble antigens and heat killed whole parasites antigens respectively may be attributed to poor presentation of antigens due to the method used in their preparation. Due to

this, there was poor sensitisation of the immune system of these animals against the virulent strain of *L. major* parasites.

However, the role of antibodies in protection against leishmaniases remains debatable. The antibodies may have contributed in elimination of the parasites indirectly either by opsonizing the virulent *L. major* promastigotes or activating the classic complement pathway which then promoted the uptake of the parasites by macrophages via Fc and/or C3b receptors. Once inside the macrophages, the infected macrophages either stimulated the activity of the protective T-cells or eliminated them on their own without involving the T-helper cells (Molyneux and Ashford, 1983).

4.7 Persistence of live attenuated parasites at the site of inoculation.

The promastigotes of the live attenuated parasites could not be traced within the macrophages after 48 days and neither were they seen on the blood smears. These parasites may have been eliminated by components of the complement system (Franke *et al.*, 1985; Pearson and Steigbigel, 1980) or by the antibody through opsonization (daSilva *et al.*, 1989). Similar results were realized by Nolan and Herman 1985 when they used serially cultured *L. donovani* parasites, all their splenic cultures obtained from the animals 120 days post infection were negative. It is possible that *in vitro* cultivation suppresses LPG and gp63 production, thus rendering the parasite susceptible to destruction by the immune system. Furthermore, preliminary study by Jackton, 1998 (Unpublished data) showed that heat-shocking the serially cultured *L. major* did not elicit production of the heat shock proteins that could make the parasites turn virulent when introduced into the animal host. This therefore

renders the live attenuated parasites a safe candidate vaccine that can be tried in the fight against leishmaniases.

CHAPTER 5: SUMMARY OF CONCLUSIONS

1 Although antibodies have not been previously implicated in protection of leishmaniases, in conjunction with the complement components they may have played a role in elimination of the live attenuated parasites.

2 Freeze-thawing and heat-killing did not produce highly protective antigens and therefore they are not the best way of preparing antigens.

3 Loss of infectivity and virulence in the serially cultured *L. major* may be due to reduction of gp63 and LPG that are associated with uptake and survival of *L. major.*

4 There may be another glycoconjugate apart from gp63 on the surface of *L. major* promastigote that is immunogenic and is not affected with prolonged *in vitro* cultivation as was found with gp36 on *L. donovani* (Palatnik *et al.,* 1993).

5 The live attenuated parasites induced the best protective immunity against the virulent *L. major*

6 Since the live attenuated parasites vaccine do not regain virulence in a normal healthy BALB/c mice and at the same time it can be easily and inexpensively produced, it may be considered for a vaccine trial.

CHAPTER 6: SUGGESTIONS FOR FUTURE RESEARCH WORK.

1. To determine whether the changes that take place in *Leishmania* promastigotes after prolonged *in vitro* culturing are genetic or phenotypic.
2. To determine whether there are more changes on the parasites after prolonged *in vitro* cultivation apart from the changes in LPG and gp 63 level.
3. To assess the infectivity and virulence of the live attenuated parasites in immunosuppresed BALB/c mice.

3 Higher dosage of the live attenuated parasites can be used to repeat the experiment in order to assess whether there will be a be stronger stimulation of the T-cells.

4 To assess whether the avirulent parasites can regain virulence in the sand fly gut.

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APPENDIX I

STATISTICAL ANALYSIS OF LESION SIZE ON THE 4TH AND 12TH WEEK POST-INFECTION RESPECTIVELY.

AT 4TH WEEK POST INFECTION

Vacination groups	Level of significance(P value)	comment
1 and 2	0.035	S
1 and 3	0.03	S
1 and 4	0	S
2 and 3	0.673	NS
2 and 4	0	S
1 and 4	0.011	S

AT 12TH WEEK POST- INFECTION

Vacination groups	Level of significance(P value)	comment
1 and 2	0	S
1 and 3	0	S
1 and 4	0	S
2 and 3	0.005	S
2 and 4	0.347	NS
1 and 4	0.005	S

S -Significant

NS- Not significant

1 -Live attenuated group

2- Heat-killed group

3 -Soluble group

4 -Controls

APPENDIX II

MEAN WEIGHTS IN GRAMS OF LIVER AND SPLEEN OF BALB/c MICE AT 14 WEEKS POST-INFECTION.

Immunized with 116^0 (Live attenuated)		Heat-killed whole parasite		Soluble antigens		PBS Control	
Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver
0.385	1.401 E	0.475	1.287 E	0.462	1.025	0.456	0.96
0.400	0.923	0.358	1.158	0.389	1.265	0.456	1.49 SE
0.494	1.000	0.550 GE	1.41	0.5	0.995	0.422	1.40
0.412	1.100	0.400	1.070	0.354	1.154	0.405	1.36
0.610 GE	1.10	0.470	1.056	0.476	1.456 E	0.403	1.17
0.351	0.870	0.508 GE	1.381	0.40	1.145	0.5	1.288
0.480	1.234	0.436	1.207	0.41	1.313	0.51	1.402 E
0.386	0.994	0.520 E	0.838	0.336	0.990	0.678	1.496 SE
0.456	1.098	0.350	0.792	0.43	0.805	0.390	1.226
0.333	1.120	0.441	1.580 GE	0.801 GE	1.80 GE	0.613	1.530 GE

GE - Greatly enlarged

SE - Slightly enlarged

E - Enlarged

APPENDIX III

BIOPSIED CULTURES OF LIVER AND SPLEEN

Heat killed WPA		Soluble antigen		Control	
Spleen	**Liver**	**Spleen**	**Liver**	**Spleen**	**Liver**
-ve	+ve	+ve	-ve	-ve	-ve
-ve	-ve	-ve	-ve	+ve	-ve
-ve	-ve	+ve	-ve	+ve	+ve
-ve	-ve	-ve	-ve	+ve	-ve
-ve	-ve	+ve	-ve	-ve	-ve
+ve	-ve	-ve	-ve	-ve	+ve
-ve	-ve	-ve	-ve	+ve	+ve
-ve	-ve	-ve	+ve	+ve	-ve
-ve	-ve	+ve	-ve	-ve	-ve
+ve	-ve	-ve	-ve	+ve	-ve

-ve no parasites were seen in the cultures

+ve there were parasites in the culture