EVALUATION OF IMMUNE ASSOCIATED CELLULAR PHENOTYPES IN LESIONS OF VERVET MONKEYS (Cercopithecus aethiops) EXPERIMENTALLY INFECTED WITH Leishmania major

BY OZWARA HASTINGS SUBA

Bsc. Hons. (Nairobi).

DEPARTMENT OF ZOOLOGY,

UNIVERSITY OF NAIROBI.

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DECLARATION

This research project is my original work and has not been presented for a degree in any other University.

DATE 21/3/96

OZWARA H. SUBA

This thesis was submitted with our approval as University supervisors,

DATE 26/3/96

DR. LUCY W. IRUNGU.,
Senior Lecturer, Department of Zoology,
University of Nairobi.
(PRINCIPAL SUPERVISOR)

DATE 16/4/96

DR. D.G.K. MANYASI
Depatment of Zoology,
University of Nairobi

and with my approval as the external supervisor,

DATE 11/4/96

DR. JOSEPH O. OLOBO.,
Principal Research Scientist Leishmaniasis Programme
and Head of Department, Institute of Primate Research.

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This work is dedicated to my parents, Joseph Suba Odiyo and Enid Kamonya Suba under whose care I have been able to reach this level of education

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ABBREVIATIONS

Abbreviation	Expanded form
AEC	3-amino-9-ethyl carbazole
ANOVA	Analysis of Variance
BCG	Bacille Calmette Guerin
B2M	ß2-microglobulin molecule bearing cells
BSA	Bovine Serum Albumin
C3	Complement factor 3
CD	Cluster of Differentiation
CH_3COOH	Acetic acid
CH_3COONa–	Sodium acetate
cm	Centimetres
CMI	Cell Mediated Immunity
con A	Concanavalin A
CTL	Cytotoxic T Lymphocytes
$CuSO_4$	Copper II sulphate
DCL	Diffuse Cutaneous Leishmaniasis
DDW (DDH_2O)	Double Distilled Water
DMSO	Dimethyl Sulphoxide
DTH	Delayed Type Hypersensitivity
ELBS	English Language Book Society
FACS	Fluorescence Activated Cell Sorter
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
g	Grammes

GLM	Generalized Linear Model
GM	γδ TCR antigen bearing cells
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
Gp 63	Glycoprotein 63
HCL	Hydrochloric acid
HLA	Human Leucocyte Antigen
H_2O_2	Hydrogen peroxide
HRP	Horse Radish Peroxidase
IBM	International Business Machines
i.d	Intradermal
IFAT	Indirect Fluorescent Antibody Test
IFN-	Interferon
Ig	Immunoglobulin
IL-	Interleukin
i.p	Intraperitoneal injection
IPR	Institute of Primate Research
i.v	Intravenous injection
kD	Kilo Daltons
KEMRI	Kenya Medical Research Institute
Kg	Kilogrammes
K_2HPO_4	Di potassium hydrogen phosphate
KLM	Killed *Leishmania major*
K/NK	Killer/Natural Killer cells
LCL	Localized Cutaneous Leishmaniasis
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MAF	Macrophage Activation Factor
mg	Milligrams

MHC	Major Histocompatibility Complex
min(s)	Minutes
ml(s)	Millilitres
mm	Millimetres
mRNA	Messenger ribonucleic acid
NaCL	Sodium chloride
NaH_2PO_4	Sodium di hydrogen phosphate
NaOH	Sodium hydroxide
nm	Nanometres
NNN	Novy Neal McNeal
P	Probability
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PHA	Phytohaemaglutinin
PKADL	Post Kala Azar Dermal Leishmaniasis
r	Radius
rpm	Revolutions per minute
SAS	Statistical Analysis Software
s.c	Sub cutaneous injection
SIV	Simian Immunodeficiency Virus
SLA	Soluble *Leishmania* Antigen
TCR	T Cell Receptor
TDR	Tropical Diseases Research
TH^1	T Helper 1
TH^2	T helper 2
TMA	T Macrophage Activation
TNF	Tumour Necrosis Factor
USSR	Union of Soviet Socialist Republics

WHO	World Health Organization
µg	Microgrammes
µl	Microlitres
µm	Micrometres
#	Number
™	Trade Mark
1N	1 molar
3bi	Inactivated complement factor 3b

ABSTRACT

Studies were conducted to evaluate immune associated cellular phenotypes in the lesion areas of vervet monkeys (*Cercopithecus aethiops*) experimentally infected with *Leishmania major*. Different immune associated cell types recruited into the lesion sites as a result of primary, secondary infections were characterized using established cross reactive antihuman monoclonal antibodies in combination with the immunoperoxidase methods.

Nine adult vervet monkeys were used in this study. The primary infection group consisted of 4 adult naive animals of either sex. The secondary infection group also consisted of 4 adult animals of either sex which had self cured from experimental *L. major* infection. One extra naive control animal was also included. Animals in both groups were inoculated with 5×10^7 stationary phase promastigotes of *L. major* and observed after every 2 weeks for lesion development. Samples from the skin and draining lymph node were obtained at six weeks post infection and used for staining.

Studies from non infected areas taken as control skin sections in all the animals revealed the presence of resident CD8 positive T cells both in the epidermis and dermal layers of the skin. These (CD8 positive T cells) were smooth in morphology with majority being in the dermal layer.

B cells, CD16 and CD4 positive cells were virtually absent in the normal skin while there was a negligible population of macrophages. These cells, including the keratinocytes only expressed MHC class 1 antigens.

A heavy cellular infiltration consisting primarily of mononuclear cells was observed at the lesion sites in both primary and secondary infection groups. Majority of these cells expressed both MHC class 1 and 2 and the $CD8^+$ phenotypes. The main cell type in the infiltration was CD8 positive T cell. CD16 positive cells were also recruited into the lesions and these had a

tendency of clustering around the hair follicles. Macrophages recruited into the lesions were not heavily laden with parasites. Some B cells were stained in the infiltration but these were fewer than T cells.

Lymph nodes draining the control skin sites recorded a lower population of cells than those draining lesions. Majority of these cells were both MHC class 1 and 2 positive. There continued to be a higher population of CD8 positive T cells than both B cells and CD4 positive T cells. The difference in CD16 positive cells between nodes draining control sites and lesions was not clear. In general, cellular changes occurring in the skin were reflected in the draining lymph nodes in both infection groups. Nodes draining lesion sites were enlarged, a feature not observed in those draining control sites.

There were positive delayed type hypersensitivity (DTH) reactions in animals with active infection and those that had self cured. This indicated that the experimental animals developed cell mediated immunity.

The results show that the $CD8^+$ T cell phenotype is the main cell type restricting parasites to the lesion site in both primary and secondary *L. major* infection in vervet monkeys. The results also show that cellular phenotypes expressing CD3, CD8, CD16 and MHC classes 1 and 2 antigens restricted parasites to the lesion site in both primary and secondary *L. major* infections in vervet monkeys.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Leishmaniasis is a disease complex caused by species of an intracellular protozoan parasite belonging to the genus *Leishmania*. Leishmaniasis is endemic in the tropical regions of America, Africa the Indian sub continent and in the subtropics of South West Asia and the Mediterranean. There are over 20 known species of *Leishmania* of which over a dozen are associated with various forms of leishmaniasis (WHO/TDR, 1993). Based on clinical patterns of disease caused by different species, the disease is grouped in three main categories namely: cutaneous, mucocutaneous and visceral leishmaniasis (Liew, 1989).

1.1.1. Taxonomy and life cycle of *Leishmania*.

The systematic position of the genus *Leishmania* as revised by Levin *et al.*, (1980) (reviewed in Lainson and Shaw, 1987); and Lainson and Shaw, (1987), is given below.

Kingdom	Protista (Haeckel, 1866)
Sub Kingdom	Protozoa (Goldfuss, 1817)
Phylum	Sarcomastigophora (Honiberg and Balamuth, 1963)
Sup phylum	Mastigophora (Deising, 1866)
Class	Zoomastigophora (Calkins, 1909)
Order	Kinetoplastida (Honigberg, 1963; Vickerman, 1976)
Sub order	Trypanosomatina (Kent, 1880)
Family	Trypanosomatidae (Doflein, 1901, Grobben, 1905)
Genus	*Leishmania* (Ross, 1903)
Sub genus	*Leishmania* for example *L.(l). donovani*
	Viania for example *L.(v). braziliensis*

Leishmania parasites are digenetic. That is they use two types of hosts (vertebrate and invertebrate) to complete their life cycle. They are also pleomorphic, existing as amastigotes in the macrophages of vertebrate hosts (Liew, 1989), and promastigotes in the vector female sandflies and in culture (Minter, 1987).

Female sandflies of the genera *Phlebotomus* in the old world and *Lutzomyia* in the new world are the vectors of *Leishmania* parasites. When the appropriate vector sandfly feeds on a host infected with *Leishmania* parasites, macrophages infected with amastigotes are taken in. These amastigotes are liberated and then enclosed within the peritrophic membrane where they undergo at least one cycle of multiplication before transforming to promastigotes (Minter,

1987). When an infected sandfly bites a susceptible host, the promastigotes are released into the host in which they are phagocytosed by macrophages within hours of entry. Within the susceptible macrophage, promastigotes transform into amastigotes completing the cycle (Molyneux and Killick-Kendrick, 1987).

1.1.2. Cutaneous leishmaniasis.

This infection is restricted to the skin. It consists of a lesion that develops at the site of the sandflies bite. This later heals leaving an ugly scar. Healing takes from 6 months to several years (Mauel and Behin, 1981). It is commonly referred to as Oriental sore in the old world, (Peters and Gilles, 1991). Cutaneous leishmaniasis is a spectral disease (Bryceson, 1969) which, by comparison with leprosy, is presumed to reflect the immune response of the individual host. Oriental sore is caused by *L. major* and *L. tropica* complex.

In the south America *L. mexicana* causes simple cutaneous leishmaniasis. The vectors the in the old world are sandflies belonging the genus *Phletobotomous* while those in South America belong to the genus *Lutzomyia* (Peters and Gilles, 1991).

Leishmaniasis recidivans: this is also referred to as lupoid, tuberculoid or allergic form. It is an aberrant form of cutaneous leishmaniasis. The condition has an overlapping distribution with the anthroponotic cutaneous leishmaniasis caused by *L. t. tropica* and is believed to be a chronic phase of the same infection. This produces lupoid or tuberculoid eruption of the skin adjacent to or in the vicinity of an apparently spontaneously healed old leishmanial lesion (Mauel and Behin, 1981).

Diffuse cutaneous leishmaniasis (DCL): this is also an aberrant form of cutaneous leishmaniasis. The condition involves large areas of the skin, particularly the extremities and other exposed parts, on which numerous papular or nodular eruptions appear. Multiplicity of lesions results from metastasis of the parasite from one site to another through the lymphatic vessels or wandering macrophages (Mauel and Behin, 1981). *L. mexicana* complex and *L.t. aethiopica* are the commonest species causing this (Mauel and Behin, 1981). *L. aethiopica* causes single sore and DCL in Ethiopia and Kenya. *P. longipes* is the vector in Ethiopia while that in Kenya is *P. pedifer* (Peters and Gilles, 1991).

1.1.3. Mucocutaneous leishmaniasis.

This is commonly known as Espundia. *L. braziliensis* causes Espundia in Brazil, Ecuador, Peru, Bolivia, Venezuela, Paraguay and Colombia. The vectors in these areas are not known except for *Lu. wellcomei* in Brazil. The infection involves metastatic spread of infection that affect the nose, pharynx, mouth, larynx, face and limbs often eating the whole organ away. It begins with a simple cutaneous ulcer that heals spontaneously. After one year or more, a papule reappears and expands destroying the cartilagenous and overlying soft tissues of the nasopharyngeal cavity (Mauel and Behin, 1981).

1.1.4. Visceral leishmaniasis.

This is universally known as Kala-azar. This form of leishmaniasis is caused by *L. donovani*, *L. infantum*, *L. archibaldi* and *L. chagasi*. As the name suggests, it involves the invasion of visceral organs of the vertebrate host by *Leishmania*. Nearly all the internal organs can be infected but the commonest are liver, spleen, bone marrow and lymph nodes (Mauel and Behin, 1981). It is fatal if untreated (Mauel and Behin, 1987).

L. donovani infection is distributed over India, China and Kenya. The vectors in these areas belong to the genus *Phlebotomous*.

Post kala azar dermal leishmaniasis (PKADL): is an aberrant form of visceral leishmaniasis that apparently follows an incompletely treated case of visceral leishmaniasis after a lapse of time varying from several months to many years. There are numerous maculopapular or nodular eruptions on various parts of the skin, particularly on the face, neck, lips or trunk. The dermal lesions may be depigmented or erythrematous macules or papules, usually non-ulcerative (Mauel and Behin, 1981). PKDL is prevalent in India and Kenya and *L. donovani* is the causative agent (Peters and Gilles, 1991).

1.1.5. Public health impact.

Leishmaniasis is a health problem in all continents of the world except Australia. The estimated number of people at risk with leishmanial infections is 350 million while there are 12 million cases, and about 80 countries reported to have the disease. Three million people suffer from various forms of leishmaniasis and the number of cases of disease each year are in the order of 1.5 million of which 500,000 are of the visceral type (WHO/TDR, 1993).

The impact of cutaneous leishmaniasis is less dramatic in mortality but it causes severe suffering in endemic areas because of the social and psychological trauma associated with disfigurement. The importance attached to this by people in endemic areas is reflected in the old practice of leishmanization where the risk of an ulcer and lesion on less exposed body parts is secondary to life long immunity (WHO/TDR, 1993).

In Kenya both cutaneous and visceral leishmaniasis do exist (Chulay *et al.*, 1985; Githure *et al* 1986b). Although the exact figures of those infected are not known, the disease is of national

importance because of its high morbidity and mortality rates and subsequent low productivity in the affected areas.

1.2. LITERATURE REVIEW

1.2.1. Parasite entry into macrophages.

Leishmania parasites parasitize the macrophages of the host. The stage of the parasite in the macrophage is called amastigote. The parasite stage in the sandfly vector and in culture that is ineffective to the host macrophages is the metacyclic promastigote. *Leishmania* promastigotes develop from a non ineffective to ineffective stage in the sandfly vector. Infectivity of *Leishmania* promastigotes from stationary phase cultures greatly exceeds that of promastigotes from log phase (Sacks *et al.*, 1984) hence the use of stationary phase promastigotes in inoculation experiments. Identical developmental changes occur during growth of promastigotes in the fly. Entry of *Leishmania* parasites into host macrophages is achieved by phagocytosis. This process involves interaction between complement factor 3 (C3) and a surface glycoprotein of the parasite (gp 63), which in turn interacts with complement factor 3b inactivated (C3bi) and mannose fucose receptors on the macrophage surface. Gp 63 is rich in mannose, has protease activity and has been named parasite surface protease. C3 is thought to facilitate close contact between the promastigote and the macrophage. Phagocytosed *Leishmania* fuse with the lysosome to form the phagolysosome (Blackwell *et al.*, 1986).

1.2.2. Survival of *Leishmania* in macrophages and other cell types.

Leishmania live in the phagolysosome of the host macrophages.
The parasite avoids destruction by the digestive enzymes of the lysosomes partly through: 1) possession of a proton pump that lowers the pH of the lysosomal vacuole leading to sub-optimal functioning of the lysosomal enzymes; 2) an unusual organelle, the megasome in some species which may alter the lysosomal environment in favour of the parasites; 3) production

and secretion of excreted factors which includes an acid phosphatase. These may change the pH or inactivate the lysosomal enzymes; 4) structural resemblances on parasite surface to lysosomal membrane proteins (Clark and Howell, 1990).

The current understanding of the immune aspects of the host/parasite relationship in leishmanial infections suggests two stages during the control of the infection: 1)an early stage in which components of the innate defence system eliminate or control parasite replication; 2) a mechanism operating late during infection in which acquired immunity regulates parasite survival in the macrophages through T-cell dependent mechanisms (Grencies, 1990).

Complement has been implicated in *Leishmania* killing in macrophages. Complement activation results in the deposition of C3 on the surface of *Leishmania* parasites. This leads to the formation of the membrane attack complex (MAC). The amastigotes of *L. major* may be killed even when an incomplete MAC is formed (Hoover *et al.*, 1985). The tendency of certain leishmanial species to disseminate may then be related to their capacity to resist complement mediated lysis (Mauel and Behin, 1987).

The inability of the macrophage to destroy *Leishmania* parasites accounts for the hold these parasites have over their hosts and contributes to the chronicity and poor prognosis of some forms of the disease (Mclaren, 1990). The transformation of promastigotes to amastigotes is thought to be an adaptive process that counters the harsh environment within the phagolysosome (Lewis and Peters, 1977). Other work has indicated that the quantity of lysosomal enzymes released into the phagosome might be important, since if these are increased through the exposure of the cells to agents, such as chloroquine, that stimulate lysosomal fusion with the vacuole, the killing potential of the cell increases (Alexander, 1981). Intracellular survival thus depends on a delicate balance between the potency of macrophage

killing mechanisms and the efficacy of the evasive strategies evolved by the parasite itself (Mauel, 1984).

Sher *et al.*, (1983), showed that parasites of healing strains of *Leishmania* were readily destroyed by lymphokine activated macrophages *in vitro*, whereas parasites of non healing strains that produced chronic infestations in mice were resistant to the same cells *in vitro*. The evasive capacity of some parasite strains may therefore account for their ability to induce a chronic disease state. *Leishmania* species causing cutaneous disease may survive the leishmanicidal activities of macrophages because of the impaired activities of these cells at temperatures below 37°C (Scott, 1985).

In studies by Fruth *et al.*, (1993), bone marrow derived macrophages from BALB/c mice, after infection with *L. major* were shown to have a greatly reduced capacity to present *L. major* derived antigen to specific T cell hybrids derived from mice immunised with those antigens. These macrophages regained normal antigen presenting capacity after pulsing with relevant peptides. This inhibition of native antigen presentation did not appear to result from decreased endocytosis or catabolism. In as much as the inhibition of presentation was not due to impaired antigen processing or unavailability of MHC class II molecules on the surface of infected cells, these results indicated that the presence of *L. major* could have interfered with intracellular loading of MHC class II molecules with antigenic peptides (Fruth *et al.*, 1993).

Leishmania amastigotes do not trigger a respiratory burst in the macrophages when phagocytosis is initiated. It has been suggested that this may be due to the effect of the parasite's surface acid phosphatase/lipase activity on the macrophage membrane or through a more generalised interference with macrophage physiology (Blackwell *et al.*, 1986). The parasites also contain superoxide dismutase and trypanothion which protects against oxidant damage.

It has also been shown that Langerhans cells can phagocytose *Leishmania* parasites and present them to immunocompetent cells (Moll, 1993). Langerhans cells do express C3bi which opsonizes *L. major* parasites for macrophages. They have been shown to have the ability to phagocytose *Leishmania* both *in vitro* and *in vivo* but this rate has been shown to be low *in vitro* compared to macrophages, hence phagocytosis is done with the purpose of presenting the parasites to T cells. They are greater T cell proliferation and lymphokine production stimulators than macrophages. They migrate from the epidermis to the site infected with *L. major*. The parasitized ones have the ability to migrate from skin to the draining lymph nodes (Moll, 1993) thereby acting in the spread of the disease.

1.2.3. Immune response to *Leishmania.*

Valuable information on the immune response to leishmanial infections has been generated mainly by using the murine model. Quite abit has also emerged from human studies and non human primate studies.

1.2.3.1. Antibody responses.

Anti-leishmanial antibodies were shown *in vitro* to lyse promastigotes in the presence of complement and to enhance phagocytosis (Pearson *et al.*, 1980). Careful quantitative studies by Poulter (1980), suggested that antibodies to *L. enriettii* in the guinea pig could have some protective role. On the other hand antibodies were shown not to be important in protection in the mouse cutaneous leishmaniasis and neither were the levels of antibodies in both resistant and susceptible strains of mice markedly different during the course of the disease, following infection with *L. tropica*. (Olobo *et al.*, 1980). Pearson *et al.*, (1983b) suggested that antibodies could play an important role in early defence or in the entry of the parasites into the

host cell. However antibodies to leishmanial parasites may be useful for diagnosis and epidemiological surveys of the disease.

Antibodies were also detected in vervet monkeys following challenge infection but had apparently no role in protection (Olobo *et al.*, 1992).

1.2.3.2. Cell mediated immune responses.

Protective immunity to leishmaniasis is mainly T cell mediated (Liew *et al.*, 1984; Gajewsky and Fitch, 1988; Heinzel *et al.*, 1989; Clark and Howell, 1990). Resistant CBA mice rendered relatively T-cell deficient by thymectomy followed by irradiation and reconstitution by syngeneic bone marrow cells were found to be less able to control *L. major* infection (Preston *et al.*, 1972). Athymic mutants of the highly resistant CBA and C57BL became totally unable to control *L. major* which progressed unabated and visceralized (Preston *et al.*, 1972).

Activated macrophages have been shown to be most important for the elimination of the parasites from the host (Pearson *et al.*, 1983a). Activation of murine macrophages *in vitro* enabled such cells to destroy intracellular *L. tropica* parasites. The methods used by several workers to achieve this included:

1) co-cultivating polyclonally activated T-lymphocytes with infected macrophages (Behin *et al.*, 1979); 2) co-cultivating *Leishmania* specific T-cells with infected macrophages (Louis *et al.*, 1982); 3) adding either supernatants of mitogen (concanavalin A) or antigen stimulated spleen cells (Titus *et al.*, 1984).

Studies on *L. major* in mice indicated that T-cells in infected mice could have resistance promoting or exacerbation effects (Mitchel and Handman 1985). The relative balance of each type of T cell was found to be determined by host genotype via major histocompatibility complex (MHC) and thus the pattern of disease in different strains of mice could be

remarkably different. In the susceptible mouse strain such as BALB/c the infection progresses from simple lesions to visceralization and death (Djoko tammou *et al.*, 1981) or remains localised and later resolved in resistant strains such as A/J mice (Zilton *et al.*, 1984).

Following self cure from cutaneous leishmaniasis, it was shown that IFN-γ and IL-2 were the important cytokines which mediated protection. Macrophages cultured with interleukin 2 (IL-2) and IFN-γ before exposure to *Leishmania* developed the ability to resist infection with *L. major*. The induction of this effector mechanism was maximal by the sixth to the eighth hour after lymphokine addition and was not dependent on the sequence of lymphokine addition (Belosevic *et al.*, 1990).

Protective immunity in cutaneous leishmaniasis is mediated by T cells that produce IFN-γ and macrophage activating factor (MAF). Macrophages become activated and intracellular parasites are killed. Parasite destruction in the recovering host is actually mediated by activated macrophages, and IFN-γ is the central cytokine mediating activation (Weiser *et al.*, 1987; Locksley and Scott, 1991). Assays performed *in vitro* have elucidated cell-mediated immune responses in cutaneous leishmaniasis. Other mechanisms such as antibodies, complement and phagocytosis by eosinophils may be secondary but the essential role of the macrophage is the most important (Clark and Howell, 1990). Although tumour necrosis factor (TNF) alone can not activate macrophages to kill *L. major*, the activity of this cytokine is markedly synergized by IFN-γ (Bogdan *et al.*,1990a). Peripheral blood lymphocytes from patients with active lesions and individuals recovered from previous infection, have been shown to undergo blast transformation in the presence of leishmanial antigens (Green *et al.*, 1983). Antigen cross-reactivity at the cellular level between *L. major* and *L. donovani* has been shown *in vitro* lymphoproliferative assay in man, and in a mouse model between *L. enriettii* and *L. major* (Mauel and Behin, 1987).

Acquired immunity against *L. major* as a result of recovery from infection or prophylactic immunisation can be transferred by T but not B-cells (Liew *et al.*, 1984). In resistant mice such as C57BL or C3H/HeN infected with *L. major*, control of infection is associated with expansion of IFN-γ and IL-2 producing T-cells in the draining lymph nodes and the appearance of delayed type hypersensitivity (DTH) to parasite antigens (Sadick, *et al.*, 1986). Progressive infection in susceptible strains such as the BALB/c strain is associated with the appearance of interleukin-4 (IL-4) and IL-5 producing T-cells (Heinzel *et al.*, 1989). The same protective immune response of C3H mice could be ablated by the administration of neutralising anti-IFN-γ monoclonal antibodies within the initial 10 days of the infection resulting in the dissemination of the cutaneous disease. Similarly, non protective response in BALB/c could be ablated by the administration of neutralising anti-IL-4 monoclonal antibody within the initial 7 days, with the establishment of the healer phenotype; and addition of anti-cytokine antibodies after 7 days was not effective (Sadick *et al.*, 1990). IL-3 has been shown to inhibit activation of infected macrophages by IFN-γ to kill amastigotes *in vitro*, particularly when combined with IL-4 (Feng *et al.*, 1988). Hence IL-3 and IL-4 serve to drive the immune system towards non protective responses. The production of granulocyte monocyte-colony stimulating factor (GM-CSF) has been associated with progressive infection (Greil *et al.*, 1988). Contributions of $CD8^+$ and γ^δ T-cells have also been shown in host response to *L. major*. Elimination of $CD8^+$ cells during *L. major* infection caused a slight delay in the clearance of the organisms in the mice (Titus *et al.*, 1987). This suggested that CD8+ could play a yet undetermined role in leishmaniasis.

Mouse T helper cells have been characterised by functional heterogeneity into TH^1 and TH^2 depending on the cytokine profile they secrete (Liew, 1989). Present evidence suggests that these different lymphokine profiles are the results of activation of either TH^1 cells that mediate healing or TH^2 cells that mediate progressive infection in mice infected with *L. major* (Bogdan

et al., 1990b; Heinzel *et al.*, 1989). The response of BALB/c mice to infection with *L. major* is not due to deletions in the T-cell repertoire, since a variety of immunomodulatory interventions, including prior sub-lethal irradiation and $CD4^+$ cell depletion, and therapy with anti IL-4 antibodies enable these mice to heal following *Leishmania* infection. Each of these procedures is associated with ablation of the predominant TH^2 cell responses that occurs in untreated mice. The ability to modulate such strikingly biased subset responses has created intense interest in this model of spectral infectious diseases (Locksley and Scott, 1991).

Within 72 hours of infection with *L. major*, the draining lymph node cells of BALB/c mice were shown to produce a biased TH^2 response characterised by the generation of IL-4 and IL-5 after antigenic stimulation *in vitro* (Scott unpublished observations). In contrast, cells from previously immunised BALB/c mice or resistant C3H generated IFN-γ.

Studies with protective and disease promoting T helper (TH) cell lines showed that they reacted to different pools of parasite antigens. A protective TH^1 clone at one time proliferated in response to a low molecular weight antigen that did not stimulate TH^2 line (Scott *et al.*, 1990).

Once induced, subsequent expansion of the TH subsets is facilitated and regulated by subset specific cytokines: IFN-γ by inhibiting the proliferation of TH^2 cells (Gajewsky and Fitch , 1988); IL-4 by inhibiting IL-2 receptor expression and IFN-γ production (Martinez *et al.*, 1990) and IL-10 by inhibiting cytokine release from TH^1 cells (Moore *et al.*, 1990). Recent evidence from studies using clones suggests that TH^1 and TH^2 subsets use different signalling mechanisms after the engagement of the T-cell receptor (Gajewsky *et al.*, 1990).

Interleukin-12, a cytokine which stimulates NK cells and T cells to produce IFN-γ appears to be critical in TH^1 differentiation and immunity to *Leishmania*. Spleen cells from *L. major*

infected C3H/HeN resistant mice made more IL-12 than cells from BALB/c susceptible mice. Further, the levels of IL-12 correlated directly with those of IFN-γ in the cultures, and anti-IL-12 monoclonal antibodies decreased the levels of IFN-γ. BALB/c mice were vaccinated with SLA (Soluble Leishmania Antigen) and IL-12 after realising that injecting IL-12 together with SLA blocked IL-4 production and increased IFN-γ in BALB/c mice. This showed that following challenge, BALB/c mice were protected, similar to C3H/HeN mice at 8 weeks (Scott, 1993, reviewed in David and Harn, 1993). Heinzel, (1993), reviewed in David and Harn, (1993), reported that BALB/c mice injected i.p with IL-12 for the first 7 days of infection showed 80% protection. This protection was partially blocked by anti-IFN-γ. Infected macrophages showed no mRNA for IL-12, but did so after stimulation with LPS (David and Harn, 1993).

Some of the immune mechanisms mediating secondary challenge with *L. major* in vervet monkeys have been studied (Olobo *et al.*, 1992), using peripheral blood lymphocytes. The studies showed that IFN-γ and DTH but not IL-4 were correlated with disease resolution (Olobo *et al.*, 1992). This information is crucial for vaccine development for leishmaniasis and extents the findings in mice to vervet monkeys.

1.2.3.3. Histopathology of *Leishmania* infections.

A number of reports have been made on the histopathology of leishmanial infections in different hosts. In guinea pigs infected with *L. enriettii*, the histology of primary lesions revealed an early infiltration of the dermis by macrophages, with a progressive shift to parasite-laden multinucleated giant cells (Bryceson *et al.*, 1970); lymphocytes and plasma cells were shown to be present throughout the evolution of the lesion with plasma cells predominating at later stages. Few or no parasites could be found extracellularly after 4 weeks of infection. Recovery was accompanied by degeneration of parasites within phagocytes and

parasite degeneration from the lesion site was complete in 12 to 16 weeks (Mauel and Behin, 1987).

Mauel and Behin, (1982a), summarised the histopathological features of cutaneous leishmaniasis in humans as described below. The disease consisted of a histiocytoma that developed at the site of the sand fly bite. In its early phase, the lesion exhibited hypertrophy of stratum corneum and of the papilla with the accumulation of mononuclear phagocytes in which the parasites multiplied. At times, free amastigotes were seen presumably liberated from ruptured macrophages. Gradually a cellular infiltrate consisting mainly of small and large lymphocytes among which many plasma cells could be observed surrounding the lesion. Ulceration followed necrosis which resulted in disintegration of the epidermis and the basement membrane. Diffuse lymphocytic infiltration with increasing numbers of plasma cells and giant cells, and numerous anastomosing capillaries marked a shift in favour of the host leading to spontaneous recovery.

Ridley and Ridley, (1983), identified 3 basic mechanisms of parasite elimination from histological analyses of human skin biopsies: 1) elimination of parasites within intact macrophages that later evolved as epithelioid cells, 2) elimination of parasites as a result of macrophage lysis and 3) necrosis at the centre of a focal mass of macrophages. They concluded that such responses depended on factors such as parasite load and the geographical origin of the leishmanial isolates but also suggested that the three responses might have been the outcome of a common immunological mechanism.

Zilton *et al.*, (1984), showed that in BALB/c mice experimentally infected with cutaneous leishmaniasis, there was an acute reaction, an infiltration of polymorphonuclear neutrophils, congestion and oedema in the first four days. Macrophages laden with parasites increased by the fourth day. By the seventh day, there was an increase in the size of the lesion with many

parasitized macrophages accumulated giving the appearance of fatty tissue. Necrotic areas free of cells began to appear but soon this was followed by a dense collection of polymorphonuclear neutrophils. Around the 14th day, there was a gross increase in the lesion size with many parasitized macrophages infiltrating the striated muscle bands and even into the dense connective tissues of the tendon sheath. By the 30th day these cells infiltrated the sebaceous glands as well.

Grimaldi *et al.*, (1984), examined acute and chronic phases of *L. mexicana mexicana* infections in Swiss webster mice. In addition to macrophages, they recorded granulocytes particularly eosinophils accumulating in the lesion during the acute phase of the disease and apparently destroying many parasites. The chronic phase was also characterised by an infiltration of granulocytes that paralleled parasite multiplication, although both eosinophils and neutrophils exhibited low level of phagocytosis of *Leishmania* throughout the infection. Parasitized macrophages were also prominent in chronic lesions.

Barral *et al.*, (1987), found T-lymphocytes interspersed amongst macrophages and comprising 48% of the cells that infiltrated lesions of cutaneous and mucocutaneous leishmaniasis patients using immunoperoxidase techniques.

Leu 3a +3b (helper phenotype) were more frequent than Leu 2a cells (suppressor/cytotoxic phenotype) in both groups. The helper/suppressor ratio was identical in both cutaneous and mucocutaneous leishmaniasis lesions. They found fewer cells expressing HLA-DR antigens in the lesions of cutaneous leishmaniasis groups while it was expressed in virtually all lymphocytes and macrophages in mucocutaneous leishmaniasis inflammatory infiltrates. On individual basis, they found the helper/suppressor ratio for cutaneous leishmaniasis patients lower in the lesion than in blood.

According to Mcelrath *et al.*, (1987), palpable skin lesions were detectable in both C57BL/6 and BALB/c mice after two weeks of infection. Central ulceration occurred in most of the experimental animals by four weeks of infection. Lesions progressively enlarged over 12 weeks; after which no increase was noted in C57BL/6 mice. It slowly healed in the next 8 to 10 weeks. BALB/c failed to heal by 25 weeks, they reached over 2 cm with non ulcerating dissemination over the face, trunk and limbs. Both BALB/c and C57BL/6 mice had their lesions showing a paucity of small round lymphoid cells on haematoxylin and eosin sections by week 8. T-lymphocyte migration was apparent in C57BL/6 by 12 to 15 weeks. BALB/c failed to show this, and only scanty number of T-lymphocytes could be demonstrated with L_3T_4, Lyt-1 or Lyt-2 subset markers by immunoperoxidase staining up to week 23 of infection. The influx of T-lymphocytes after 12 weeks in C56BL/6 was associated with a reduction in lesion size and healing of the cutaneous ulceration.

Ridley, (1987), on the other hand always found a mononuclear cell response that was always associated with some lymphocyte and plasma cell infiltration which was sometimes heavy. Using these cells he classified the spectrum for cutaneous leishmaniasis into five groups. Ulceration occurred mainly in lesions with a moderate parasite load, and parasite laden macrophages were usually found in the sub-epidermal zone. They probably precipitated ulceration and after destruction of the epidermis, they were ejected from the lesion (Ridley, 1987). The release of enzymes from ruptured macrophages, formation of immune complexes *in situ* or activation of the complement were among factors that could explain the necrotic processes in affected tissues (Mauel and Behin, 1987). The tissue damage seen in cutaneous leishmaniasis in humans may result from the multiplication of parasites and destruction of surrounding cells, though it may also proceed in the total absence of the parasites (Clark and Howell, 1990).

Ridel *et al.*, (1988), detected a high proportion of cells bearing the K/NK phenotypes (Natural Killer cells) using immunoperoxidase techniques in skin biopsies from patients infected with *L. braziliensis guyanensis*. They suggested that these cells could play a crucial role in the local control of parasite dissemination.

In both susceptible BALB/c and resistant CBA/J mice an increase in γ^{δ} T cells was observed after s.c. infection. In chronically infected BALB/c mice, these cells could represent up to 35% of the $CD3^{+}$ cells in the spleen. In CBA/J mice, the percentage of γ^{δ} T cells returned to lower level after resolution of lesions. Further experiments confirmed that correlation existed between multiplication of the parasites and expansion of γ^{δ} T cells in the spleen. In BALB/c mice infected for 4 months, these cells represented 80% of the blast population and a large proportion of them expressed the α-chain of the CD8 molecule. In both BALB/c and CBA/J mice, blocking of γ^{δ} TCR resulted in the development of larger lesions that contained increased numbers of parasites. This treatment significantly delayed the healing of cutaneous lesions in the otherwise resistant CBA/J mice. Taken together these results indicated that γ^{δ} T cells were expanded during experimental infection in mice with *L. major* and could be involved in host defence against this parasite (Rosat *et al.*, 1993)

Rhesus monkeys resisted re-infection with *L. mexicana* while lesions were still developing. These animals produced lesions that were similar to those in man. They were found to be readily infected with *L. mexicana* and *L. braziliensis* (Lainson and Bray, 1966).

Owl monkeys developed primary and satellite cutaneous lesions that persisted up to 52 weeks post infection with *L. b. panamensis*. Some of them demonstrated acquired resistance when challenged i.d. The immunological responses of these animals to this infection were similar to those of humans with localised cutaneous leishmaniasis in many respects (Lujan *et al.*, 1986a).

Inoculation of vervet monkeys with 1×10^7 promastigotes in 100µl of saline resulted in nodule formation at 49 days post infection and began resolving by 84 days post infection. The lesions in re-challenge animals became positive for parasites by the 14th day post infection, the lesions were smaller in size and did not ulcerate. In the primary infection, the lesions disappeared after 16 weeks but in secondary infection the lesions resolved within 6 to 8 weeks (Githure *et al.*, 1987).

In cutaneous and diffuse cutaneous leishmaniasis, Nilsen and Mshana (1987), found higher numbers of Leu 2^+ and Leu 3^+ cells in localised cutaneous leishmaniasis (LCL) than in diffuse cutaneous leishmaniasis (DCL) in human patients in Ethiopia. Leu 3a+b/Leu 2a ratios were the same. No differences were found in the numbers of HLA-DR and DP expressing cells in the granulomas. Lower numbers of IL-2 receptor expressing cells were found in DCL than LCL lesions suggesting interference in the activation of the T cells. IL-2 containing cells were absent in DCL but present in LCL lesions. Keratinocytes above the LCL but not DCL expressed HLA-DR but not DQ suggesting a lower IFN-γ production in the DCL granulomas. The number of Langerhans cells (Leu 6^+) was higher in the epidermis of DCL than LCL while a lower number was seen in the dermis. In general they found that LCL skin biopsies showed massive infiltration of both the reticular and papillary dermis. The infiltrates consisted of granulomas composed of macrophages and lymphocytes. Plasma cells were often seen as dense clusters at the periphery of the granulomas. *Leishmania* amastigotes were evident in all of the sections but in small numbers. HLA-DQ positive dendritic epidermal cells were however much fewer than the Leu 6^+ ones. There were fewer parasites in LCL lesions than DCL indicating a more effective immune response. Further evidence of the effectiveness of this type of response was shown in the lesion localisation and self healing nature of the disease, and positive skin test reaction to *Leishmania* antigen and *in vitro* lymphocyte responses to similar antigens.

An effective immune response in leishmaniasis in terms of localising the lesion and parasite clearance as was seen in LCL lesions took place despite the apparent equal numbers of both suppressor/cytotoxic and helper/inducer T cells. The monoclonal antibody Leu 2a was not able to distinguish between suppressor and cytotoxic T cells and hence it was possible that the cells detected in LCL were predominantly T cytotoxic and those in DCL suppressor T cells (Nilsen and Mshana 1987).

Nilsen and Mshana (1987) also found in their studies that HLA-DR and DQ expression in the dermis was similar in both LCL and DCL. Due to the fact that IL-2 receptors appear at a slightly later stage of T cell activation, the reduced number of cells that expressed this antigen in DCL lesions suggested that there was an interference in the process of activation of T cells in these patients. Activated T cells release mediators such as IFN-γ which are known to be able to induce keratinocytes to express HLA-DR antigen on their surfaces. These cells did not express HLA-DQ on their surfaces.

1.2.3.4. Lymph node involvement in leishmaniasis

The removal of popliteal lymph node prior to foot pad infection with *L. tropica*, caused a considerable exacerbation of the disease in BALB/c and B6D2 mice. Increased severity was associated with an initial inhibition of induction of DTH and a delay in the emergence of acquired resistance. Lymph node removal did not however compromise the effector arm of the acquired immune response, nor prevent the eventual suppression of DTH that has been shown to occur in the BALB/c during leishmanial infection (Poulter and Pandolph, 1982).

It has been clearly demonstrated that interruption of the lymphatic drainage of the injection site predisposed the host to develop DCL. It was felt therefore that variations in clinical disease resulting from different routes of infection could be a reflection of the variations in the lymphatic drainage from these sites Kadivar and Soulsby, (1979), (reviewed in Poulter and Pandolph, 1982).

Poulter and Pandolph, (1982), demonstrated that removal of popliteal lymph node caused a more rapidly developing and severe *L. tropica* infection in both the susceptible BALB/c and resistant B6D2 mice. By week 8 the majority of the BALB/c without the draining lymph node developed metastatic lesions while infection in the intact mice remained localised. Persistent lesions also developed in B6D2 mice without the draining lymph node. More rapid parasite multiplication at the lesion site was observed in animals without the draining lymph node but by week 8 the number equalled that in intact animals.

In the absence of the draining nodes, DTH was present in the BALB/c during infection with *L. tropica*. Higher levels of DTH developed in foot pad infections but this reactivity declined after 4 weeks of infection. In B6D2 mice without draining nodes, mice infected with *L. tropica* in the foot pad had retarded DTH but reached equivalent of intact animals by week 8. These mice also failed to show resistance to challenge by week 3 compared to intact animals. From these experiments it was shown that levels of DTH expressed were not affected by the absence of the draining node. It was also found out from this work that the loss of draining node failed to influence the subsequent course of infection. Hence it was concluded that after 2 weeks of infection, the acquired response had been induced and the course of *L. tropica* infection predetermined. The results clearly demonstrated that the interruption of lymphatic drainage considerably delayed induction of DTH and resistance to infection with *L. tropica*, while removal of the draining node prior to infection also blocked initial induction of DTH. These results further implied that while inhibiting the induction of DTH to *L. tropica*, removal of

draining node did not inhibit the emergence of the suppressor mechanisms responsible for the eventual loss of cell mediated immunity to *L. tropica* infection (Poulter and Pandolph, 1982).

Highly resistant C57BL/10 and intermediately resistant DBA/2 mice infected sub-cutaneously with *L. amazonensis* in the hind foot pad subsequent to the removal of the draining popliteal lymph node, developed greatly exacerbated infections compared to the sham operated controls or to mice infected in the contralateral foot pad. The majority of the mice in which the draining nodes were removed prior to infection developed metastases, lost their DTH and some died as compared to control groups (Reed *et al.*, 1985). Two thirds of consecutively untreated cutaneous leishmaniasis patients early in their disease had enlarged lymph nodes. Patients with enlarged lymph nodes had higher anti-leishmanial immune responses than those without such involvement. They also had higher IgG levels and stronger DTH. Sixty-two percent of lymph node cultures were positive for *Leishmania*. This work stressed the importance of *L. braziliensis* as an agent involved in the early invasion of the lymphatic system (Barral *et al.*, 1992).

Lymph node involvement during cutaneous leishmaniasis was reported more than 90 years ago (Splendor, 1912, reviewed in Barral *et al.*, 1992). Some lymph node enlargement was observed in some patients with leishmaniasis in Saudi Arabia. A similar high frequency of lymphadenopathy associated with leishmaniasis was reported in American cutaneous leishmaniasis with 30% of the isolates from the lymph nodes becoming positive for *Leishmania* (Pessoa and Barreto 1948, reviewed in Barral *et al.*, 1992).

1.2.3.5. Delayed type hypersensitivity (DTH)

T cell mediated macrophage activation is exemplified by a characteristic skin test reaction and is termed DTH. This is a state of reactivity characterised by inflammatory reactions starting

macroscopically 6-12 hours after antigen administration in a previously sensitised individual, reaching their peak after 24-72 hours. This is characterised histologically by lymphocytic and mononuclear cell infiltration (De weck, 1992).

DTH response to leishmanial antigen is a widely applicable indicator of previous exposure to either leishmanial infection or antigens in the mouse model and human hosts (Mauel and Behin, 1982b). DTH responses have been assessed in squirrel monkeys (*Saimiri sciureus*) challenged with *L. donovani* (Dennis *et al.*, 1986). These were negative throughout primary infection but developed strong DTH 7 weeks after challenge infection. Subsequent challenge with *L. braziliensis panamensis* revealed strong DTH in all animals in association with cutaneous lesion resolution.

The leishmanin skin test is similar to mantoux test. The reaction is positive in late oriental sore, in late East African and Mediterranean kala azar and in South American leishmaniasis but negative or weakly positive in Indian kala azar. The DTH becomes positive early in infection with *L. tropica* and *L. mexicana*, but this does not denote immunity to re-infection unless the leishmanial lesion has completely healed. In *L. braziliensis* the test becomes positive during the active phase of infection. In *L. donovani* however, it may not become positive until 6 to 8 weeks following recovery from the disease (Manson Bahr, 1963, reviewed in Ali and Ashford, 1993a). PKDL cases give weak positive or negative reactions suggesting that the immune mechanisms is incomplete. The test is negative in active untreated cases of DCL but may become positive after extended courses of treatment with pentavalent antimony compounds (Cahill, 1965, reviewed in Ali and Ashford, 1993a). A positive DTH is expected to indicate cryptic (active but asymptomatic) infection with leishmaniasis. The leishmanin test has been used to quantify transmission of leishmaniasis (Leeuwenberg *et al.*, 1983, reviewed in Ali and Ashford, 1993a).

Positive and strong DTH responses were elicited in vervet monkeys following secondary infection with *L. major*. These animals had previously been vaccinated with leishmanial antigen exposed to primary infection and allowed to self cure. The DTH responses were measured following intradermal injection of 5×10^7 fixed promastigotes per animal before re-challenge, during active infection and following self cure. The responses were highest during the active infection and lowest after self cure (Olobo and Reid, 1993).

Positive but weak DTH to *L.b. panamensis* were detected at 8 weeks post infection in owl monkeys. These were stronger at weeks 20 and 27. The readings were found to be maximal between 48 and 72 hours. After challenge, the tests remained positive when tested at weeks 25 and 37, (Lujan *et al.*, 1986).

Animals previously experimentally infected with the Kenyan or Iranian strain of *L. major* and had self cured 1 to 48 months prior to DTH test gave positive DTH response. The response was identical between the Kenyan and the Iranian strain. It was apparent from the studies that DTH response to *L. major* antigen could persist for at least 48 months after self cure from the cutaneous disease and these responses did not wane over this period (Olobo and Reid, 1993).

Although DTH test fails to distinguish between cutaneous from visceral leishmaniasis, it is a good tool for epidemiological surveys of the infection. A positive leishmanin reaction is believed to remain so for almost the rest of life, probably indicating an immune state which prevents further development of disease (Moddaber, 1989; Ali and Ashford, 1993b).

1.2.4. Reservoir hosts and animal models.

L. major a causative agent of human cutaneous leishmaniasis is primarily a parasite of rodents. Different species of rodents serve as reservoir hosts in different parts of the world:

Rhombomys opimus in southern Russia and Iran, *Psamomys obesus* and *Meriones crassus* in Israel, *Meriones hurrianae* in India, *Mastomys erythroleucus* and *Tatera gambiana* in Senegal, *Tatera spp. Mastomys natalensis*, *Taterilus emini* and *Aethomys kaiseri*, *Xerusrutilus sp.* in Kenya, *Arvicanthus sp.* in Senegal and Ethiopia and dogs in Egypt and Saudi Arabia (Githure *et al.*, 1986b).

Pathogenesis of leishmaniasis is determined by two sets of factors (Mauel and Behin, 1987); those related to the parasite itself and those related to the host. Parasite related factors include the species and the sub species involved, its antigenicity and virulence, the initial number inoculated into the host and perhaps its association with a particular species of vector. Host factors involve the ethnic and genetic background immune status, nutritional status etc.

Mice infected with *Leishmania* parasites have been used as models for the study of the human disease caused by these parasites (Zilton *et al.*, 1984; Howard *et al.*, 1980; Olobo *et al.*,1980). Depending upon the parasite species and the mouse strain chosen, a considerable spectrum of disease pattern can be produced. It has been shown that inbred mouse strains of non permissive phenotypes repeatedly display relative resistance to both visceral and cutaneous leishmaniasis (Zilton *et al.*, 1984).

Resistance in mice is displayed as either resolution of infection within several weeks after intradermal injection of promastigotes (and solid resistance to re-infection) or apparent host non permissiveness (Mitchel *et al.*, 1980). BALB/c mice on one end are highly susceptible to infection with *L. major* the causative agent of cutaneous leishmaniasis. The disease progresses from simple cutaneous lesion to visceralization and ultimately becomes fatal in this strain. The infection in these animals produces lymphadenopathy, splenomegaly, anaemia and hyperglobulinaemia (Djoko-Tammou *et al.*, 1981), similar to the human visceral disease (kala-azar) caused by *L. donovani*.

The susceptibility of BALB/c mice to *L. major* is essentially dose independent being demonstrated with as few as 20 parasites. Following infection, lesions extent progressively but more slowly in DBA/1 and DBA/2 mice. Strains A, C57BL/6 and CBA are relatively resistant to as much as 2×10^7 promastigotes leading to the arrest of lesion growth within three weeks and subsequent gradual healing (Howard *et al.*, 1980). The C3H/He and CBA/H strains of mice also exhibit resistance (Olobo *et al.*, 1980). Lesions metastasize and tend to visceralize in BALB/c infected with cutaneous leishmaniasis but remain localised in the resistant A/J strain. The relatively susceptible BALB/c mice develop a nodular foam-cell type of lesion and progressive depression of delayed type hypersensitivity response to leishmanial antigens while the A/J mouse strain develop mixed fibrosing and encapsulating reaction and develop and maintain positive delayed type hypersensitivity responses to leishmanial antigens (Zilton *et al.*, 1984).

Inoculation of *L. enriettii* into bare tracts of skin (ear, nose feet, shaven areas) of the guinea pig produces a cutaneous ulcer similar to the human oriental sore. This has led to the use of the model to some extent for immunologically related studies (Bryceson *et al.*, 1970).

1.2.4.1. Primates as models of leishmaniasis.

Phylogenetic closeness of non human primates to man make them more attractive models for studying leishmaniasis. Some non human primates including rhesus (*Macaca mulata*) (Lainson and Bray, 1966), Cebus (*Cebus apella apella*) and squirrel monkeys (*Saimiri sciureus*) (Lainson and Shaw, 1977) have varying degrees of susceptibility to infections with *L.*

braziliensis or *L. mexicana*. Cebus monkeys experimentally infected with *L. braziliensis* were protected when challenged with *L.m. amazonensis*, but reversing the order of inoculation did not protect them (Lainson and Shaw, 1977). The Owl monkey (*Aotus trivigatus*) was shown to develop single cutaneous lesions after inoculation on the nose with promastigotes of *L.b. panamensis* and *L.m. panamensis* (Christensen and De Vasquez, 1981). Wolf, 1976also found immunity to *L. tropica* in *Macaca mulata* to be different from that in humans.

Squirrel monkeys experimentally infected with either *L.b. braziliensis* or *L.b. panamensis* developed ulcerated lesions that persisted. PBMC numbers increased following infection. Cultured PBMC from infected animals proliferated in response to parasite antigens. Responses of PBMC to mitogens were not suppressed. Elevated levels of *Leishmania* specific IgM and IgG were obtained (Pung and Kuhn, 1987), following the infection.

Owl monkeys were found to be susceptible hosts for *L.b. panamensis* whereby the dorsal base of the tail was described as a useful site for the induction of a cutaneous lesion. The lesions developed were similar to those of localised human cutaneous leishmaniasis except for common occurrences of satellite lesions in the monkey. This may therefore be a satisfactory model for the study of the pathogenesis and immunological responses to cutaneous leishmaniasis (Lujan *et al.*, 1986b). Christensen and Vasquez, (1981), established that Owl monkeys were susceptible to both *L. braziliensis* and *L. mexicana*. These animals became DTH positive during the infection.

The vervet monkey (*C. aethiops*) has been found to be naturally infected with *L. major* (Binhazim *et al.*, 1987), follow up experimental studies showed this species as a suitable model for human cutaneous leishmaniasis (Githure *et al.*, 1987). The disease course in these animals has been shown to be similar to that in humans (Githure *et al.*, 1987). Following re-

challenge with an appropriate dose of homologous parasites, the animals just as in humans were immune to re-infection (Anjili *et al.*, in press).

Limited studies have been carried out on a variety of non human primates to determine their susceptibility to visceral leishmaniasis. Monkeys were used as models for visceral leishmaniasis (Nicolle and Manccanx, 1910, reviewed in Lainson and Bray, 1966). These workers showed that *L. infantum* infection in monkeys conferred immunity to *L. tropica* and vice versa. Other workers successfully infected a monkey recovered from *L. tropica* with *L. donovani* (Perrot *et al.*, 1927), (reviewed in Lainson and Bray, 1966). Monkeys reported to be susceptible and to develop fulminating visceral leishmaniasis include *Macaca mullata*, (Shortt, 1923); *Saimiri sciureus*, (Chapman and Hanson, 1981b); *Macaca fascicularis*, (Meleney, 1925); *Aotus trivigatus*, (Chapman *et al.*, 1983); *Cercopithecus aethiops*, (Kirk, 1945); and *Galago senegalensis senegalensis*, (Sati, 1963). Several new world primate species such as the Owl monkey (*Aotus trivigatus*) (Chapman *et al.*, (1981a), and marmoset (*Callithrix jacchus jacchus*) (Marsden *et al.*, 1981), have been found to be highly susceptible to experimental *L. donovani* infections. Fulminating visceral leishmaniasis developed after intravenous and intraperitoneal inoculation. All the three species have since been used in anti leishmanial chemotherapy studies (Madindou, *et al.*, 1985; Chapman *et al.*, 1983).

Vervet monkeys (*Cercopithecus aethiops*), Sykes monkeys, (*C. immitis*) and baboons (*Papio cynocephalus*) were found to support low grade experimental *L. donovani* infections for periods ranging between 4 and 8 months. They subsequently appeared to self cure. Hepatic histiocytic nodules similar to those observed in asymptomatic human visceral leishmaniasis were observed (Githure *et al.*, 1986b). Recent studies have shown that following intradermal inoculation of *L. donovani* promastigotes to vervet monkeys, some animals self cured while others developed the disease and died (Gicheru et al., in press).

Studies of Dennis *et al.*, (1985), identified the squirrel monkey as a moderately susceptible host for *L. donovani*. This study also identified it as having a sustained course of visceral leishmaniasis and usually recovered from the disease. Visceral leishmaniasis in squirrel monkeys was found to have certain clinical haematologic and pathologic characteristics similar to those seen in visceral leishmaniasis in man. In the following year, the same group found out that majority of squirrel monkeys (*Saimiri sciureus*) intravenously inoculated with 5×10^7 amastigotes of *L. donovani* per body weight recovered from the infection. The recovered animals demonstrated acquired resistance when challenged with an intravenous inoculation of 1.0×10^8 amastigotes per kg body weight (Dennis *et al.*, 1985). When challenged intradermally with 2.2×10^7 promastigotes of *L. braziliensis panamensis*, all the animals developed cutaneous lesions (Dennis *et al.*, 1985). Reactivity of peripheral blood leukocytes from infected squirrel monkeys to PHA was depressed 2 to 10 weeks after infection while the reactivity to Con A was unaffected. Squirrel monkeys subsequently challenged with *L. b. panamensis* developed persistent cutaneous lesions indicating a lack of resistance to the heterologous challenge (Dennis *et al.*, 1985). Madindou *et al.*, (1985), did studies on the chemotherapy of visceral leishmaniasis in squirrel monkeys and concluded that the squirrel monkey is susceptible to *L. donovani*. They then suggested that it could be a useful model for certain types of chemotherapeutic and immunological studies. In addition, this model could be used to compare virulence of different lines or isolates of the same or different species of *Leishmania*.

Owl monkeys were shown to develop fulminating visceral leishmaniasis following intravenous injection of 3.22×10^7 *L. donovani* promastigotes (Chapman *et al.*, 1981a).

Squirrel monkeys that had recovered from experimental *L. donovani* infection developed primary and satellite lesions after challenge infection with *L.v. panamensis*. This indicated that squirrel monkeys recovered from *L. donovani* were still susceptible to *L.v. panamensis* and

hence there was no cross immunity between the two parasites in these animals (Lujan *et al.*, 1990).

1.2.5. Relationship between immune systems of non human primates and man.

Due to their phylogenetic relationship to man, non human primates have provided useful models for the examination of immunological mechanisms involved in infectious disease, autoimmunity and transplantation (Mills, 1992). There is a considerable homology in MHC systems and other immune systems of man and non human primates. Many of the reagents such as mouse monoclonal antibodies and recombinant interleukins specifically developed for the studies of the human immune system, show variable cross reactivity with non human primates (Mills, 1992). Furthermore, immunological techniques for the assessment of humoral and cell mediated immune responses in man can be used with little modification for similar studies in non human primates (Mills, 1992). Cross reactivity studies with lymphocytes from non human primates have shown that certain B and T cell determinants are highly conserved in evolution whereas others are confined to a few primate species. In general the number of epitopes shared with humans correlates with the evolutionary distance of the non human primate species from man (Mills, 1992). A murine monoclonal antibody (FN18) raised against rhesus macaque T cells, believed to be specific for the equivalent of the human CD3, reacted with the T cells form the Cercopithecidae but not with lymphocytes from man, apes or Ceboidea (Mills, 1992). The CD4 and CD8 determinants, associated with MHC class 1 and 2 restricted T cell recognition of antigen, are highly polymorphic in non human primates. Nine epitopes have been detected on the human CD4 and the majority are present on the apes and old world monkeys except in the Cynomolgus macaque where only 30 to 40% are shared with man (Mills, 1992). Although the CD8 is very well conserved in apes and certain old world monkeys, the epitope expressed in the new world monkeys in particular is highly polymorphic, with most antibodies displaying a different phylogenetic pattern (Mills, 1992).

Non human primates have considerable variation in the CD4:CD8 ratio where it is often less than 1:1 whereas in normal humans it is usually 2:1. Mouse antihuman B cell markers display phylogenetic patterns in non human primates with some conserved on majority of them (Mills, 1992). Monoclonal antibodies against human HLA antigens can be used to detect MHC antigens on the cells of many non human primate species. The anti HLA A-B-C monoclonal antibody W6/32 reacts with class 1 antigens of apes, and old world monkeys. The association of the class 1 molecules with β_2-microglobulin is conserved in higher primates (Mills, 1992). In comparison with the mouse, the genomic organisation of MHC of man and non human primates is very similar. Serological analysis of rhesus macaque lymphocytes revealed no evidence for the third locus analogous to HLA-C. The MHC class 2 loci which are very difficult to analyse with serological typing reagents have not been so well investigated in non human primates (Mills, 1992). Two dimensional gel electrophoresis and restriction length polymorphism analysis have suggested the existence of DR, DQ and DP like loci in chimpanzees and rhesus macaque and DR and DQ loci in cynomolgus macaque, (Mills, 1992).

1.2.6. The Skin as an immunological organ.

The skin is the largest human body organ. It functions as a general defence system. Various cell types are responsible for the immune reactivity of the skin. The cells are involved in the homing of immune cells (endothelial cells, keratinocytes), either in natural immunity (macrophages, NK cells, neutrophils) or in acquired immunity (antigen presenting cells, T cells, mast cells). The complexity of the cells in the skin and their interactions during host defence is commonly referred to as Skin immune system (Kapsenberg and Bos, 1992).

Components of humoral immunity are related to keratinocytes, tissue macrophages, monocytes, granulocytes, mast cells, fibrinolysins, anti-microbial peptides, complement

peptides, eicosanoids and cytokines. While those related to acquired immunity include Langerhans cells, secretory immunoglobulins, interleukins, interferons, colony stimulating factors and other cytokines like TNF. Keratinocytes secrete several cytokines non specifically upon non-specific stimulation, while the dermal perivascular unit is a major site of inflammatory and immunological reactivity (Bos and Kapsenberg, 1993).

Normal human epidermis long dismissed as merely a protective covering for the body is now recognised as a complex immunological unit. Langerhans cells have been demonstrated as potent antigen presenting cells for a variety of T cell responses while keratinocytes upon stimulation can secrete several cytokines (Stingl *et al.*, 1989).

Dendritic skin cells include melanocytes, merkel cells, tissue macrophages, dermal dentrocytes, indeterminate cells and Langerhans cells. Immune response dendritic cells are in groups of lymphoid, tissue and epithelium. The lymphoid group includes cells in the lymphoid environment like interdigitating (reticulum) cells of lymph nodes and veiled cells in lymph vessels. The tissue group comprises cells of the connective tissue, indeterminate cells and dermal dentrocytes. Epithelium dendritic cells are found in the epidermis of the skin and include Langerhans cells as the major representative (Kapsenberg and Bos 1992).

Langerhans cells trap antigen in the epidermis and carry it to the draining lymph nodes where they present the peptide fragments as lymphoid dendritic cells to T cells (Bos and Kapsenberg, 1993).

It is possible that keratinocytes are involved in many different inflammatory and immunological skin diseases as non specific activators, responding to a wide variety of injurious events. As shown by Baker *et al.*, (1991), (reviewed in Bos and Kapsenberg, 1993), keratinocytes act as proinflammatory signal transducers responding to non specific external

stimuli with the production of inflammatory cytokines, adhesion molecules and chemotactic factors. Histologically the perivascular area of post capillary venules of papillary dermis, deep dermis and skin appendages contain the highest concentration of immune response related cells of the integument. In this area, mast cells, monocytes, macrophages and T cells are frequently present and tissue dendritic cells may be observed (Bos and Kapsenberg, 1993).

Human epidermis also contains occasional T cells mainly $CD8^+$. In mice, the majority of the epidermal T cells are $CD3^+4^-8^-$ with γ^δ TCR. γ^δ positive T cells are between 5 and 10% in humans which is the same range in peripheral blood (Kapsenberg and Bos, 1992).

The papillary dermis, close to the epidermis, contains a capillary network and harbours various bone marrow derived cell types involved as effector cells in natural and acquired immune reactivity of skin. A recently recognised aspect of potentially reactive connective dermal tissue is the occurrence of perivascular located T cells. These T cells contact antigen presenting cells scattered around capillary vessels. A substantial population of these are $CD4^+$ of the memory phenotype with an αß T cell receptor (Kapsenberg and Bos 1992). The dominant epidermal T cell receptor in mouse is γ^δ and this population is referred to as the dendritic epidermal T cell (Stingl *et al.*, 1989).

There is as yet no direct evidence to consider the skin as an independently operating immune organ. The lack of ability to mount a separate local primary antigen specific T cell response, the virtual absence of B cells, B cell follicles and germinal centre formation clearly distinguish skin from connective tissues that contain well organised areas of lymphoid tissue. The skin provides an immunisation site giving rise to generalised T and B cell priming, as mounted in the skin draining lymphoid organs. The skin also participates in the efferent phases of both systemic and local immune reactivities, providing a reliable reflection of the sensitive status, evident from prick, patch or scratch tests. The presence of dermal perivascular $CD4^+$

lymphocytes, is an interesting aspect of normal human skin. They may be considered a normal feature of potentially reactive connective tissue. These T cells probably represent a pool of antigen specific memory T cells that can be activated by the local antigen presenting dendritic cells at a very early phase of a secondary DTH (Kapsenberg and Bos, 1992). T cells have been routinely detected in normal non lesional, clinically normal skin, where the epidermal T cells are $CD5^+$ and react to anti αß TCR antibodies. Occasionally a few of them react to γ^δ TCR antibodies, especially in follicular epithelium and prevalent in the dermis. Other cells are dendritic and occasionally stain with UCHL1, the anti-memory T cell antibody. In the peripheral blood, almost all T cells are $CD7^+$ while only 15 to 30% of the epidermal ones are. $CD8^+$ T cells are the predominant ones in the epidermis (ratio 3:2 with $CD4^+$) and this is pronounced in the sole. Staining with UCHL1 antibodies showed that resting epidermal T cells belong to the memory phenotype. The epidermal T cells could also have a predilection for the external root sheath of hair follicles and the acrosyringial epithelium of sweat ducts. The dermal T cells are often clustered under the basement membrane usually in concentric layers of 2 to 4 cell layers thick. These cells appear to be in close association with Langerhans cells. The majority of the epidermal T cells are basally located and express the memory phenotype and are in a resting state (are $HLA\text{-}DR^-$). More than 50% express the αß TCR while a small population are γ^δ positive (Foster *et al.*, 1990).

In the peripheral blood, there are twice as many helper/inducer as cytotoxic/suppressor T cells and only 56 and 40% respectively are $CD45RO^+$ (Ashmore *et al.*, 1989, reviewed in Foster *et al.*, 1990). A subpopulation of T cells previously sensitised to antigen in the cutaneous area specifically "home" to the skin and remain there in a resting state in readiness, until coming into contact with the appropriate antigen (Foster *et al.*, 1990).

Cutaneous population of lymphocytes express a 200 kD cell surface glycoprotein recognised by monoclonal antibody HECA-452 present on about 16% of peripheral blood lymphocytes

including CD4 and $CD8^+$ lymphocytes (17 and 11% respectively). This was confirmed not to be a conventional activation antigen (Picker *et al.*, 1990).

1.3. Justification

Research into human infections with leishmaniasis has always been done in other animals (models) because of the difficulty of experimenting with humans due to ethical reasons. Studies in immunology and chemotherapy of cutaneous leishmaniasis have been done extensively in the mouse model (Howard *et al.*, 1980; Sadick *et al.*, 1986, 1990; Heinzel *et al.*,1989). However, the phylogenetic closeness of non human primates to man make them more attractive models for the study of human cutaneous leishmaniasis. Studies in the old world cutaneous leishmaniasis have shown that vervet monkeys (*C. aethiops*) can naturally habour cutaneous leishmaniasis caused by *L. major* in Kenya (Binhazim *et al.*, 1987). Subsequent studies confirmed that the disease could be transmitted experimentally to the monkeys through needle inoculation (Githure *et al.*, 1987), and infected sandfly bite (Lawyer *et al.*, 1990).

It has been recently shown that a number of antihuman monoclonal antibodies do cross react with the vervet monkey system (Olobo, 1992). These monoclonal antibodies in combination with immunoperoxidase techniques has greatly simplified enumeration of lymphocyte subsets (Olobo, 1992). The availability of these markers made this study possible.

Before developing the vervet money as a model for vaccine development studies against cutaneous leishmaniasis, it is important to understand the basic immune response provoked by the infection in the vervet monkeys especially at the lesion site infected with the parasites. This

is important for effective design of vaccine studies, and the proposal was designed to address this problem.

1.3.1. Aim and objectives.

The aim of the study was to characterise the local cellular response induced by the parasite at the lesion sites after primary and secondary infections with *L. major* parasites in vervet monkeys. This would enable us clarify the mechanisms that naturally limit or encourage the infection.

The objectives of the study were:

1. Determine the cellular phenotypes induced at the lesion sites following primary and secondary infection with *L. major* in vervet monkeys.
2. Determine the cellular phenotype induced in lymph nodes draining lesion sites following primary and secondary infection with *L. major* in vervet monkeys.
3. Compare the cellular phenotype changes at the lesion sites and draining lymph nodes between primary and secondary infections with *L. major* in vervet monkeys.

CHAPTER 2

MATERIALS AND METHODS.

2.1. Acquisition, care and maintenance of experimental animals.

Adult vervet monkeys (*Cercopithecus aethiops*) of either sex were used in this study. These were classed as adults for they had three molar teeth, well developed breasts in case of females and colour of scrotum in case of males (navy blue scrota). These animals were trapped from *Leishmania* non endemic areas in Siaya. They were then quarantined at the Institute of Primate Research for three months during which they were examined and treated for any infections such as haemoprotozoan and gastrointestinal parasites and also tested for SIV. Only normal healthy animals were used. Each monkey was housed in an individual squeeze-back cage,
0.6×0.6×0.68 metres high (Plate 1). They were maintained on a balanced commercial non human primate diet (Unga, Nairobi). This was supplemented with fruits, vegetables and additional ascorbic acid. Water and mineral salts were provided *ad libitum* (Olobo and Reid, 1990). The animals were examined by the Institute's veterinary staff on a daily basis for any signs of illness.

A total of nine adult animals of either sex were used. There were four animals in each study group. There were two study groups namely primary and secondary infection groups. One extra naive animal was included as control. The primary infection group animals were naive before they were in experiments. The secondary infection group consisted of animals that had self cured from primary *L. major* infection. The period between self cure and secondary infection was 24 months.

Plate 1. Vervet monkey with cutaneous leishmaniasis in a squeeze back cage.

2.2. Preparation of complete Schneider's Drosophila medium.

Three types of growth media were used for *Leishmania* promastigote culture. These were Schneider's Drosophila (Sigma, UK), M199 (Sigma, UK) and NNN. Schneider's Drosophila medium was prepared as described below. 500 ml of incomplete medium (Sigma, UK) was obtained. This was supplemented with 20% heat inactivated FBS batch 0890134 (BDSL, UK). This had been thawed at 37°C and decomplemented at 56°C for one hour in a water bath. Other supplements included 100 µg/ml of gentamycin (BDSL, UK) as an antibiotic and 0.5µg/µl of 5-fluorocytosine (BDSL, UK) as an antifungal agent. The complete medium was sterilised using a 0.45µm and 0.2µm pore size flowpore™ (Flow labs, UK) filters. The sterile complete medium was filtered into a sterile glass bottle container which had earlier been autoclaved at 121°C for 15 minutes in a Bioclave™ (Germany). 70% ethanol (Kobian, Kenya) was used to wipe around the bottle top followed by wrapping in parafilm (ANC, USA). The medium was then labelled with the name, date of preparation and initials of the person who prepared it. This was stored at 4°C and used within 2 weeks.

2.3. Preparation of complete M199 medium.

The M199 medium powder (Sigma, UK) which had been stored at 4°C was dissolved according to the manufacturer's directions. 90% of the final required volume of water at 15-20°C was measured. While gently stirring, the powder was added without heating the water, until dissolved. The original package was rinsed with a small known volume of water to remove all traces of powder and added to the rest of solution. To the media, 2.0 grammes of sodium bicarbonate (cell culture tested) (Sigma, UK) was added for each litre of final volume of media being prepared, and stirred gently until dissolved. While continuing to stir, the pH of the media was adjusted to 0.1 - 0.3 pH units below 7.2 (the desired final pH of media) This

was done since the pH may rise during filtration. pH adjustments were done using 1N HCL or 1N NaOH as recommended. The remaining volume of water was added to make the final volume. Addition of supplements, sterilisation and storage was the same as for Schneider's Drosophila media.

2.4. NNN Medium

This medium was first prepared by Novy, McNeal and Nicolle in the early parts of this century. Several modifications have been made to the original one but the one in my use was a modification of the one prepared by Jadin and Wery, (1963), reviewed in Marin *et al.*, (1982).

The medium consisted of blood agar with liquid overlay. It had a solid phase of nutrient agar and defibrinated rabbit blood. To the sterile melted agar, the defibrinated blood was added and prior to solidification, the tube in which the blood and agar were poured was tilted in such a way as to allow the surface of the solid phase to be at an angle when the culture tube was kept upright. The tilted tubes were incubated for 24 to 48 hours to ensure that they were sterile prior to the addition of the liquid overlay which was complete Schneider's Drosophila medium.

2.5. Parasites and culture.

Leishmania major Kenyan strain NLB 144 (Githure *et al.*, 1987), obtained from the Nairobi *Leishmania* bank at KEMRI was used. This strain, whose complete isolation code is MHOM/KE/83/NLB-144B was originally made from the skin of a patient from Marigat in Baringo District of Kenya (Mebrahtu *et al.*, 1992).

Parasites for infecting experimental animals, preparing the material for DTH tests were cultured in either complete Schneider's Drosophila or M199 media.

Primary cultures were obtained from the lesion sites of previously infected vervet monkeys using a tuberculin syringe (Becton Dickinson, USA) fitted with 16mm long gauge 27 needle. Sterile PBS was injected into the edges of the lesion (the centre was avoided to reduce fungal and bacterial contamination), and drawn back into the needle. This was repeated three times and the final saline drawn into the syringe was dispensed into the primary NNN culture media. Before dispensing, the mouth of the culture tube was flamed, and so was the dispensing needle to control contamination. The culture was then incubated at 26°C in an incubator (Astro™, UK) and observed daily under an inverted microscope (Leitz, Germany) for parasite growth and any contamination. When the primary culture reached stationary phase (determined from growth curve), it was expanded into T25 cell culture flasks (Corning™, UK) to a maximum medium capacity of 10ml. Five ml of either complete Schneider's Drosophila or M199 medium was dispensed into it using a Sterilin™ sterile serological pipette (Flow labs, UK). 500µl of primary parasite culture was obtained under sterile conditions and dispensed into the T25 flask with medium using a Sterilin™ serological pipette. If the culture being expanded was not a primary one, 10^5 stationary phase promastigotes/ml were dispensed instead. The flask tops were then tightly replaced and wiped with 70% ethanol before placing into the incubator. The parasite culture flask was then labelled with the strain, the passage, number inoculated, date of culture inoculation and initials .

Promastigotes for growth curve determination were seeded at 10^5/ml, observed and counted in a haemocytometer (Weber scientific, UK) on a daily basis as described below.

More media was added to the cultures after every three to four days to obtain higher parasite harvests and sometimes bigger culture flasks (T50 or T75) were used. In all instances, promastigote cultures were made only up to the 10^{th} passage (Sadick *et al.*, 1984).

2.5.1. Parasite counting.

To obtain a standard growth curve, promastigotes were counted from the cultures on a daily basis under the haemocytometer. All the promastigotes from the four 16 grid areas were counted and their average determined. The parasites were counted in ×10, ×100 and occasionally ×1000 dilution's depending on the density. Before counting, the promastigotes were fixed in 10% formalin, to stop motility. 10µl aliquot of fixed parasites were dispensed under the haemocytometer coverslip and counted. The final average figure arrived at was multiplied by a constant of 10^4 (haemocytometer counter) the dilution factor and the volume in the culture flask to obtain the total number in culture.

2.5.2. Parasite freezing.

Extra cultures of promastigotes were grown for preservation for future use. These were obtained from the first few passages and logarithmic phase parasites. Preservation was done as a precautionary measure incase the growing cultures got contaminated or the animals with the infection lost through accidental deaths. This is normally experienced at times (Olobo, personal communication). It is also easier and faster to begin promastigote growth from a preserved culture than from infected animals.

Preservation was done by freezing in liquid nitrogen (East African Oxygen, Kenya) using the procedure standardised in our laboratory. The Promastigotes to be frozen were harvested as described elsewhere (section 2.7), washed in sterile PBS, counted and adjusted to densities between 10^{6-7}/ml. These promastigotes were made into a pellet and mixed with 1ml of freeze mix. The freeze mix was prepared by mixing 90% of sterile heat inactivated FBS with 10% DMSO (Fisher scientific, USA). Parasite/freeze mixture was kept in sterile 1.8 ml nunc™ vials (Nunc, Denmark), which had been chilled at -20°C. These were kept on ice for 5 minutes to lower their temperature gradually before transferring them to -70°C for an overnight stay and finally to liquid nitrogen the following day.

2.6. Determination of protein concentration.

This was done using a modified method of Lowry that gives linear photometric responses (Hatree, 1972). 1mg of BSA was dissolved in 1ml of distilled water to make the standard (1µg/µl). Dilution's of the standard were done to give a range of 10µg to 100µg of BSA/ml. A blank was set with sterile PBS only. The unknown protein of interest (sonicated whole stationary phase promastigotes) was taken as neat and thereafter diluted two fold starting at ½ up to 1/10 in sterile PBS. 0.9 ml of solution A (see appendix 1) was added to all the tubes including the standards, blank and promastigote lysate and incubated in a water bath at 50°C for 10 minutes. The tubes were removed and allowed to cool to room temperature. All the tubes were then treated with 0.1 ml of solution B (see appendix 1), mixed and allowed to stand at room temperature for at least 10 minutes. Three ml of solution C (see appendix 1) were rapidly forced into all the tubes and vortexed (Fisons, USA) to ensure mixing. The tubes were again incubated at 50°C in a water bath for 10 minutes and then cooled to room temperature. The samples were read in a Cecil 6000 series (CE 6600R™) (Cambridge, UK) photospectrometer at a wavelength of 720nm. The curves of the standards were obtained automatically from the spectrophotometer and optical densities of the samples read at the

same wavelength and recorded manually. The optical density of the unknown protein was used to find the corresponding mass by constructing a line from the optical density axis to meet the graph line and dropping it down to the mass of BSA axis. The other method used was to calculate the masses using the equation used by the spectrophotometer to draw the curve for the standards.

2.7. Parasite inoculation into experimental animals.

The 8 animals to be inoculated with parasites were individually caged. They were anaesthetised using 10mg of Ketamine™ (OBDI, INDIA) per Kg bodyweight. The right and left eye brow ridges were shaven using an Oster™ (Milwaukee, USA) shaver and disinfected with 70% ethanol. Transparent safety goggles were worn to protect the eyes against accidental parasite spill during inoculation. The right brow ridges were inoculated intradermally with 5×10^7 stationary phase promastigotes in 50µl of sterile PBS. The left side was inoculated with 50 µl of PBS alone, as control. Inoculation was done using a 1 ml tuberculin syringe fitted with 16mm long 27 gauge needle. The animals neither shared the syringes nor injection needles. Three control BALB/c mice were inoculated on the hind foot and observed as control for parasite virulence (Githure *et al.*, 1987).

Parasites for inoculation were prepared as described hereafter. Second passage late stationary phase promastigotes (determined by observing the cultures and the numbers from cultures used in plotting the growth curve) were harvested. Harvesting was done by centrifugation at 2500rpm in a Beckman TJ-6R™ centrifuge (Beckman, USA) and washed three times in sterile PBS. They were counted as described elsewhere and adjusted to 5×10^7 per 50µl of sterile PBS. Most of the promastigotes were in large clumps and also had reduced motility compared

to those of log phase. The counts from cultures for the growth curve were also used in determining cultures ready for harvesting.

2.8. Delayed type hypersensitivity (DTH) tests.

This was performed to determine the degree of cell mediated immunity in experimental animals. It was done using the method described by Olobo and Reid, (1993). Two groups of 4 monkeys each were used. One group consisted of the animals that were used in the primary infection group. The other group was made up of animals from the secondary infection group. The first group was tested during active infection as determined by ulcerated lesions and positive cultures obtained from them. The second group was tested 2 months after self cure as determined by 5 consecutive negative cultures obtained from the lesion sites 14 days apart.

The animals were each anaesthetised with 10mg of ketamine per Kg body weight. The right and left lateral thorax regions were shaven and disinfected with 70% ethanol. Each animal was intradermally inoculated with 5×10^7 formalin fixed promastigotes in 100µl of sterile PBS using 1ml tuberculin syringe fitted with a 16mm long 27 gauge needle (Olobo and Reid, 1993). Different syringes and needles were used for each animal. The left lateral thorax region was inoculated with 100µl of sterile PBS alone and processed as control for DTH.

Leishmanin antigen used for the tests was prepared as follows: late stationary phase promastigotes cultured in Schneider's Drosophila medium were harvested and counted as described elsewhere (section 2.7). After determining the number, they were killed in 1% formalin (BDH, UK) in PBS for 1 hour at 4°C. They were then washed twice in PBS and adjusted to 2×10^8 per ml of 1% formalin in PBS for storage at -70°C until the day of testing. On the experimental day, they were thawed at 4°C followed by washing three times in sterile

PBS. After the last wash, they were adjusted to 5×10^8 per ml in sterile PBS, from which 100µl was used to inoculate each animal.

The results were read after 48 and 72 hours by measuring the degree of skin induration. The induration was measured using a vernier calliper and also traced on lead acetate paper and the average diameter in mm determined. An induration diameter of more than 5 mm was regarded as positive (Olobo and Ried, 1993).

2.9. Lesion observation.

Lesion development in both primary and secondary infection groups were observed after every 2 weeks. Measurements on lesion sizes were taken on the major and minor diameters of the lesions with the aid of a calibrated vernier calliper. The lesions were also traced on lead acetate paper and the area determined using the formula $\pi r1r2$ (where r1 and r2 are major and minor radii of the lesion respectively) as described by Wilson *et al.*, (1979).

Information about the lesion development was scored on the attached sheet (see appendix 2). The state of the lesions were described according to the seven point classification of the IPR and KEMRI standardised procedure (see appendix 3). Control sites on vervet and BALB/c mice were also observed for any lesion development.

2.10. Surgery.

Skin biopsies were surgically removed from the sites of parasite inoculation and their corresponding control sites. One of the lymph nodes draining the site of surgery in the skin was also removed. This was done for both primary and secondary infection groups. Their corresponding control sites were also operated. This was done by trained veterinary pathologists at the Institute of Primate Research using standard surgical facilities.
The animals to be operated on, were anaesthetised as before on 10mg Ketamine. The sites to be operated were shaven and disinfected with Betadine™ (Basel, Switzerland).

For skin surgery, two parallel incisions of about 1 cm long were made at the required site using size 24 sterile surgical blade (Feather™, India). The distance between them was about ½ cm. The rectangular flap of skin biopsy was removed by blunt dissection using clamp forceps. Blades were changed at every operation site and other pieces of equipment disinfected thoroughly in 70% alcohol. The operated site was widened into an elliptical shape and sutured in a simple interrupted pattern using standard suture material (Vicryl™, USA) of size 2.0. The sutured site was sprayed with Terramycin™ to disinfect it. Suturing was done for each site before starting an operation on the other one. Sterile gloves and face masks were worn throughout the operations. The gloves were thoroughly disinfected in 70% ethanol after every operation to avoid contamination.

Lymph node biopsy was obtained as described hereafter. The site of the animal containing the lymph node of interest was shaven and disinfected as described for the skin biopsy. The node was traced with fingers and held in place. An incision was made into the skin covering the lymph node using size 24 surgical blade as above. The muscles underlying the incised skin and covering the lymph node of interest were also opened up. The blood and lymph vessels serving or draining the node were held using artery forceps and tied using suture material to stop any bleeding. The node of interest was then removed after which the inner muscles were

sutured first in a simple interrupted pattern followed by the incised skin. A small opening was left on one end to allow excess lymph to flow out during healing. The area was sprayed with Terramycin™ and left to heal.

2.11. Processing of biopsy samples

Biopsy samples were frozen within 5 minutes from the time of removal in OCT compound™ (Tissue tek II, USA) to ensure they remained fresh. Each biopsy sample was trimmed into two pieces and preserved in different nunc™ cryovials (Nunc, Denmark) to replace any that may be spoiled before or during sectioning.

After obtaining the biopsy from each animal, it was immediately trimmed using size 24 surgical blade into two smaller pieces. These were immediately transferred into cryotubes in which some OCT compound™ had been poured. These tubes had been previously labelled with the animal number, date of surgery, the type of organ and whether it was from a control or infected site, and finally initials. After dipping in OCT compound in the tubes, they were immediately transferred into a smaller beaker with iso-pentane (BDH, UK). The iso-pentane inside the beaker was kept in liquid nitrogen until crystals started forming before the biopsies in the cryotubes were immersed. Alternatively the cryotubes could be immersed directly into liquid nitrogen in absence of iso-pentane. After staying in iso-pentane for about 5 minutes, the biopsies were kept on dry ice and immediately transferred to -70°C until the day of sectioning. The biopsies were also sometimes kept in liquid nitrogen if -70°C facilities were lacking.

The lymph nodes were immediately dissected into two equal parts after removal, and placed into different cryovials, before being frozen. The freezing procedure was similar to that of skin biopsies.

2.12. Sectioning of biopsy samples.

One end frosted slides (Chance™, UK) to be used were cleaned in advance by making the first wash in a Five star™, detergent (Labchem, Kenya), followed by thorough rinsing with tap water. The slides were then dipped for one minute in 1N chromic acid (May & Baker, UK), to remove any greasy material on them. This was followed by thorough rinsing in running tap water, to remove the chromic acid, demineralized water and a final rinse in double distilled water. They were then dried using a hair drier. After the drying, the slides were dipped for one minute in gelatin-chromo-alum solution (see appendix 1), drained and left to dry overnight in a warm place not exceeding 40°C.

Sectioning was done using a frigocut 2800™ cryostat machine (Reijhert-jhung, Germany) which had been set at -20°C, (Rita *et al.*, 1987). The machine had previously been set to defrost automatically at midnight to avoid interruptions during sectioning.

On the day of sectioning, the required samples were retrieved from -70°C. These were thawed under running tap water, making sure that water did not get into the biopsies. Water trapped inside biopsies may form crystals during sectioning, which may interfere with the results. After thawing, some OCT compound was poured onto the metal tissue block holder inside the cryostat (for faster freezing). The biopsy was then embedded in the OCT compound on the block at a plane of choice. It was left to cool in the machine for at least one hour, to equilibrate with the temperature inside the machine. The cutting knife (Leitz, Germany) was set at an angle of 40°C as recommended by the manufactures; the thickness of the sections set at 7μm after which cutting proceeded. The sections were picked from the knife using the slides which had been at room temperature. Because of the low temperatures of the sections, they easily stuck onto the slides without folding. The slides with sections were left to warm at room temperature for at least 30 minutes, during which the slides were labelled with the date

of sectioning type and condition of the tissue and the animal number. A diamond pencil was used to mark around the sections on the slides. The sections were then dehydrated using acetone at 4°C for 5 seconds at room temperature, after which the slides were then wrapped individually in aluminium foil (Fay, UK), transferred into an air tight polythene bag and preserved at -70°C until needed. Some self indicating silica gel, 4-6 mesh (Griffin & George, England) was poured into the polythene bag containing the slides to avoid the slides sticking together which could result in the deterioration of quality during storage.

2.13. Isolation of peripheral blood lymphocytes.

The animal from which blood was to be obtained was anaesthetised with 10mg/Kg body weight of ketamine™, (OBDI, India). The inguinal region was then shaved and 5ml of blood obtained from the femoral vein using a 19 gauge needle. This was diluted in equal volume of Alsever's solution (see appendix 1). 5ml of diluted blood was overlaid on 3ml of Ficoll-paque ™ (Pharmacia, UK) and centrifuged at 2500rpm (Beckman™ TJ-6R) for 30 minutes at room temperature. The lymphocytes were harvested using a sterile pipette and washed 3 times in sterile PBS by centrifugation at 1500rpm (Beckman™ TJ-6R) for 10 minutes at 4°C. Purified washed lymphocytes, were counted within 3 minutes in a haemocytometer and adjusted to 5×10^5/ml. The trypan blue dye (Sigma, UK) exclusion method was used during counting to asses the viability. Only the live cells were counted. 200µl cell suspensions containing 1×10^5 cells were used to prepare cytospins. They were spun in a cytospin (Shandon, UK) at 600rpm for 5 minutes, the slides removed and dried 1½ feet away from a light house (75 watt bulb), (Philips, Kenya) for 1 hour. They were then wrapped in aluminium foil and placed into a polythene bag with self indicating silica gel, labelled and preserved at -70°C.

2.14. Monoclonal antibodies.

Well characterised antihuman monoclonal antibodies which cross react with vervet monkey system, available from commercial sources and gifts were used. Two anti rhesus monkey antibodies (FN18,GM12) were also included. The antibodies used recognise vervet monkey mononuclear cells of the following subpopulations: Pan T-cells, a suppressor/cytotoxic T cell subset, helper/inducer T cell subset, B cells, monocytes and MHC class 1 and 2 antigens, γ^{δ} TCR, Langerhans cells and cells bearing the β_2-microglobulin molecule (Olobo, 1992), (Table 1).

These antibodies were diluted and stored according to the directions of the manufactures. They were stored in working dilution's in aliquots of 25µl to avoid unnecessary freezing and thawing and loss of activity. Their sources and specificity's are listed below (Table 1).

Table 1

Monoclonal antibodies used in this study.

Antibody	Specificity	Source.
FN18	CD3	M. Jonker, TNO Primate Centre, The Netherlands.
UCHM1	CD14	P.C.L Beverly, ICRF, London, UK.
B9:12:1	MHC class 1	S. Carrel, LICR, Switzerland.
B7:12:2	MHC class 2	S. Carrel, LICR, Switzerland.
GM12	B cells	M. Jonker, TNO Primate Centre, The Netherlands.
UCHL1	CD45RO	P.C.L Beverly, ICRF, London, UK.
Leu 11b	CD16	Becton Dickinson, USA.
Leu 2a	CD8	Becton Dickinson, USA.
Leu 3a	CD4	Becton Dickinson, USA.
L243	MHC class 2	P.C.L Beverly, ICRF, London, UK.
W6/32	MHC class 1	P.C.L Beverly, ICRF, London, UK.
2ß2M	β_2 Microglobulin	S. Carrel, LICR, Switzerland.
UCHT2	CD5	P.C.L Beverly, ICRF, London, UK.
δTCS1	γ^{δ} T cell receptor	T cell sciences, USA.
OKT6-FITC	CD1	Amersham, UK.

Note that except for FN18 and GM12, all antibodies used were against the human system.

2.15. Immunoperoxidase staining.

The modified method of Modlin Li *et al.*, (1985), was used for cell staining. The slides were retrieved from -70°C and left at room temperature for at least 20 minutes before staining began. All the buffers and BSA used in staining were centrifuged in a suprafuge 22™ (Hereaus, Germany) at 16000rpm for 30 minutes to remove aggregates. For each staining, one slide in which the primary antibody had been omitted was included as a control for non specific staining. All incubations were done at room temperature. The biopsies were fixed in 100% chilled acetone (kept overnight at -20°C) for 10 minutes. This was followed by washing with agitation 3 times for 5 seconds each time in PBS with 0.015 Molar thiomersal. The slides were transferred to a jar containing H_2O_2-azide mixture (see appendix 1) and incubated for 10 minutes to block endogenous peroxidase in the cells (Chin Yang Li *et al.*, 1987). From this step and subsequent ones, all the incubations were done in a moist chamber to avoid drying. After incubation, the biopsies were washed 3 times in cold PBS (at 4°C) with thiomersal for 5 minutes each time. They were then incubated in 3% BSA for 15 minutes to block non specific binding sites. Excess BSA was removed by washing twice in cold PBS with thiomersal for 5 minutes each time. 25µl of the appropriately diluted primary antibody (Table 1) were applied onto each biopsy and incubated for 1 hour. The unbound antibody was flicked off and washed twice with PBS with thiomersal for 5 minutes each round. 25µl of the secondary antibody (biotin labelled goat anti mouse IgG, Sigma, UK) diluted 1:40 was applied onto each biopsy and incubated for 25 minutes. Excess unbound antibody was flicked off, followed by washing twice in PBS with thiomersal for 5 minutes each round. 25µl of Extravidin™ conjugated to HRP enzyme (Sigma, UK) diluted 1:100 was applied followed by incubation for 15 minutes. Excess unbound avidin was flicked off, followed by washing twice in the same buffer for 5 minutes each time. The slides were then stained with the chromogen substrate mixture 3-

amino-9-ethyl carbazole, (Sigma, UK); dissolved in dimethylformamide and mixed with 30% H_2O_2, (see appendix 1) for 5 minutes in the dark, to avoid destruction of reaction product (red in colour). Excess stain was washed off with acetate buffer (see appendix 1). The biopsies were counterstained with Mayer's haematoxylin (Sigma, UK) for 1 minute. Excess haematoxylin was washed off under running tap water for 10 minutes. The biopsies were mounted in glycerol NaCl mountant (see appendix 1), coverslips (Chance, UK) placed on and the edges sealed with nailvarnish (Luron, UK). The slides were allowed to dry at room temperature for 30 minutes before counting positive cells (refer to the section on positive cell counting).

Slides with peripheral blood lymphocytes were obtained from -70°C and equilibrated at room temperature for at least 20 minutes. The cells were fixed in chilled 100% acetone for 4 minutes. This was followed by washing the slides once in cold PBS, PBS at room temperature and at 37°C respectively for 5 minutes. The cells were then incubated in H_2O_2-azide mixture for 10 minutes. From here onwards all incubations were done in a moist chamber. Excess azide mixture was removed by washing twice in cold PBS at room temperature. Non specific binding of the primary antibody was blocked by incubating the cells for 15 minutes in 3% BSA in PBS with thiomersal. Excess BSA was washed off once in cold PBS and twice in PBS at room temperature for 5 minutes each round. 25µl of primary antibody were applied to the cells and incubated for 1 hour. Excess antibody was flicked off, followed by washing twice in cold PBS for 5 minutes each time. The secondary antibody (biotin labelled goat anti-mouse IgG) diluted 1:40 was applied to the cells and incubated for 25 minutes. Unbound secondary antibody was flicked off and washed using cold PBS once, followed by warm PBS once for 5 minutes each round. 25µl of Extravidin™ conjugated to HRP were applied to the cells and incubated for 15 minutes. Excess unbound avidin-HRP was flicked off followed by washing once in PBS at 37°C for 5 minutes each round. From here onwards the procedure went on as described for the biopsies.

2.16. Cell counting.

The videoplan™ (Kontron, Germany) image analysis system was used to scan through the slides to determine whether the staining was specific or not and to count the percentage of positive cells in the samples. This computer programme involved a video camera (Panasonic, UK) which transferred the image from the slide under the microscope into the computer. The image was then magnified and transmitted to the coloured computer monitor. It could magnify images by factors of up to 10^6. This rendered clustered cells wide apart for much easier and more accurate analysis.

Slides with fewer and non clustered cells were counted under a Leitz Autholux™ II teaching microscope (Leitz, Germany). A calibrated eyepiece with 100 divisions was used. Only the cells under the divisions were counted (Mcelrath *et al.*, 1982). A minimum of 200 cells counted was used to calculate the percentage of positive ones (Olobo, 1992). The positive cells had red surfaces showing the colour of the chromogen substrate reaction product. Negative cells only had the blue colour of the counterstain. The ×40 objective was the most preferred for counting cells from the skin biopsies as they were fewer, but that of ×100 for those from the lymph node.

Counting cells from the skin biopsies was started with the epidermis and finished with the hypodermis. Where more than two hundred cells were arrived at before the whole cross section had been counted, 400 or 600 cells were counted followed by averaging to get the number positive out of 200.

In the case of the lymph node, 200 cells were counted each from the cortex, deep cortex and medulla regions after which the average was determined. This was done to give a representation of the distribution of the positive cells in the node.

2.17. Photography.

Photographs were taken using a Leitz Autholux II™ camera system fitted onto the microscope. The ×40 and ×100 objectives were used to take pictures of the samples using Kodak gold II™ film (Kodak, Kenya) for colour prints of ASA 200 and 400. These were printed at the Kodak processing centre, Nairobi. External photographs were taken using a Nikon F301™ (Nikon, Japan) Camera.

2.18. Immunofluorescence staining.

Indirect immunofluorescence method (IFAT) was used to stain for CD1 positive cells in the skin and PBMC. The edges of the cells or tissues on the slides were dried using absorbent paper. The tissues were incubated in PBS containing 10% FBS for 30min in humid chamber to avoid drying. They were then washed three times in PBS + 2.5% FBS + 0.1% sodium azide (the indirect immunofluorescence buffer). 10µl of commercial OKT6-FITC (Orthodiagnostics, USA) conjugated antibody was applied per tissue section and incubated for 1 hour at 4°C in a humid chamber as live cells tend to pinocytose the surface antigen at room temperature. This was followed by washing three times as above. The slides were mounted in 10% glycerol/PBS. A coverslip was placed and the edges sealed with nailvarnish. They were viewed under a Leitz Dialux 22 EB™ fluorescent microscope (Leitz, Germany) which had been switched on 15 minutes earlier to allow the bulb to emit enough fluorescent light. Cells were viewed at ×500 magnification. The controls involved omitting the primary antibody step and staining on a slide without the tissue.

2.19.1. Data collection and presentation.

Data was collected on, the weight of the animals during the course of the experiments to determine if there were any changes attributed to the inoculated material. Lesion size measurements were also taken after every 2 weeks in the whole period of the experiments. The number of parasites in the cultures used in constructing the growth curve were counted daily for 2 weeks. The number of positive cells out of an average of 200 cells counted after immunoperoxidase staining, were also recorded for analysis. This also included the control slides for non-specific staining.

DTH measurements were also taken after 48 and 72 hours. Data on lesion sizes was presented as lesion areas, while that from DTH sites as the average diameter of the skin induration after 48 and 72 hours. Data from parasites in culture was presented as the number/ml of culture media with a growth curve illustrating this (Figure 1). Positive cells from slides were presented as the mean and percentage in 200 cells with figures on the percentages illustrating this (Figures 2-10).

2.19.2. Data analysis and graphics.

Data was analysed using 2 way ANOVA in the GLM procedure, Wilcoxson T test and Mann Whitney U test statistical techniques. Two way ANOVA was used to compare inter-animal and inter-antibody variation in the mean number of phenotypes stained in each experimental group. Wilcoxson T test was used to determine any significant difference in the number of positive phenotypes stained by the different antibodies between controls and their corresponding experimental sites. Mann Whitney U test was used to determine any significant difference in the % of cells stained between different sites.

Spearmans rank correlation test (Siegel, 1956), was used to determine any correlation in positive cell counts between controls and their corresponding experimental sites. This was done with an intention of determining if the results obtained at the control sites were influenced by the outcome at the experimental sites. Tables of Siegel, (1956) were used for all non parametric tests.

Several IBM compatible computer programmes were used in data analysis and graphics. Harvard graphics™ and Lotus freelance graphics™ programmes were used in the construction of figures. Word perfect™ release 5.1 and Microsoft word 6.0 programmes were used in word processing. SAS™ and Statgraphics™ release 2.01 programmes were used in data analysis. Macintosh compatible Statworks™ programme was also used to some extent in data analysis.

CHAPTER 3

RESULTS

3.1. Growth of *L. major* promastigotes.

L. major promastigotes were in early log phase by the third day, staying in log phase until the seventh day. The promastigotes were in early stationary phase from the seventh day, remaining in stationary phase until the ninth day when the numbers started to decline hence entering the decline phase (Figure 1). The highest number of parasites obtained was 10^8 per ml of culture medium, which occurred on the seventh day.

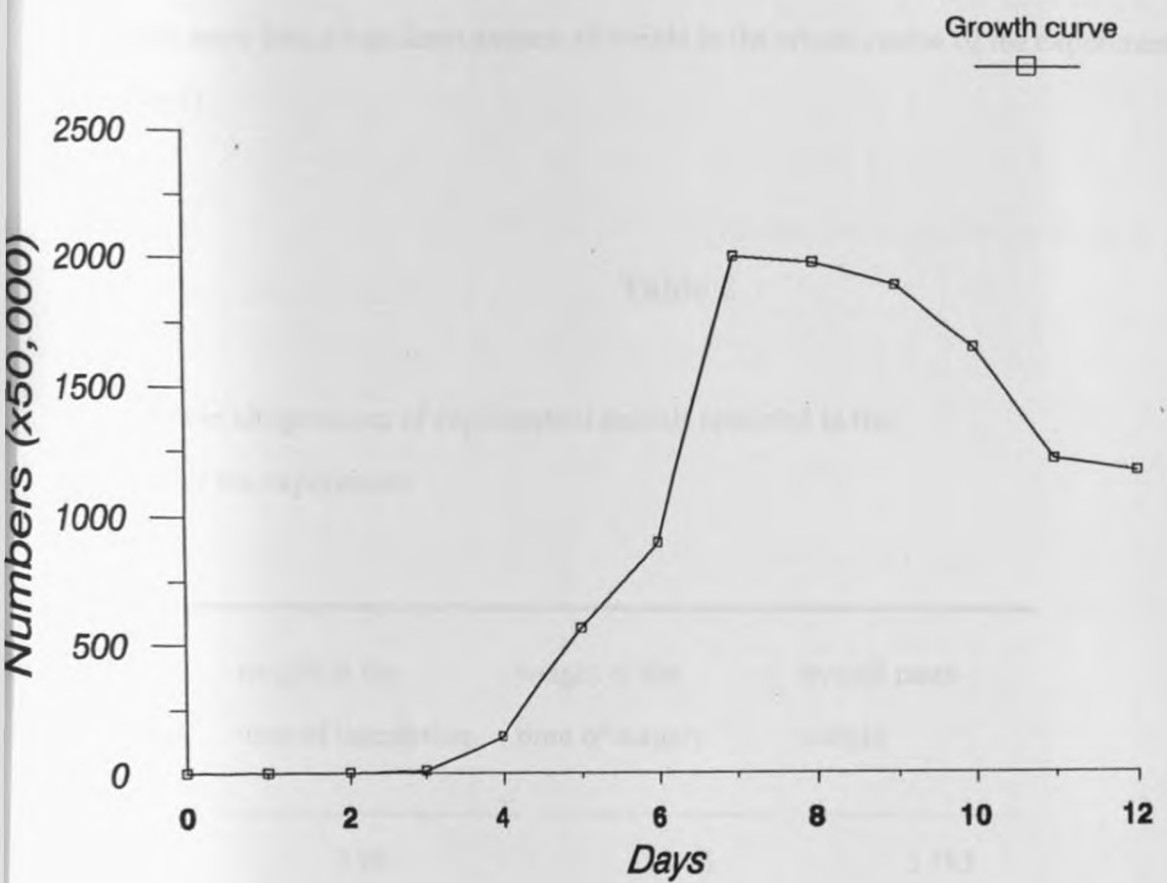


Figure 1. Growth curve for *L. major* in Schneider's Drosophila media.

3.2. Animal weight changes.

Animals were weighed before any work was done with them. This was with an intention of finding out the effect of the infection on the weights of the animals. All the experimental animals never lost a significant amount of weight in the whole course of the experiments (Table 2).

Table 2

Weights in kilogrammes of experimental animals recorded in the course of the experiments.

Animal number	weight at the time of inoculation	weight at the time of surgery	overall mean weight
365	3.00	3.05	3.183
1430	4.00	3.95	4.113
1484	3.00	3.00	3.178
1479	4.40	4.40	4.733
1547	2.00	2.35	2.200
1562	1.90	2.00	1.957
1566	2.30	2.80	2.579
1568	2.00	2.25	2.114

3.3. Delayed type hypersensitivity (DTH) response.

. All the animals used in these experiments were DTH positive after 48 and 72 hours (Table 3). A DTH value of 5 mm and above was considered positive (Olobo and Reid, 1993). Animals with active infection (determined through open ulcers and positive cultures) had DTH values significantly higher than those of the self cured group ($P<0.05$). DTH values from control sites inoculated with PBS alone were less than 5mm and significantly lower than those from experimental sites ($P<0.05$).

Table 3

DTH values of vervet monkeys with active infection and those self cured from experimental infection with *L. major*.

Animal number	Diameter in mm		Infection level
	control	experimental	
1547	3.4	26.2	Active
1562	2.3	15.9	"
1566	4.1	25.6	"
1568	2.9	30.2	"
365	2.6	12.3	Self cured
1484	4.0	13.5	"
1479	3.2	13.6	"
1430	2.3	10.2	"

3.4. Lesion development and sizes.

Lesions developed in experimental animals only at areas previously inoculated with 5×10^7 live promastigotes (on the right brow ridges). Lesions appeared to be restricted to the site of parasite inoculation as no multiple lesions were observed. There was a progressive increase in lesion sizes from the time of inoculation to that of surgery. After this, it became difficult to get a true picture as the shape of the lesion was distorted following surgery. Lesions tended to reduce in size from the time of surgery, until they healed. Lesion sizes obtained at the time of surgery were significantly higher than those obtained at 2 weeks after parasite inoculation ($P<0.05$), (Table 4).

Animals undergoing primary infection had bigger lesions as compared to those with secondary infection while lesions in secondary infected animals healed faster than in the primary group.

All BALB/c control mice developed lesions which later disseminated to the legs and had to be sacrificed. The infection later visceralized in these animals as was shown by positive spleen and liver cultures.

Table 4

Lesion areas in mm^2 of experimental animals Two weeks after parasite inoculation and at the time of surgery (Six weeks post infection)

Animal numbers	size at 2 weeks post infection	size at 6 weeks post infection (time of surgery).
365	10.996	41.823
1430	0.000	45.240
1484	0.000	32.798
1479	16.164	55.220
1547	13.132	41.825
1562	0.000	45.240
1566	5.773	66.492
1568	15.174	63. 421

3.5. Immunoperoxidase staining.

Preliminary studies were carried out to determine whether the buffers and reagents for the immunoperoxidase staining were viable. These studies were also extended to determine storage conditions for slides that could not be processed immediately after sectioning.

There was no significant difference in the cell counts between freshly prepared slides and those that had been preserved at -70°C, for over Two years, using the same antibodies on the same animals ($P>0.05$). It was evident from this work that the buffers and the reagents could yield the expected results using my experimental set up. It was also concluded that slides that could not be processed immediately could be stored at -70°C until the day of processing. Staining of biopsies from experimental animals therefore proceeded using these reagents and buffers in the avidin-biotin immunoperoxidase methods.

Control slides for each of the antibodies involved omitting the primary antibody step but following the rest of the steps. All the control slides stained negative or contained light subtle staining that did not interfere with interpretation of results (Plate 12). The cells stained positive with the counterstain only, but not 3-amino-9-ethyl carbazole (the chromogen). Positive cells stained red (the colour of the chromogen substrate reaction). Staining was more characteristic around the membranes of the cells indicating that surface antigens were being stained.

3.6. Cross reactivity of monoclonal antibodies.

Five anti human monoclonal antibodies which had never been tested for their cross reactivity with the vervet monkey system were to be incorporated into this study. These were L243 which stains MHC class 2, W6/32 which stains MHC class 1, UCHT2 which stains CD5, δ TCS1 which stains γ^{δ} T cell receptor and OKT6-FITC which stains CD1 (Table 1). L243, W6/32 and UCHT2 were tested for their cross reactivity using peripheral blood mononuclear cells from animals used in the secondary infections in combination with immunoperoxidase methods. The remaining 2 antibodies were used directly in control experiments.

L243 cross reacted with the vervet monkey system (Plate 2) while W6/32 reacted with almost all the cells (Table 5). UCHT2 did not stain any vervet monkey cells. The results of the other 2 antibodies had already been discussed elsewhere. Both of them cross reacted and were used in control experiments. Due to its staining pattern, L243 was incorporated into the study while W6/32 was left out because of the uniformity in its staining pattern that would have made data analysis difficult.

Table 5.

Percentages of vervet monkey cells which cross reacted with respective anti-human antibodies.

Animal number	Antibodies		
	L243	W6/32	UCHT2
365	43	99.5	0
1430	53.5	100	0.5
1484	44.5	100	1
1479	45	99.5	0

Note that W6/32 stained nearly 100% of the cells. A minimum of 200 cells were counted before determining the percentage of positive ones.

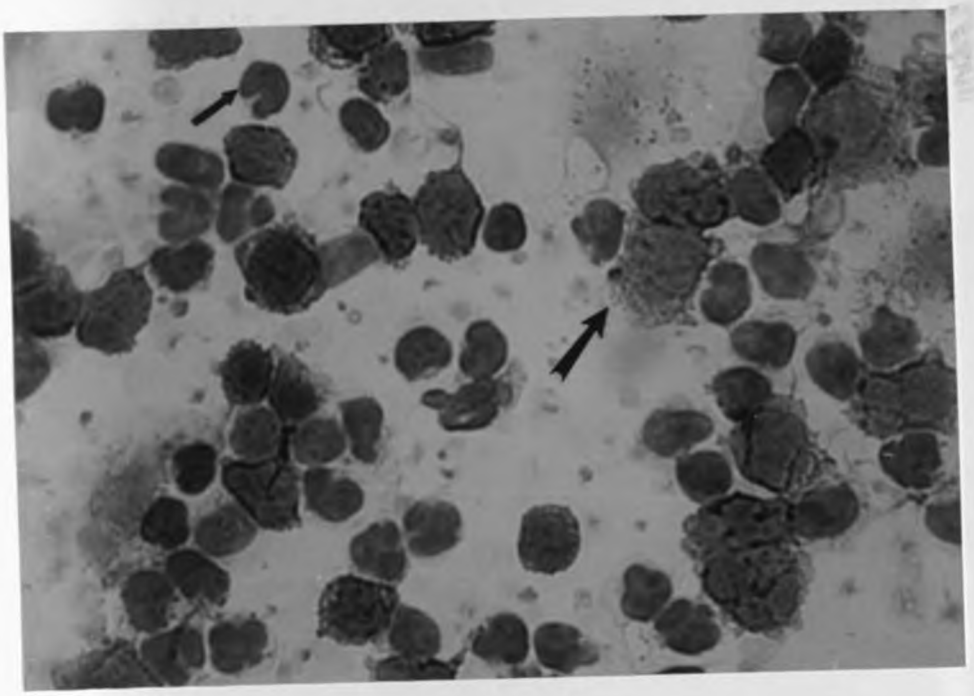


Plate 2. Peripheral blood lymphocytes of vervet monkeys (×500) stained with the antibody L243. This showed that this anti human antibody which stains for MHC class 2 molecules cross reacted with the vervet monkey system. Note that some lymphocytes did not stain. The thin arrow points at a negative cell while the bold one points at a positive cell.

3.7. Immunofluorescence staining.

There was positive immunofluorescence staining with OKT6-FITC antibody (Plate 3). It was difficult to count individual negative cells whose brown margins could not be clearly seen. Both of the control staining methods yielded negative results. This anti human antibody specifically cross reacted with the vervet monkey system. Staining of peripheral blood lymphocytes with this antibody yielded negative results.

3.8. General pathology following primary and secondary infection with *L. major.*

Skin sections from *L. major* infected sites were thicker in size as compared to those from control sites. There was a tendency for more bleeding from ulcerated lesion sites than control sites on the same animals. This was probably due to inflammation as a result of the presence of the parasites. Lymph nodes draining lesions were larger than those draining control sites.

There was a heavy infiltration of cells at the lesion sites during active infection. This was more pronounced during the primary infection. At the time of surgery (42 days after parasite inoculation), there was no evidence of many macrophages laden with parasites. Most cells in the lesion sites were mononuclear in type and CD3 positive cells (pan T cells). This was more prominent in the primary infection group (Plate 4).

There were caseous necrotic areas free of cells in, the lesions during active infection. This was particularly observed in sections from lesion sites of animals in the primary infection group (Plate 5).

There were some lymphocytes resident in the normal skin. The majority were found in clusters in the dermal layer just under the stratum basale layer of the epidermis (Plate 6). These stained positive with FN18 and leu 2a antibodies, suggesting that they were predominantly T cells that expressed the CD8 antigen. Similarly, lymphocytes were stained in the epidermal layer of the normal skin. These were interspersed amongst keratinocytes and had a smooth membrane morphology (Plate 7). They also stained positive with FN18 and leu 2a antibodies meaning they were mainly CD8 positive T cells.

Preliminary results of staining with δTCS1 antibody (anti γ^{δ} T cell receptor) in the dermal layer of the skin where it was used, showed that the antibody cross reacted with the vervet monkey system (Plate 8).

Keratinocytes did not stain with any of the antibodies used in all the experiments except for B9:12:1, (anti-MHC class 1). The layer of these cells was eroded in ulcerated lesions.

Cells around the hair follicle in the skin and in the epidermis from infected sites tended to express MHC class 2 (DP) antigens. The same cells stained positive for CD16 antigen as shown by the monoclonal antibody leu 11b (Plate 9).

There was epidermal layer hyperplasia in the skin at ulcerated lesions. This feature gave the appearance of fatty tissue (Plate 10).

Lymph nodes draining skin lesion sites had their cortices infiltrated with MHC class 2 (DP) expressing cells. The majority of the macrophages stained in these sections were in the medullary region (Plate 11) and these had no evidence of parasites in them. CD3 positive cells were uniformly distributed in the nodes draining control sites while they tended to be clustered along the trabeculae sinuses in sections from nodes draining infected sites. B cells were not stained in the epidermis of the normal skin as was shown by the antibody GM12 (anti B cells).

Plate 3. Specific immunofluorescence staining with OKT6-FITC antibody

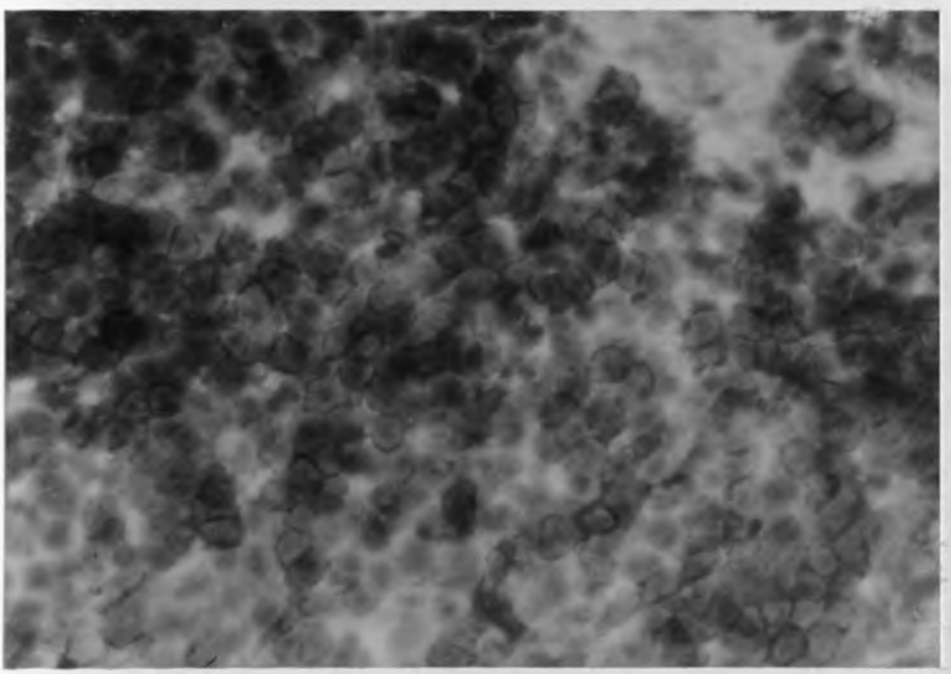


Plate 4. The lesion in the skin heavily infiltrated with CD3 positive cells. Note the high population of cells.

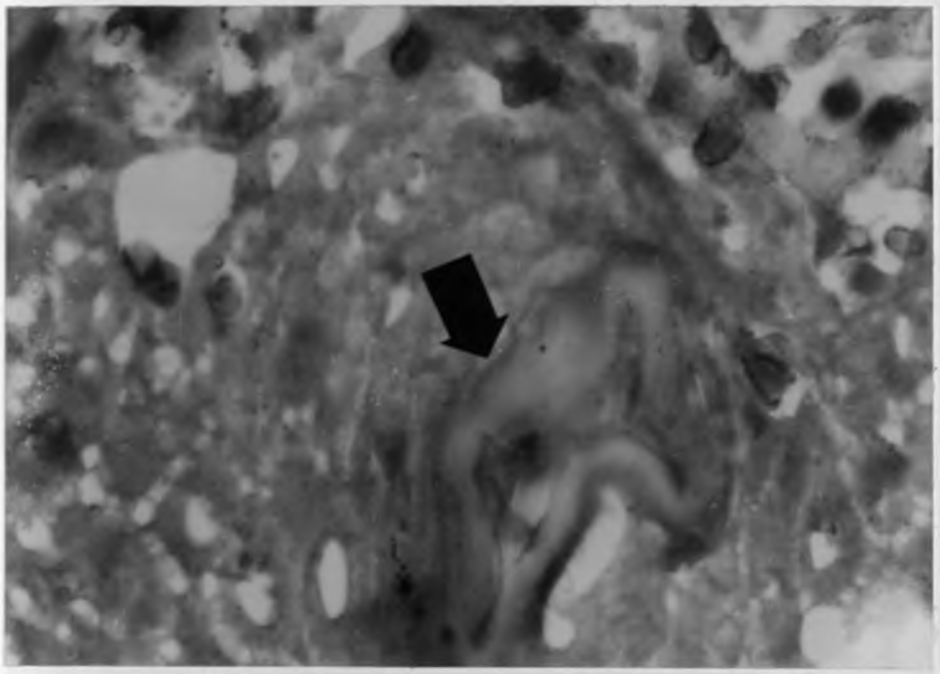


Plate 5. A section of the skin lesion showing a caseous necrotic area (arrow).

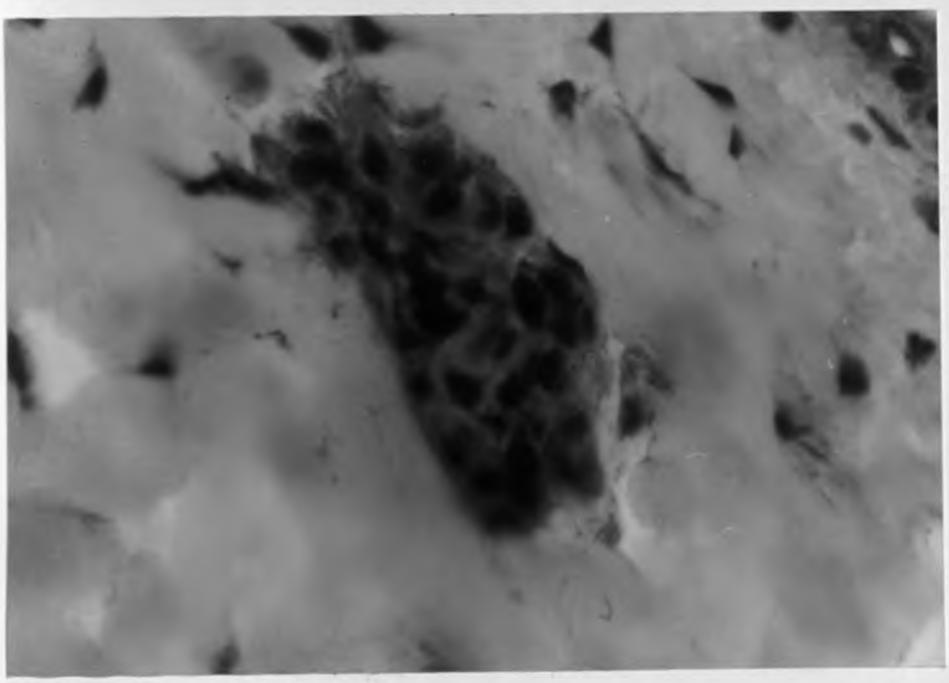


Plate 6. $CD8^+$ cells (×500) in a cluster in the dermis of a naive control vervet monkey.

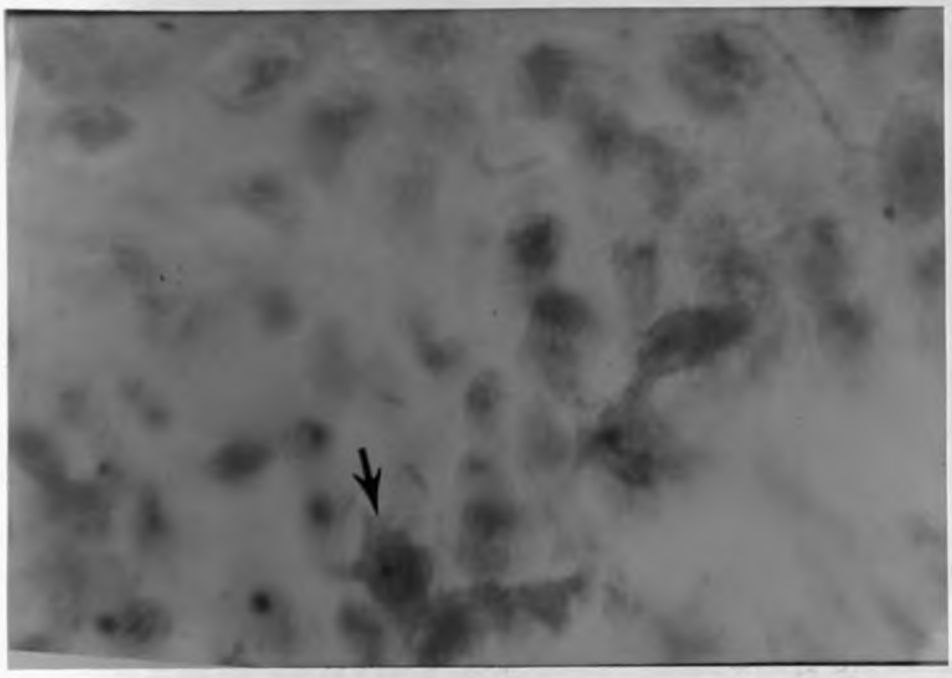


Plate 7. $CD8^+$ cells (×1000) in the epidermis of the naive control vervet monkey. The arrow denotes a positive cell.

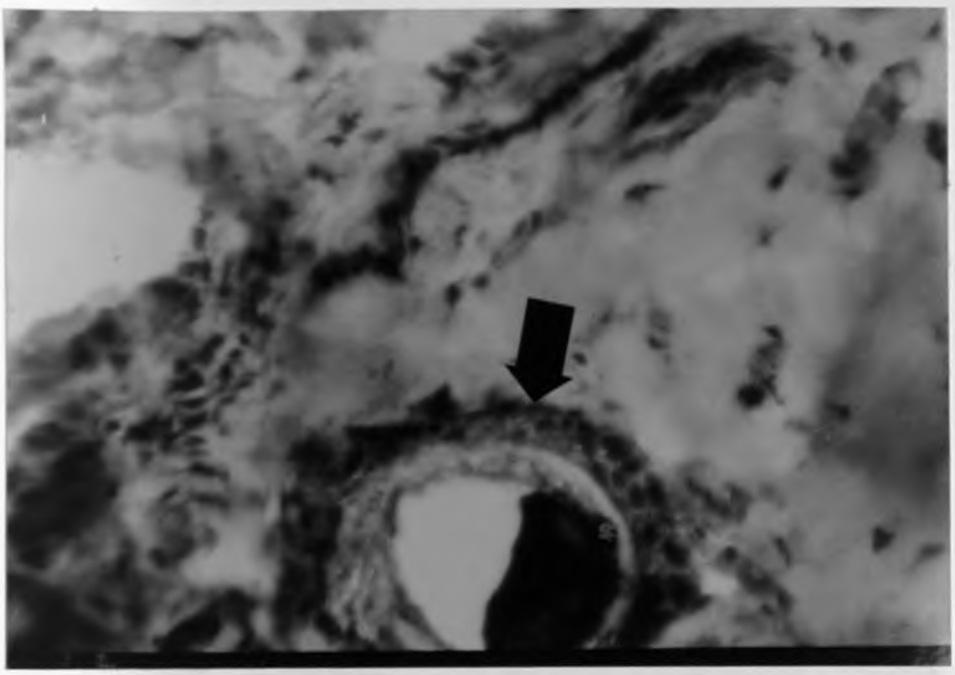


Plate 8. A section from the skin of a naive vervet monkey stained with an anti human γ^{δ} TCR antibody. This shows some cross reactivity between this antibody and the vervet monkey system. The bold arrow shows a hair follicle.

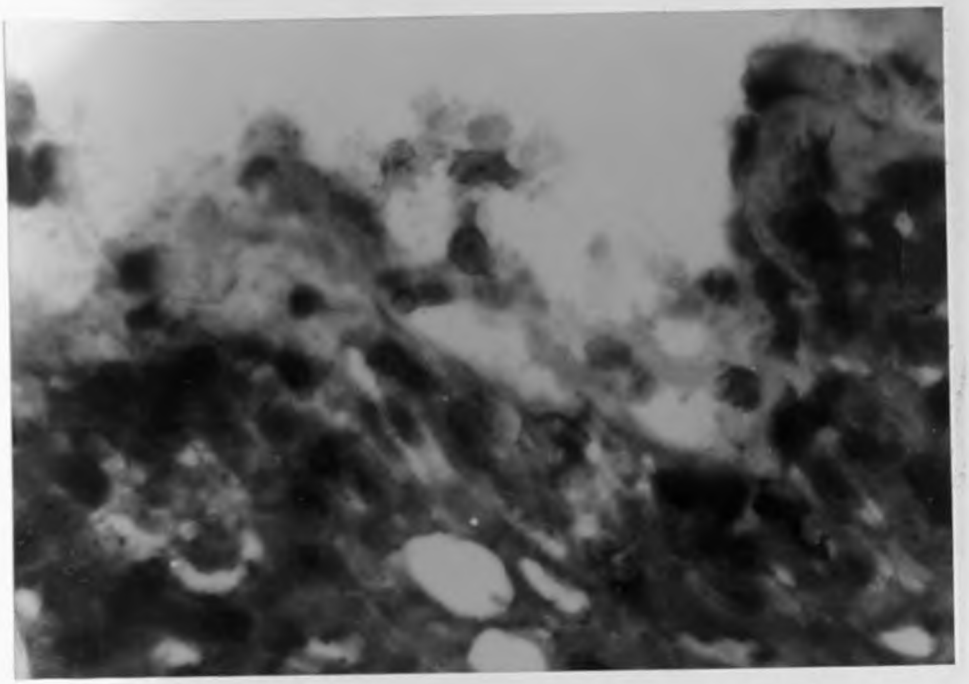


Plate 9. CD16 positive cells in the *L. major* lesion in the epidermis during primary infection.

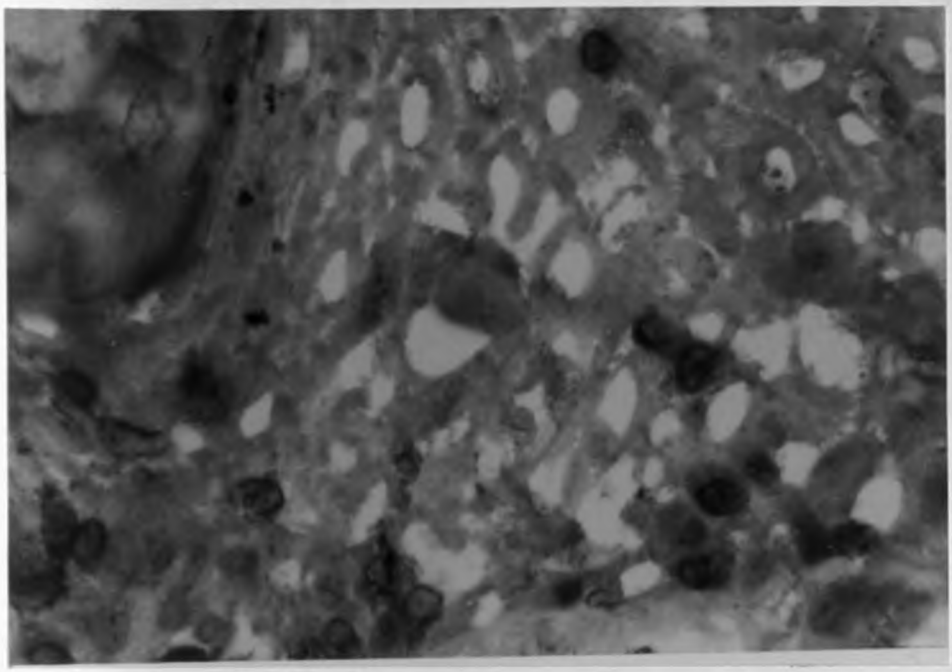


Plate 10. Hyperplasia of the epidermis in a lesion with ***L. major***.

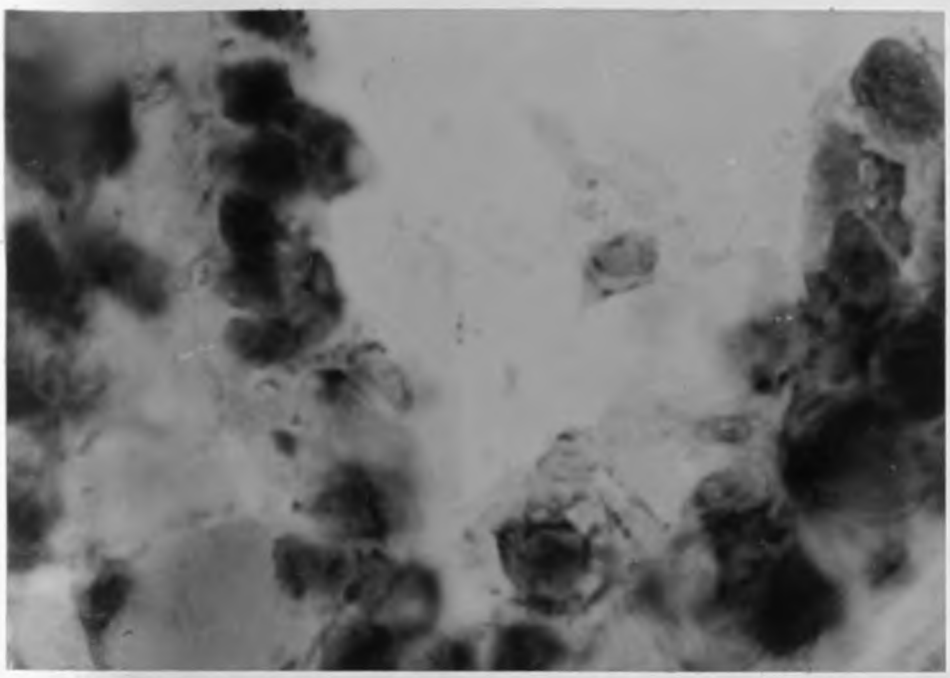


Plate 11. CD 14 positive cells in the medulla of a lymph node draining a skin lesion site

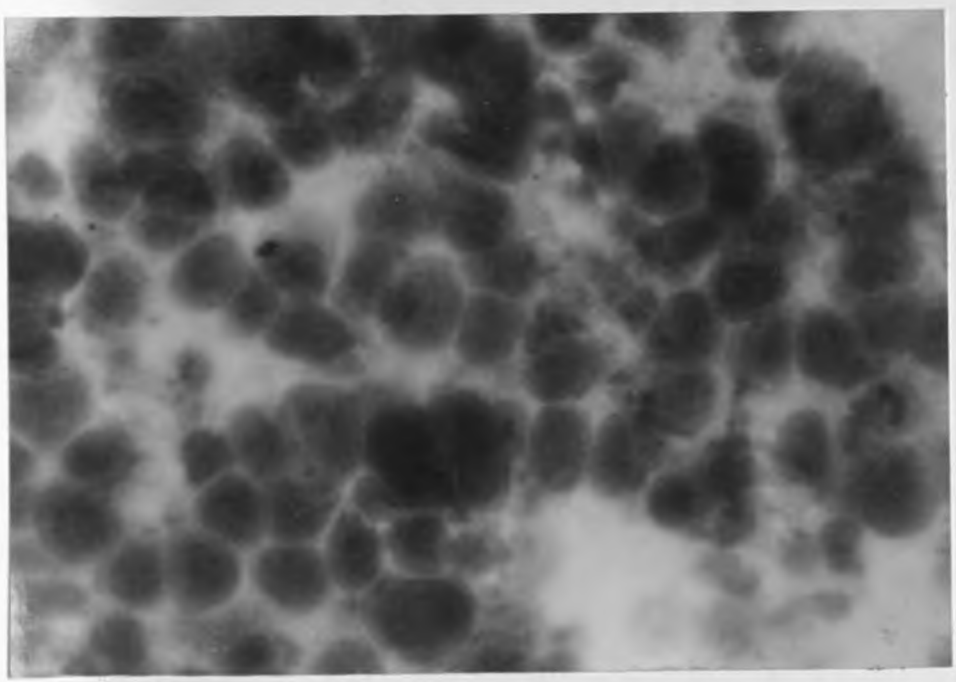


Plate 12. Control sections from the lymph node in which the primary antibody (anti-CD8) was omitted.

3.9. Analysis of immune associated cell types in vervet monkeys as a result of infection with *L. major*.

In all the animals used in these experiments, the antibodies stained percentages of cells significantly different from one another ($P<0.0001$). Different antibodies stained in different proportions in all the sites. In control sites in the skin and their draining lymph nodes, the skin recorded significantly fewer immune associated cells than their draining lymph nodes ($P<0.0001$). In lesion sites however, the skin recorded immune associated cells similar to their draining lymph nodes ($0.132\leq P\leq 0.4383$). Percentages of cells stained for each antibody in each animal in every group were not significantly different ($0.05\leq P\leq 0.7348$) except for lesion sites in animals undergoing secondary infection. In this group, one animal (vervet 1479) stained a slightly higher percentage of cells than the others ($P=0.0531$). This information showed that there were no significant differences in percentages of cells stained by the same antibody in different animals at the same site. Inter-animal variation was therefore minimum in respect to the percentage of cells stained for each antibody. There were therefore similarities in the percentages of cells stained for each antibody in different animals.

3.9.1. Percentages of immune associated cells in the skin of vervet monkeys undergoing primary *L. major* infection.

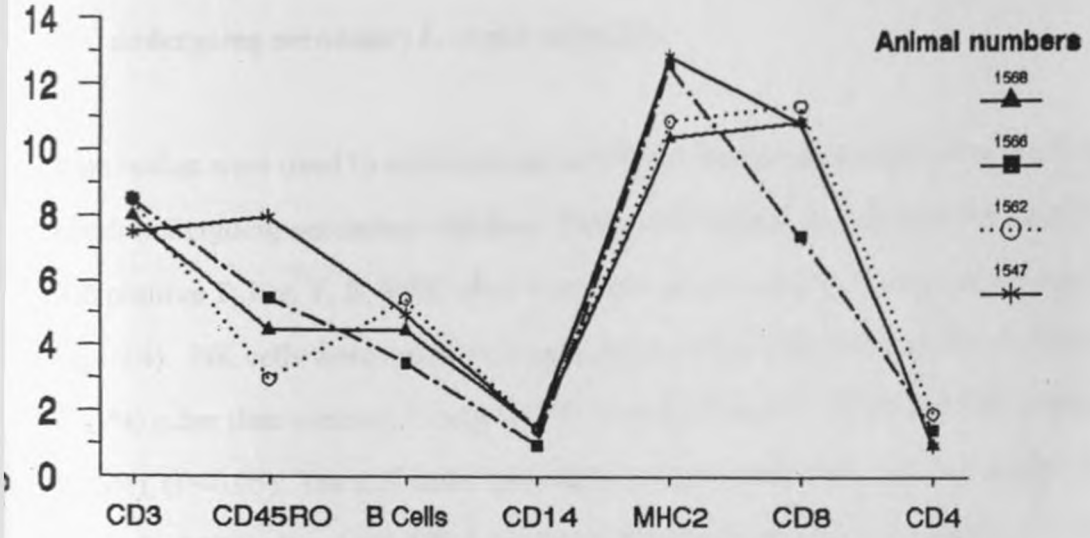
Normal skin sites opposite to lesion areas were stained for immune associated cellular phenotypes and presented as controls.

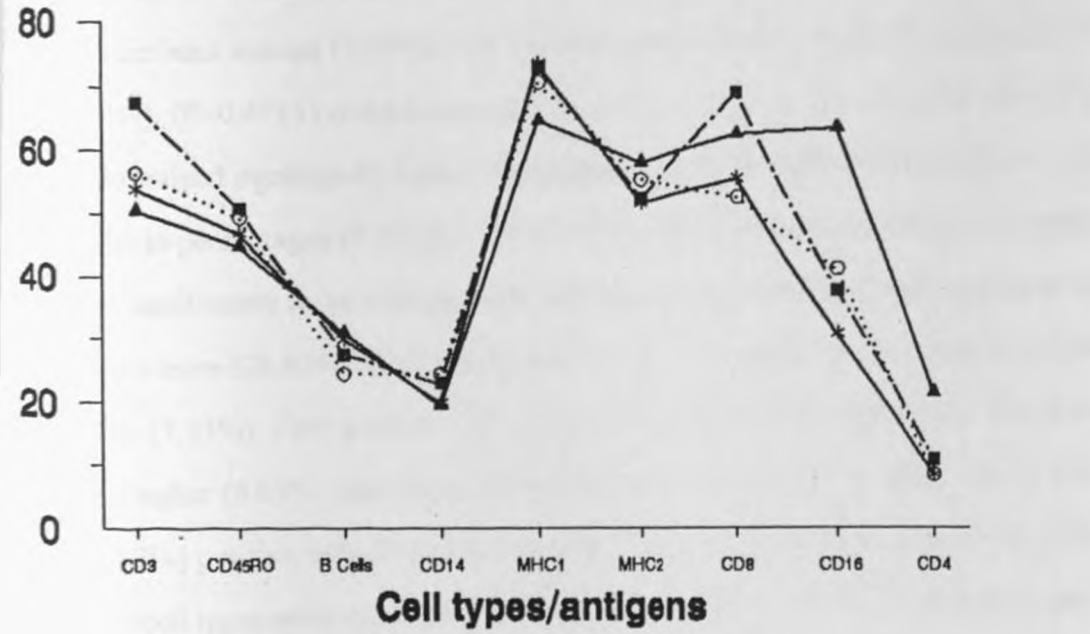
The control sites in the skin of vervet monkeys undergoing primary infection (Figure 2) were stained with antibodies against CD4, CD8, Pan T, B, MHC class 2, memory T cells and

macrophages. CD4 positive T cells were the least stained (1.38%) except for macrophages that stained similar percentages (1.38%), (P=0.9002). CD8 positive T cells were more than the others (10.25%) but not significantly different from Pan T cells (8.13%), (P=0.1457) and MHC class 2 positive cells (11.76%), (P=0.6457) in the percentages of cells recorded. Memory T cells were few in these sites (5.25%), but their percentages were similar to those recorded for B cells (4.63%), (P=0.7698). Pan T cells stained in higher percentages (8.13%) than the rest of the cell types other than MHC class 2 positive (11.76%), (P=0.0569) and CD8 positive T cells (10.25%), (P=0.1457). B cells were stained in lower percentages (4.63%) except for memory T cells whose staining (5.25%) was not significantly different (P=0.7698). MHC class 2 positive cells were stained in higher proportions (11.76%) than all the other cells except for Pan T (8.13%), (P=0.0569) and CD8 positive T cells (10.25%), (P=0.6457) which were recorded in similar percentages. Macrophages were negligible at these control sites (1.38%). They were similar to CD4 positive T cells (1.38%), (P=0.9002).

Lesion sites in animals undergoing primary infection (Figure 3) were stained with antibodies against NK, CD4 and 8 positive T, Pan T, B, MHC class 1 and 2 positive, memory T cell types and macrophages. Percentages of NK cells stained (43.63%) were similar to memory T cells (48.13%), (P=0.278) but more than macrophages (21.75%) and CD4 positive T cells (12.38%), (P<0.0001). NK cells were stained in significantly lower percentages than the rest of the cell types (P<0.02). CD8 positive T cells were similar in percentages stained (60.38%) to Pan T cells (57.13%), (P=0.4485) and MHC class 2 positive cells (54.75%), (P=0.3191). Pan T cells were higher than other cell types in the percentages stained (57.13%), (P<0.032) except for CD8 positive T cells (60.38%), (P=0.449) and MHC class 2 positive cells (54.75%), (P=0.359). B cells were fewer than all the other cells (28.25%) other than macrophages (21.75%), (P=0.119). They were however significantly more than CD4 positive T cells (12.38%), (P<0.0002). MHC class 1 positive cells stained in higher percentages (70.88%) than all the other cells (P<0.013). MHC class 2 positive cells were also significantly

more than the other cell types (54.75%), ($P<0.02$) other than CD8 positive T cells (60.38%), ($P=0.319$), Pan T cells (57.13%), ($P=0.8110$) and memory T cells (48.13%), ($P=0.056$). Memory T cells stained significantly higher (48.13%) than macrophages (21.75%) and CD4 positive T cells (12.38%), ($P<0.04$), but fewer than the other cell types ($P<0.05$). They were similar in percentages to NK cells (43.63%) and MHC class 2 positive cell types (54.75%), ($0.2136 \leq P \leq 0.2777$). Macrophages were amongst the least stained (21.75%), ($P<0.02$), being similar in percentages to B cells (28.25%), ($P=0.119$). CD4 positive T cells were the least stained (12.38%), ($P<0.0001$).





Top, **Figure 2.** Percentages of positive cells in skin sections from control sites in vervet monkeys undergoing primary *L. major* infection.

Bottom, **Figure 3.** Percentages of positive cells in skin sections from lesion sites of vervet monkeys undergoing primary *L. major* infection.

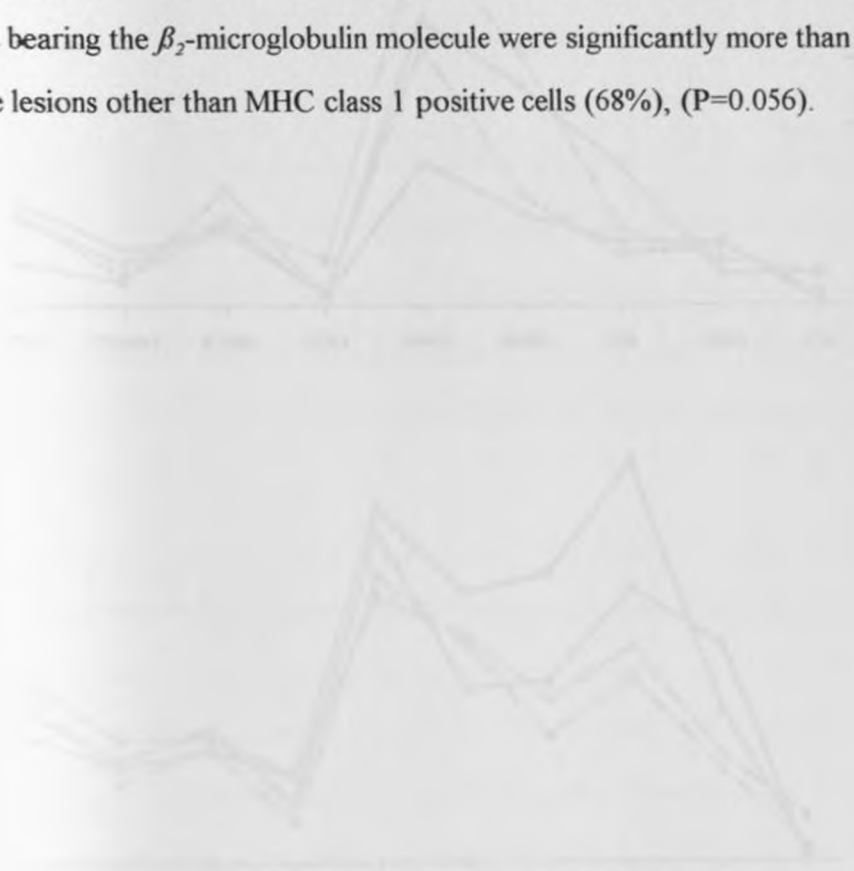
3.9.2. Percentages of immune associated cells in the skin of vervet monkeys undergoing secondary *L. major* infection.

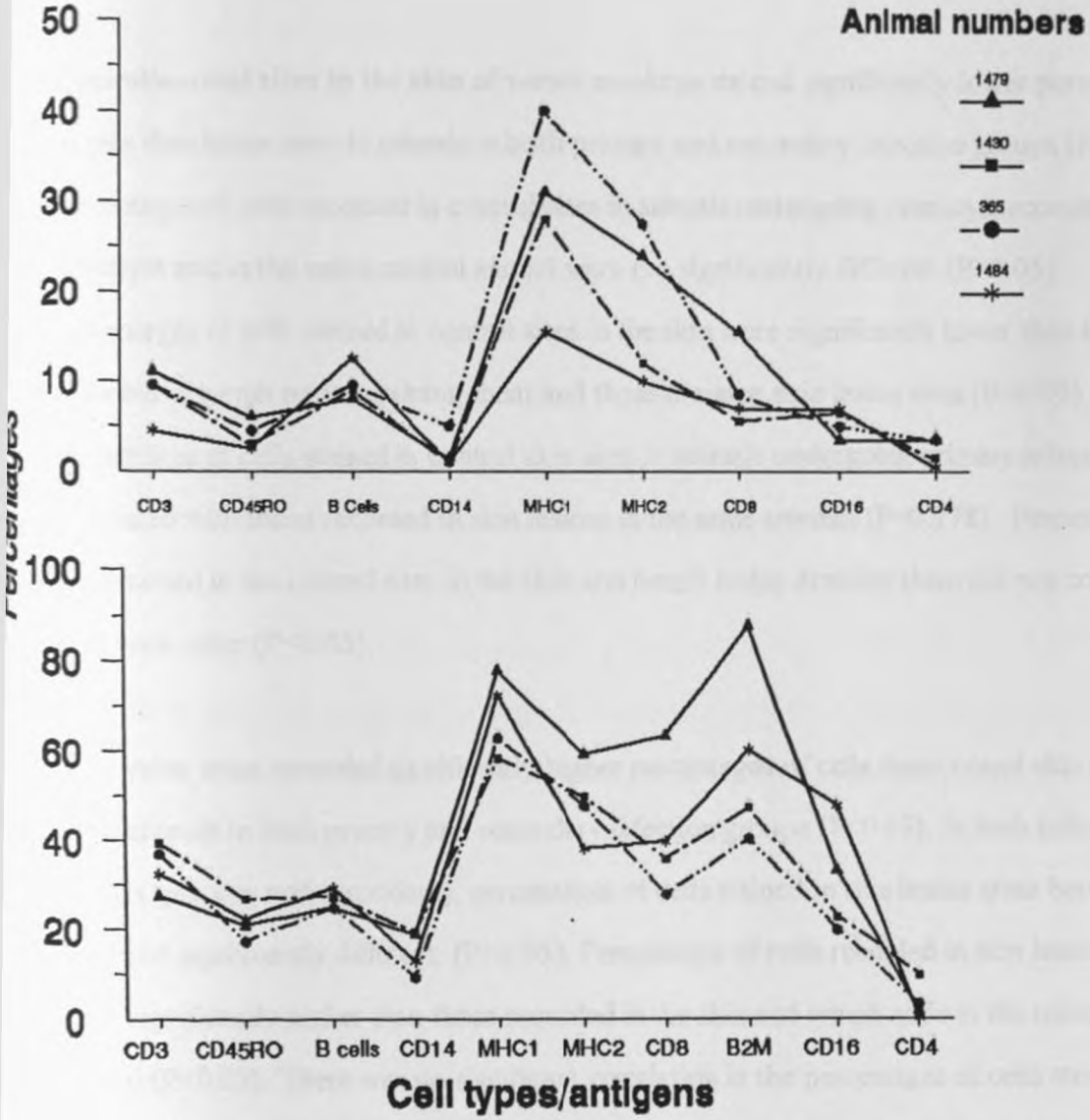
10 antibodies were used to stain immune cells in the **control/normal sites in the skin** of animals undergoing secondary infection. These were antibodies that stain for NK, CD4 and CD8 positive T, Pan T, B, MHC class 1 and 2 positive, memory T cells and macrophages (Figure 4). NK cells were recorded in percentages lower than those of other cell types (7.33%) other than memory T cells (5.33%), macrophages (2.13%), and CD4 positive T cells (2.25%), ($P<0.05$). The difference between it and other cell types was not significant ($P>0.05$) except for MHC class 1 (28.63%) and 2 (18.37%) positive cells. CD4 positive T cells again were the least stained (2.25%). Staining with this antibody was similar to memory T cells (5.33%), ($P=0.4711$) and macrophages (2.13%), (0.5763). The rest of the antibodies in the group stained significantly higher percentages of cells ($P>0.05$). CD8 positive T cells were similar in percentages (9.13%) to NK (7.33%), Pan T (8.63%) and B (9.75%) cells. They were significantly more than the other cell types other than MHC class 1 positive cells that stained more (28.63%), ($P<0.0001$). Pan T cells were similar in percentages stained (8.63%) to NK (7.33%), CD8 positive T (9.13%) and B cells (5.32%), ($P>0.05$). The percentages were higher (8.63%) than those of the other cell types except for MHC class 1 (28.63%) and 2 (18.37%) positive cells ($P>0.05$). Memory T cells were significantly fewer (5.33%) than the other cell types other than CD4 positive T cells (2.25%), ($P=0.471$) and macrophages (2.13%), ($P=0.914$). B cells were not significantly different (5.32%) from NK (7.33%), CD8 (9.13%) and Pan T (8.63%) cells ($P>0.05$). The B cells were fewer than MHC class 1 positive cells (28.63%), ($P<0.0001$). MHC class 1 positive cells were stained in higher percentages than others (28.63%), ($P<0.023$). MHC class 2 positive cells stained a significantly higher percentage of cells (18.37%) than the other cells ($P<0.04$) except for MHC class 1 positive (28.63%) cells, ($P<0.0001$). The probability values for MHC class 1 positive cells against the MHC class 2 positive showed that the former was significantly higher (28.63%) than the latter

(18.37%). Macrophages were significantly fewer (2.13%) than the other cell types (P<0.02) except for CD4 positive T cells (2.21%), (P=0.407) and memory T cells (5.33%), (P=0.56).

11 antibodies were used in staining cell types in **lesion sites in the skin** of monkeys undergoing secondary infection. Antibodies used included those that stain for cells bearing β_2-microglobulin molecule, NK, CD4 and 8 positive T, Pan T, B, MHC class 1 and 2 positive, memory T cells and macrophages (Figure 5). The percentages of cells stained by the different antibodies were compared with those within the group. Macrophages stained significantly different percentages of cells (15.25%) from the other cell types in the group (P<0.01) other than memory T cells (22%), (P=0.1278). Memory T cells were significantly fewer in percentages stained (22%) than the other cell types (P<0.04) except for B cells (26.75%), (P=0.2870), macrophages (15.25%),(P=0.1280) and CD4 positive T cells (4%), (P<0.0001). MHC class 2 positive cells were significantly more stained (48.94%) than the other cells (P<0.033) apart from NK cells (31.13%), (P=0.235), CD8 positive T cells (41.89%), (P=0.207) and Pan T cells (34.13%),(P=0.617). MHC class 1 positive cells were recorded at 68%, staining similar to MHC class 2 positive cells (48.94%), (P=0.137). B cells stained differently from the rest of the cell types (26.75%), (P<0.01) other than NK cells (31.13%), (P=0.336), and Pan T cells (34.13%), (P=0.1). Pan T cells stained higher than most of the other cells (34.13%), (P<0.007) apart from NK cells (31.13%), (P=0.491), CD8 positive T cells (41.89%), (P=0.079) and MHC class 2 positive cells (48.94%), (P=0.617). They stained fewer than MHC class 1 positive cells (68%), (P<0.0001) and cells bearing the β_2-microglobulin molecule (59%), (P<0.0001). Cells bearing the CD8 T cell antigen were stained in significantly higher percentages than the other cells (41.89%), (P<0.02) other than Pan T cells (34.13%), (P=0.079) and MHC class 2 positive cells (48.94%), (P=0.207), but lower than MHC1 (68%) and β_2-microglobulin (59%). Pan T cells stained fewer percentages than cells bearing MHC class 1 antigens (68%), (P<0.0001) and the β_2-microglobulin molecule (59%), (P<0.0001). CD4 positive T cells were stained in significantly lower percentages (4%)

than the other cell types (P<0.012). NK cells stained significantly similar percentages (31.3%) to Pan T cells (34.13%), (P=0.49), B cells (26.75%), (P=0.336) and MHC class 2 positive cells (48.94%), (P>0.05). NK cells stained in lower percentages than MHC class 1 positive cells (68%), (P<0.0001), and cells bearing the β_2-microglobulin molecule (59%), (P<0.0001). Cells bearing the β_2-microglobulin molecule were significantly more than the other cell types in these lesions other than MHC class 1 positive cells (68%), (P=0.056).





Top, **Figure 4.** Percentages of positive cells in skin sections from control sites in vervet monkeys undergoing secondary *L. major* infection.

Bottom, **Figure 5.** Percentages of positive cells in skin sections from lesion sites in vervet monkeys undergoing secondary *L. major* infection.

3.9.3 Percentages of cells stained in skin areas compared to other sites.

Control/normal sites in the skin of vervet monkeys stained significantly lower percentages of cells than lesion sites in animals in both primary and secondary infection groups ($P<0.05$). Percentages of cells recorded in control sites in animals undergoing primary, secondary infections and in the naive control animal were not significantly different ($P>0.05$). Percentages of cells stained at control sites in the skin were significantly lower than those recorded in lymph nodes draining them and those draining skin lesion sites ($P<0.05$). The percentages of cells stained in control skin sites in animals undergoing primary infection correlated with those recorded in skin lesions in the same animals ($P=0.778$). Percentages of cells stained in the control sites in the skin and lymph nodes draining them did not correlate with each other ($P<0.05$).

Skin lesion areas recorded significantly higher percentages of cells than control skin sites in the same animals in both primary and secondary infection groups ($P<0.05$). In both infection groups (primary and secondary), percentages of cells stained in skin lesion areas between them were not significantly different ($P>0.05$). Percentages of cells recorded in skin lesion areas were significantly higher than those recorded in the skin and lymph node in the naive control animal ($P<0.05$). There was no significant correlation in the percentages of cells stained between skin lesion areas and control skin sites in the same animals ($P<0.05$). There was no significant correlation in percentages of cells stained between skin lesion areas and lymph nodes draining them in the same animals ($P<0.05$). Skin lesion sites did not stain significantly different percentages of cells from lymph nodes draining them in both primary and secondary infection group animals ($P>0.05$). Cells recorded in skin lesion sites were significantly similar to those recorded in lymph nodes draining control skin sites ($P>0.05$).

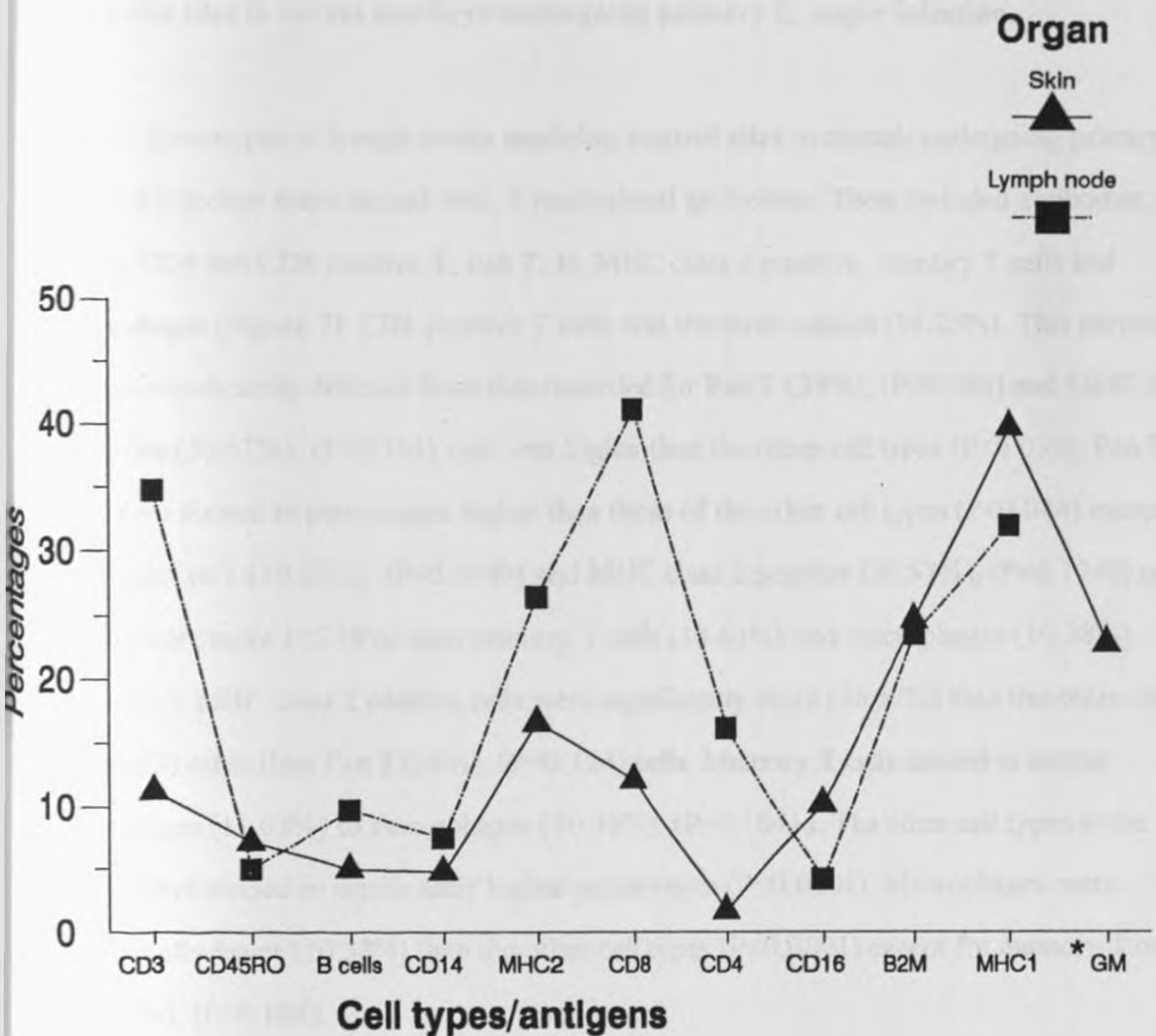
3.9.4. Immune associated cell types in the naive control animal

Immune associated cells in the skin in the **naive control** animal were stained with 12 antibodies. These antibodies recognised NK, CD4 and 8 positive T, Pan T, B, γ^{δ} TCR bearing T, MHC classes 1 and 2 positive, memory T, β_2-microglobulin molecule bearing cells and macrophages (Figure 6). Cells bearing the β_2-microglobulin molecule were recorded in high percentages (25%). This percentage was similar to that recorded for T cells bearing the γ^{δ} TCR (23%), ($P=0.7055$) and MHC class 2 positive cells (18.5%), ($P=0.1924$). The rest stained in significantly fewer percentages except for MHC class 1 positive cells that stained more (40%), ($P<0.05$). NK cells were stained in percentages similar (10.5%) to those recorded for the other cell types ($0.0707 \leq P \leq 0.8383$) other than MHC class 1 positive (40%), γ^{δ}TCR positive T (23%) and β_2-microglobulin bearing (25%) cells ($P<0.0152$). CD4 positive T cells were stained in similar percentages (1%) to macrophages (2%), ($P=0.4567$), memory T cells (7%), ($P=0.2277$), B cells (6%), ($P=0.3125$) and NK cells (10.5%), ($P=0.0707$). The rest of the antibodies in the group stained significantly more cells ($P<0.0325$). CD8 positive T cells stained in percentages similar (12%) to NK (10.5%), Pan T (11.5%), B (6%), and memory T cells (7%), ($0.1433 \leq P \leq 0.8649$). The other cells stained in significantly different percentages ($P<0.05$). Pan T cells were similar in percentages stained (11.5%) to most of the other cell types ($0.1616 \leq P \leq 0.8649$) except for β_2-microglobulin molecule bearing (25%), γ^{δ} TCR positive T (23%) and MHC class 1 positive (40%) cells ($P<0.05$). B cells stained in similar percentages (6%) to several other cell types including NK (10.5%), CD8 positive T (8%), Pan T (11.5%) and memory T (7%)cells, ($0.0806 \leq P \leq 0.8383$). The percentages in other cells were significantly different ($P<0.05$). γ^{δ}TCR bearing T cells were similar in percentages stained (23%) to those bearing the β_2-microglobulin molecule (25%), and MHC class 2 positive cells (18.5%), ($0.1118 \leq P \leq 0.7085$). The remaining cell types stained in lower percentages ($P<0.05$). Cells bearing the MHC class 2 antigens were recorded in percentages similar (18.5%) to those bearing β_2-microglobulin molecule (25%), and γ^{δ}TCR positive T cells

(23%), ($0.1118 \leq P \leq 0.4982$). The other cells were recorded in significantly less percentages apart from MHC class 1 positive cells (40%) that stained more ($P<0.05$). MHC class 1 positive cells stained more than the other cell types ($P<0.05$). Memory T cells were similar in percentages stained (7%) to NK (10.5%), CD8 positive T (12%) and Pan T (11.5%) cells, ($0.1191 \leq P \leq 0.8383$). The other cells stained in significantly different percentages ($P<0.03$). Macrophages were stained in percentages similar (2%) to those recorded for NK (10.5%), CD4 positive T (1%), B (6%), and memory T (7%) cells ($0.1433 \leq P \leq 0.7856$). The rest of the antibodies in the group stained differently ($P<0.0466$).

In the lymph node draining the normal skin site in the naive control animal, 11 monoclonal antibodies which stained for β_2-microglobulin, NK, CD4 and 8 positive T cells, Pan T, B cells, MHC class 1 and 2 positive, memory T cells and macrophages (Figure 6). Cells bearing the β_2-microglobulin molecule were similar in percentages stained to MHC class 2 positive cells (26.5%), ($P=0.3247$) and CD4 positive T cells (16.5%), ($P=0.0563$). The other cell types were significantly different ($P<0.029$). NK cells (4%) were similar to B cells (10%), memory T cells (5%) and macrophages (7.5%), ($0.1338 \leq P \leq 0.8210$) but significantly different from the rest ($P<0.03$). CD 4 positive T cells were stained in a similar percentage (16.5%) to those recorded for β_2-microglobulin bearing (23.5%), and MHC class 2 positive (26.5%) cells ($0.0563 \leq P \leq 0.3247$). The rest of the antibodies stained differently ($P<0.0029$). CD8 positive T cells were similar in percentages stained (41%) to Pan T cells (35%), ($P=0.0958$). Anti-Pan T cells stained percentages of cells similar (35%) to those recorded for CD8 positive T (41%), ($P=0.0958$), MHC class 1 (37%) and 2 (26.5%) positive cells ($0.4715 \leq P \leq 0.6195$). B cells were similar in percentages stained to NK (4%), memory T (5%) and macrophages (7.5%), ($0.089 \leq P \leq 0.5281$). The other cells stained significantly higher percentages ($P<0.01$). Higher percentage of MHC class 1 positive cells were stained compared to the rest (37%) other than Pan T cells (35%), ($P=0.4715$). MHC class 2 positive cells were comparable to cells bearing

the β_2-microglobulin molecule and CD4 positive T cells (P=0.3247). Memory T cells were similar in percentages stained to NK (4%), (P=0.8210), B (10%), (P=0.1978) cells and macrophages (7.5%), (P=0.4993). The other cell types stained differently (P<0.05). Macrophages were similar to NK (4%), (P=0.3697), B (10%), (P=0.528) and memory T (5%), (P=0.4993) cells.



* GM stands for Gamma delta
TCR positive T cells

Figure 6. Percentages of positive cells in the skin and the lymph node draining it in the naive animal.

3.9.5. Percentages of immune associated cells in lymph nodes draining normal/control and lesion sites in vervet monkeys undergoing primary *L. major* infection.

Cellular phenotypes in **lymph nodes draining control sites** in animals undergoing primary *L. major* infection were stained with, 8 monoclonal antibodies. These included antibodies against CD4 and CD8 positive T, Pan T, B, MHC class 2 positive, memory T cells and macrophages (Figure 7). CD8 positive T cells was the most stained (39.25%). This percentage was not significantly different from that recorded for Pan T (38%), ($P=0.589$) and MHC class 2 positive (36.57%), ($P=0.751$) cells, but higher than the other cell types ($P<0.039$). Pan T cells were stained in percentages higher than those of the other cell types ($P<0.044$) except for CD8 positive T (39.25%), ($P=0.5999$) and MHC class 2 positive (36.57%), ($P=0.1240$) cells. B cells were more (25.38%) than memory T cells (13.63%) and macrophages (10.38%), ($P<0.001$). MHC class 2 positive cells were significantly more (36.57%) than the other cells ($P<0.039$) other than Pan T (38%), ($P=0.124$) cells. Memory T cells stained in similar percentages (13.63%) to macrophages (10.38%), ($P=0.1641$). The other cell types in the group were stained in significantly higher percentages ($P<0.0001$). Macrophages were significantly fewer (10.38%) than the other cell types ($P<0.0001$) except for memory T cells (13.63%), ($P=0.164$).

The different cell types in **lymph nodes draining lesion sites** in animals undergoing primary infection were stained with 8 antibodies against CD4 and CD8 positive T cells, Pan T cells, B cells, MHC class 2 positive cells, memory T cells and macrophages (Figure 8). Macrophages were significantly fewer (13.63%) than the other cells ($P<0.002$). CD4 positive T cells stained in similar percentage (27.5%) to memory T cells (35.88%), ($P=0.065$). CD8 positive T cells stained in similar percentages (56%) to Pan T cells (64.75%), ($P=0.078$), B cells (49.13%), ($P=0.095$) and MHC class 2 positive cells (65.69%), ($P=0.477$). The anti-CD8 antibody stained significantly different percentages of cells from the rest of the antibodies in this group

(P<0.005). Pan T cells were similar in percentages (64.75%) to MHC class 2 positive cells (65.69%), (P=0.287) and CD8 positive T cells (P=0.078). B cells were significantly similar (49.13%) to CD8 positive T cells (56%), (P=0.095) and different from the rest (P<0.02). MHC class 2 positive cells were significantly similar in percentages stained (65.69%) to CD8 positive T (56%), (P=0.477) and Pan T (64.75%), (P=0.298) cells. The other cells stained significantly different percentages (P<0.03).

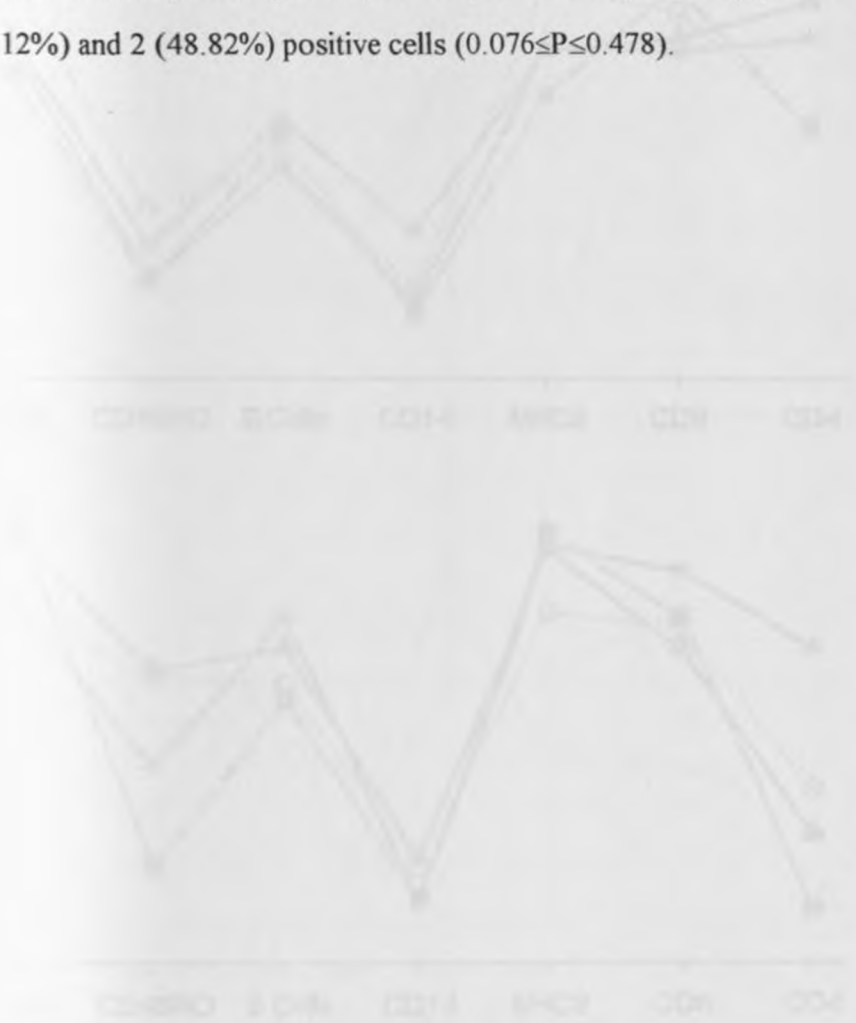
3.9.6. Percentages of immune associated cells in lymph nodes draining normal/control and lesion sites in vervet monkeys undergoing secondary *L. major* infection.

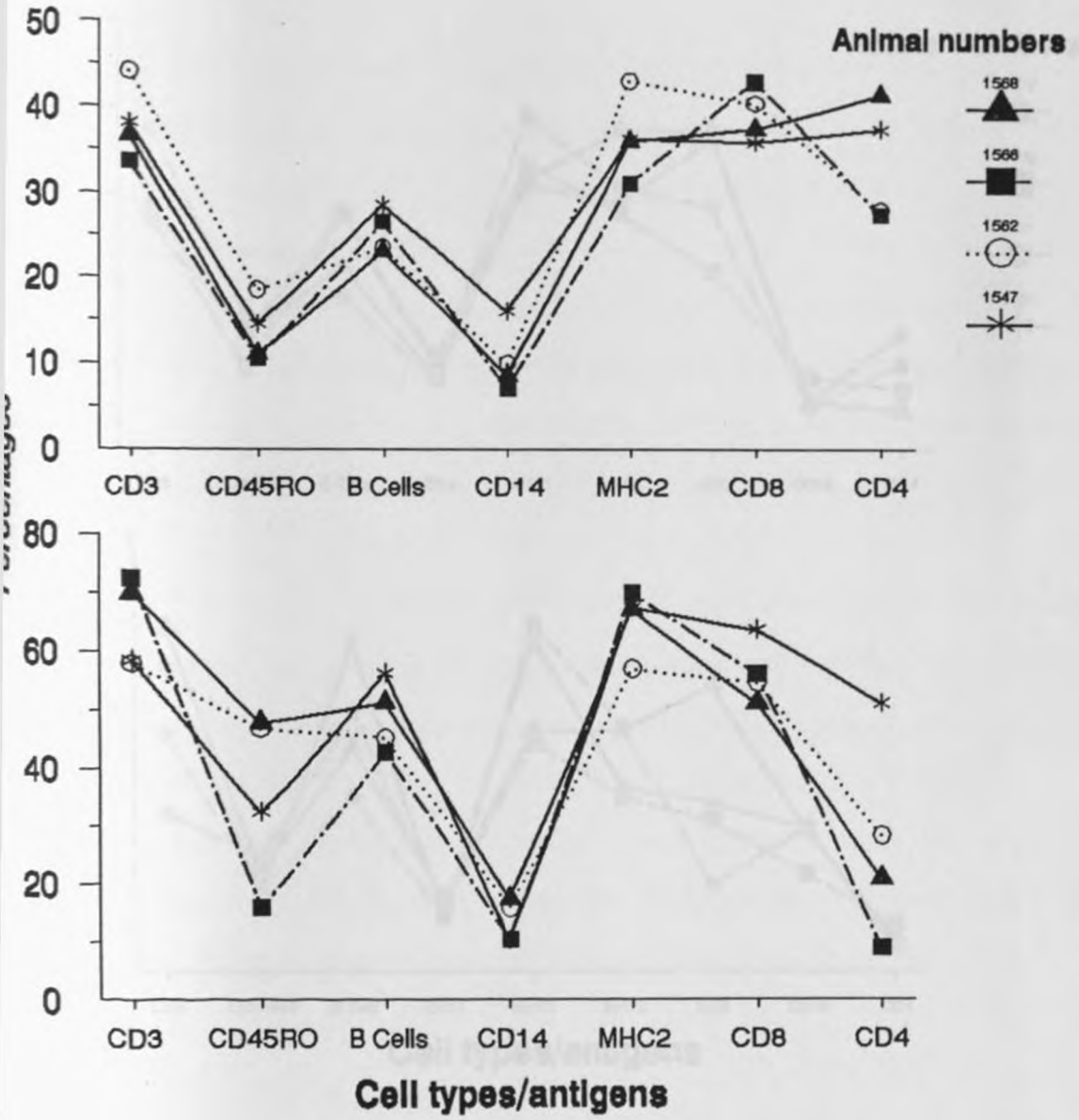
Lymph nodes draining control sites in animals undergoing secondary *L. major* infection were stained with 10 monoclonal antibodies that stained for NK, CD4 positive T, Pan T, B, MHC class 1 and 2 positive, memory T cells and macrophages (Figure 9). NK cells stained similarly (6.13%) to CD4 positive T cells (9.88%), (P=0.226) and macrophages (9.38%), (P=0.1323). The rest of the cells stained significantly higher proportions (P<0.0003). Pan T cells (30%), MHC class 1 (33.13%) and 2 (31.26%) positive cells were similar to CD8 positive T cells (30.88%), (0.204≤P≤0.956) in the percentages stained. The rest of the cells stained differently (P<0.02). Pan T cells were recorded in percentages similar (30%) to those recorded for MHC class 1 (33.13%) and 2 (31.26%) positive cells (0.1421≤P≤0.2482). The rest recorded lower percentages (P<0.006). B cells were (23.63%) similar to MHC class 2 positive cells (32.26%), (P=0.1231) in percentages stained. The other cells were recorded in significantly different proportions (P<0.006). MHC class 1 positive cells stained in similar percentages (33.13%) to CD8 positive T (30.88%), (P=0.956), Pan T (30%), (P=0.2483) and MHC class 2 positive cells (31.26%), (P=0.751). The rest of the cells were stained in significantly lower percentages (P<0.035). MHC class 2 positive cells stained in similar proportions (31.26%) to MHC class 1 positive cells (33.13%), (P=0.571), Pan T cells (30%), (P=0.1421) and CD8 positive T cells (30.88%), (P=0.78). The other cells were recorded in

significantly lower percentages ($P<0.02$). CD4 positive T cells were stained in similar percentages (9.88%) to NK cells (6.13%), ($P=0.226$) and macrophages (9.38%), ($P=0.7651$). The rest of the cells were significantly more ($P<0.01$). Memory T cells were stained in lower percentages (13%) than those recorded for the other cell types ($P<0.012$) except for NK cells that were fewer (6.13%), ($P<0.0003$). Macrophages were recorded in similar proportions to NK cells (6.13%), ($P=0.1323$) and CD4 positive T cells (9.88%), ($P=0.7651$). The rest of the antibodies in the group stained cells in higher percentages ($P<0.027$).

The cellular phenotypes in **lymph nodes draining lesion areas** in animals undergoing secondary infection were evaluated using 10 antibodies that stained for NK, CD4 and 8 positive T, Pan T, MHC classes 1 and 2 positive, memory T cells and macrophages (Figure 10). MHC class 1 positive cells were stained in proportions not significantly different (66.13%) from Pan T (59.88%), ($P=0.214$), MHC class 2 (48.82%), ($P=0.578$), and B cells (57.13%), ($P=0.076$). NK cells were recorded in percentages similar (30.38%) to CD8 positive T (40%), and MHC class 2 positive (48.82%) cells ($0.056\leq P\leq 0.185$). The rest of the cell types were recorded in proportions different from NK cells ($P<0.001$). CD4 positive T cells were recorded in similar percentages to macrophages (14.13%), ($P=0.1880$). The rest of the cell types were stained in significantly higher percentages from CD4 positive cells ($P<0.0001$). CD8 positive T cells were similar in percentages stained (40%) to MHC class 2 positive cells (48.82%), ($P=0.7023$). The percentage CD8 positive cells was however different from those of other cells types stained at these sites ($P<0.001$). B cells were stained in similar percentages (57.13%) to Pan T cells (59.88%), ($P=0.589$), MHC class 1 (66.13%) and 2 (48.94%) positive cells ($0.076\leq P\leq 0.360$). MHC class 2 positive cells were recorded in percentages similar to CD8 positive T, (40%), ($P=0.7023$) and MHC class 1 positive (66.13%), ($P=0.578$) cells. The rest of the cells types were stained in lower percentages ($P<0.0002$). Memory T cells were recorded in lower percentages (22%) than the other cell types except for NK (30.38%), ($P=0.558$), CD4 positive T (7.5%), ($P<0.0001$) cells and

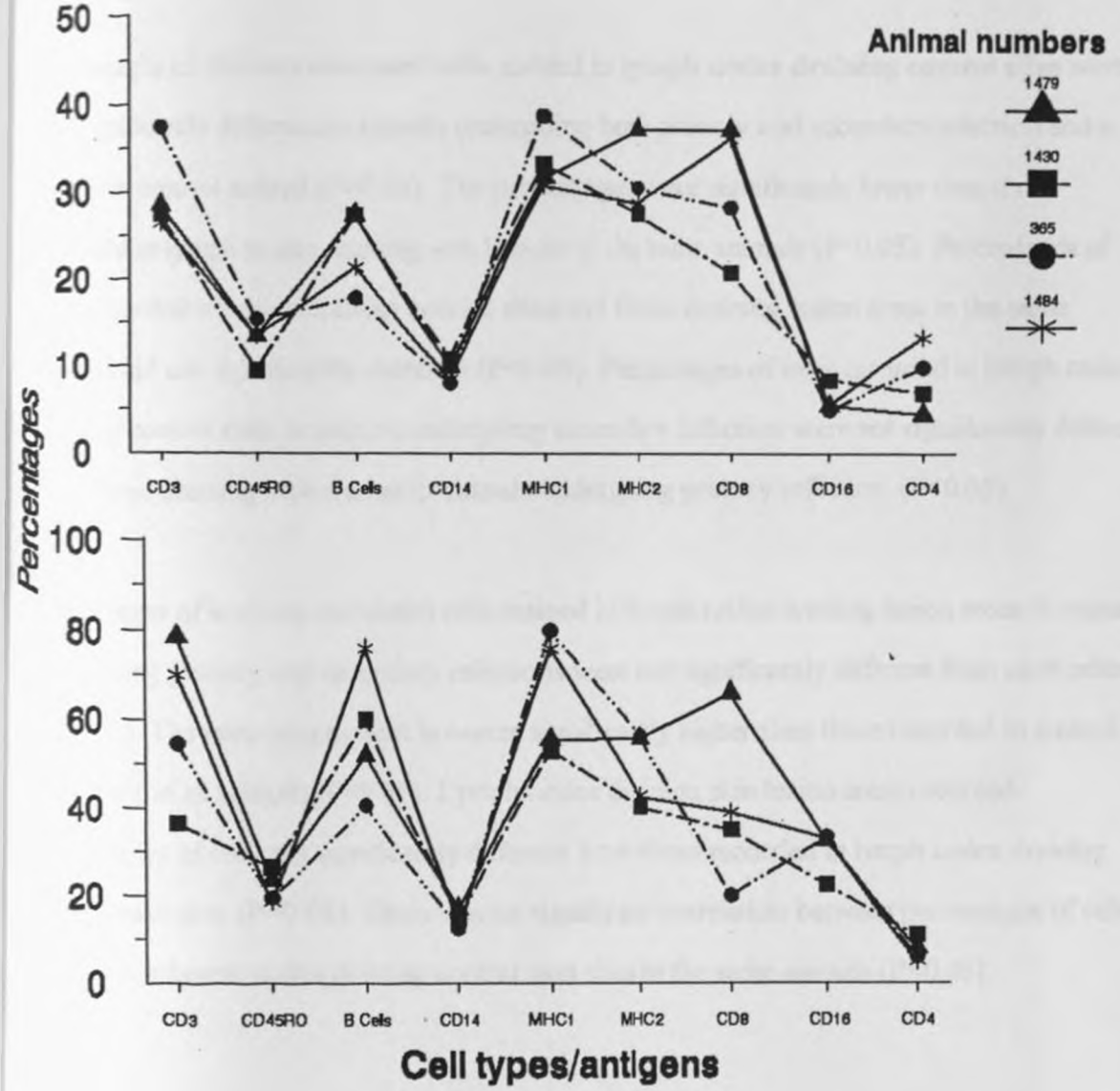
macrophages (14.13%), (P=0.1241). Macrophages stained in similar percentages (14.13%) to CD4 positive T (7.5%) and memory T cells (22%), (0.1241≤P≤0.1880). Pan T cells were stained in similar percentages to (59.88%) to B cells (57.13%), (P=0.589), MHC class 1 (66.12%) and 2 (48.82%) positive cells (0.076≤P≤0.478).





Top, **Figure 7.** Percentages of positive cells in sections from lymph nodes draining control sites in vervet monkeys undergoing primary *L. major* infection.

Bottom, **Figure 8.** Percentages of positive cells in sections from lymph nodes draining lesion sites in vervet monkeys undergoing primary *L. major* infection.



Top, **Figure 9.** Percentages of positive cells in sections from lymph nodes draining control sites in vervet undergoing secondary *L. major* infection.

Bottom, **Figure 10.** Percentages of positive cells in sections from lymph nodes draining lesion sites in vervet monkeys undergoing secondary *L. major* infection.

3.9.7. Percentages of immune associated cells in lymph nodes compared to other sites

Percentages of immune associated cells stained in **lymph nodes draining control sites** were not significantly different in animals undergoing both primary and secondary infection and in the naive control animal ($P>0.05$). The percentages were significantly lower than those recorded in lymph nodes draining skin lesions in the same animals ($P<0.05$). Percentages of cells recorded in nodes draining control sites and those draining lesion areas in the same animals did not significantly correlate ($P<0.05$). Percentages of cells recorded in lymph nodes draining control sites in animals undergoing secondary infection were not significantly different from those draining lesion areas in animals undergoing primary infection, ($P<0.05$).

Percentages of immune associated cells stained in lymph nodes draining lesion areas in animals undergoing primary and secondary infections were not significantly different from each other ($P>0.05$). The percentages were however significantly higher than those recorded in control skin areas in all animals ($P<0.05$). Lymph nodes draining skin lesion areas recorded percentages of cells not significantly different from those recorded in lymph nodes draining control skin sites ($P>0.05$). There was no significant correlation between percentages of cells recorded in lymph nodes draining control skin sites in the same animals ($P<0.05$).

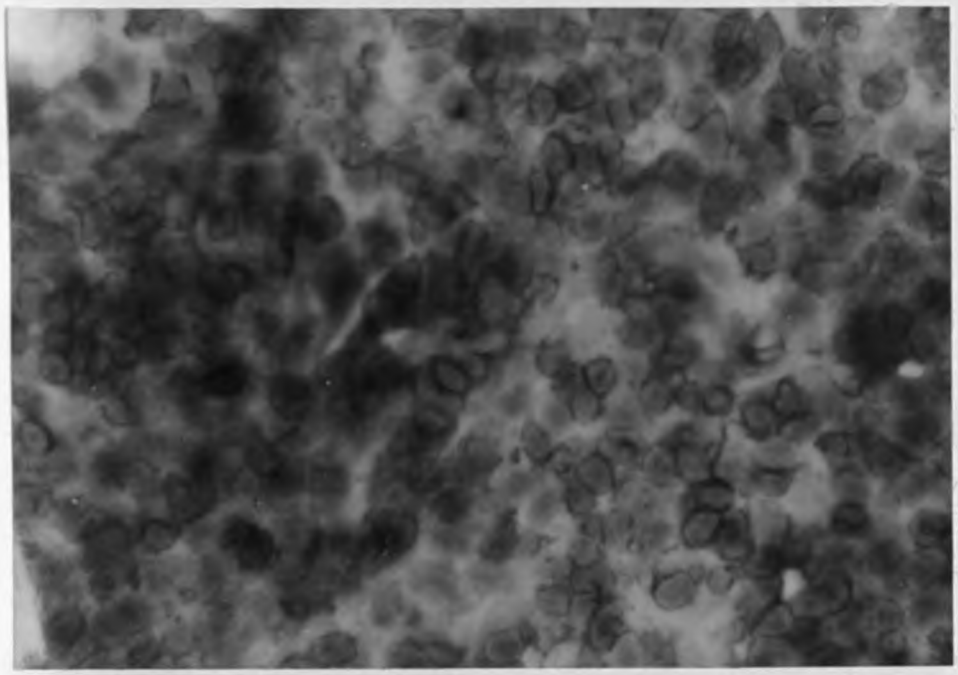


Plate 13. $CD8^+$ cells in *L. major* primary infection lesion.

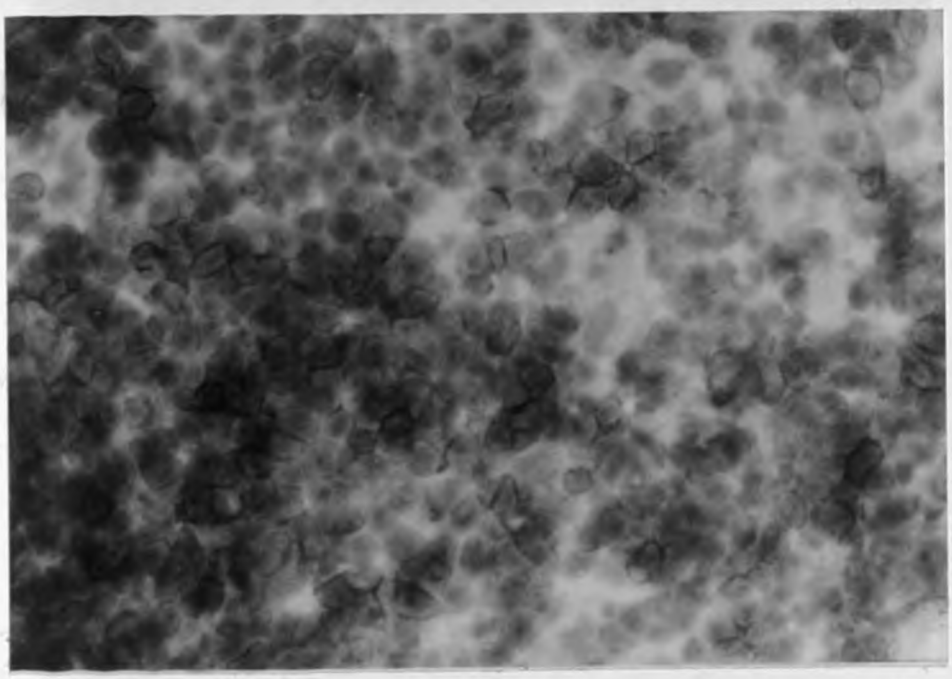


Plate 14. B cells in *L. major* primary infection lesion. Compare the intensity with that of pan T cells (Plate 4).

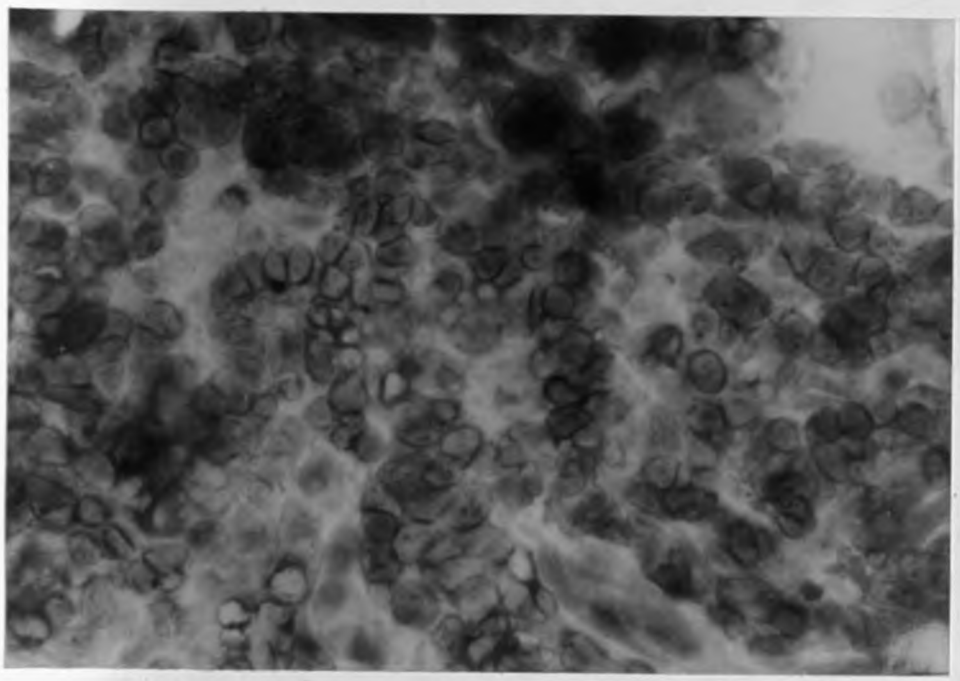


Plate 15. MHC class 2 positive cells in the lymph node draining primary infection lesion.

CHAPTER 4

DISCUSSION

4.0. Discussion.

The primary infection group animals in my experiments developed lesions faster than the secondary infection group animals. These lesions in primary infection group of animals ulcerated and took a longer time to heal. This was probably because these animals had not been exposed to leishmaniasis before and hence were naive immunity-wise. They required a longer time to develop protective immunity but the animals in the secondary infection group already had immunity against *L. major* and hence mounted a secondary response (Olobo *et al.*, 1992). This probably led to the smaller lesions and subsequently taking a shorter time to heal. The immunity to this infection in these animals may be life long as it has been confirmed that they remain DTH positive 48 months after self cure (Olobo and Reid, 1993). The lesions in these animals, just as in humans, were restricted to the site of parasite inoculation. The excessive bleeding observed from the lesion sites during surgery as compared to control sites could be attributed to the reaction of the various components of the skin to the infection. This was probably through disorganisation of the connective tissues of the dermis, hyperplasia of the epidermis and also to blood vessel involvement as was found out in lesions due to cutaneous leishmaniasis in humans (Ridley, 1987). The same sections from the lesion sites were thicker, an effect that could be attributed to strong epidermal hyperplasia, endothelial cell

proliferation and connective tissue degeneration associated with fibrocytosis or fibrosis. These features are prominent in ulcerated lesions of cutaneous leishmaniasis in humans (Mauel and Behin, 1987; Barral *et al.*, 1987), and were confirmed on the sections during cell counting (Plates 5 and 10) in this study.

That anti-human MHC class 2 (L243), γ^{δ} T cell receptor (δTCS1), CD1 (OKT6) and MHC class 1 (W6/32) antibodies cross reacted with the vervet monkey system is encouraging. These antibodies add to the list of the antihuman monoclonal antibodies that cross react with the vervet monkey system (Olobo, 1992). This increases the amount of reagents available for the immunological studies against both cutaneous leishmaniasis and other related diseases in these animals. It should be borne in mind that the unavailability of reagents for use in the primate system was a major contributory factor to the initial slow studies in the vaccine development and testing against leishmaniasis in primates (WHO/TDR, 1993). The anti γ^{δ} antibody cross reacts with the vervet system will enable studies into cells expressing this receptor, given that this type of T cell has been observed to increase during infection with cutaneous leishmaniasis in BALB/c mice and thereby subsequently being suspected to play a yet undefined role in host protection (Rosat *et al.*, 1993). It would therefore be of interest to find out if this also happens in vervet monkeys not to mention humans. This allowed for more studies on these cells that would not otherwise have been possible in humans due to ethical reasons. It would also be interesting to find out the extent of these cells in the normal skin of vervet monkeys in order to compare with what is reported in humans. The idea here is to find the extent to which the immune system of the vervet monkey is similar to that in humans. These studies are on going. The OKT6 antibody will also be of great use as the epidermal Langerhans cells have of late been implicated in antigen presentation of *Leishmania* parasites from the lesion sites in the skin to the draining lymph node (Moll, 1993). That W6/32 reacted with almost all the cells

(Table 5) making it difficult to incorporate it into my studies is not strange as Su-Ling *et al.*, (1993), found a similar situation in cynomolgus monkeys (*Macaca fascicularis*). Failure of the UCHT2 antibody (anti CD5) to cross react with the vervet system could imply that this antigen was not well conserved during evolution. The cross reactivity of L243 antibody enabled us to substitute it for B7:21:2 hence enabling a continuity of the studies on MHC class 2.

There was a heavy cellular infiltration at the lesion site during *L. major* infection in animals undergoing both primary and secondary infection (Plate 4). Most of the cells in the lesions were mononuclear. There was however no evidence of macrophages heavily laden with parasites. This is similar to the picture during similar studies in humans (Ridley, 1987), where it was found that lesions in cutaneous leishmaniasis infection had a granulomatous reaction with most cells being mononuclear and the macrophages not heavily laden with parasites. This is probably an indication of an active immunity as macrophages in diffuse cutaneous leishmaniasis (DCL) lesions were heavily laden with parasites, a condition known to be the outcome of a defective immunity (Mauel and Behin, 1987). In the DCL patients DTH was absent while in my experiments the animals were DTH positive during active infection being an indication of active immunity. From immunoperoxidase staining, the majority of the mononuclear cells were CD3 positive lymphocytes (Plate 4). This was the same picture that emerged in humans (Ridley, 1987). This infiltration of lymphocytes into the lesion site has also been shown to be less prominent in DCL lesions (Mauel and Behin, 1987), another indication of an active immune response.

That there were several caseous necrotic areas free of cells in the lesions during active infection (Plate 5) is similar to Barral *et al.*, (1987), who reported such findings in lesions of human patients suffering from American cutaneous leishmaniasis. This finding therefore suggests common features in some histopathological features between old world and new world cutaneous leishmaniasis. Ridley and Ridley, (1983), reported same findings in human patients. Similar findings have also been observed in BALB/c mice (Zilton *et al.*, 1984), infected with *L. major*. This implies that this feature is a common outcome in lesions from infections with cutaneous leishmaniasis.

There were some lymphocytes resident in the normal skin of vervet monkeys. Normal skins of humans and mice have been confirmed to harbour resident lymphocytes that are suspected to be effector cells in natural and acquired immune reactivity of the skin (Kapsenberg and Bos, 1992). The human dermal tissue has also been found to have perivascular located T cells that are likely to be contacting antigen presenting cells scattered around capillary vessels. Similarly, I recognised the majority of these lymphocytes in normal skin being in clusters in the dermal tissue of vervet monkeys (Plate 6). These lymphocytes were shown to belong to the T cell lineage as they expressed the CD3 and CD8 antigens. It was therefore possible that they performed the same functions as suggested for similar cells in the dermis of normal human skin. Lymphocytes were also seen in the epidermal layer of the skin interspersed amongst keratinocytes. These cells were smooth in morphology and positive for CD3 antigen hence they were T cells. Lymphocytes have also been found to be present in the epidermis of normal mice skin. They were dendritic in morphology and subsequently designated dendritic epidermal T cells (Stingl, 1989). The type present in mice therefore differ with those in the

vervet monkey epidermis, which are smooth in morphology. Epidermal T cells in the mouse were found to be predominantly expressing the γ^{δ} T cell receptor (Stingl, 1989). The predominant receptor of those in the vervet monkey skin is yet to be characterised but γ^{δ} T cells are present (Plate 8). The T cells in the human epidermis were found to be smooth in morphology and predominantly expressing the αß T cell receptor (Foster *et al.*, 1990).

Although the dermal T cells in humans belong to the CD4 phenotype (Foster *et al.*, 1990), I found out that the ones in the vervet monkey predominantly express the CD8 antigen. However results with the anti CD4 antibody (leu 3a) were inconclusive. Olobo, (1990), and Su-ling *et al.*, (1993), showed that there was little or no cross reaction between available anti human CD4 markers and vervet or cynomolgus monkeys respectively and this could explain results obtained in these studies. Specific anti monkey CD4 monoclonal antibodies are required to clearly resolve this problem. The epidermal T cells in humans were as well found to predominantly express the CD8 antigen (Foster *et al.*, 1990), similar to what I found in vervet monkeys. The epidermal T cells in humans were occasionally found to express the γ^{δ} T cell receptor. There was some evidence from my work that some of the cells in the normal skin of vervet monkeys stained positive with this antibody (Plate 8). An important piece of information required here is the proportion of the two receptors amongst the normal skin dwelling T cells so as to find out whether the vervet monkey system has similarities to the mouse or the human one. Humans have twice as much CD4 positive cells in the peripheral blood as there are CD8 positive cells (Foster *et al.*, 1990). The epidermal T cells in normal human skin were found to be HLA-DR negative which meant that they were in the resting

state (Foster *et al.*, 1990). I found the ones in the normal vervet monkey skin not reacting to anti human MHC class 2 antibodies. They could probably have been in the resting state.

The keratinocytes from the epidermis from both control and lesion sites expressed MHC class 1 antigens only. These cells have been shown to be secretors of several cytokines upon non-specific stimulation (Bos and Kapsenberg, 1993). Nilsen and Mshana, (1987), found that keratinocytes in cutaneous leishmaniasis lesions expressed HLA-DR but not DQ and therefore suggested that they were activated. From my studies, keratinocytes did not stain with antihuman MHC class 2 antibodies. This information does not agree with what was reported by earlier workers (Nilshen and Mshana, 1987). It was difficult to conclude whether they were not activated or the vervet monkey keratinocytes express MHC class 2 type of antigens that antihuman antibodies could not cross react with. Information on whether they were activated or not, is important as it would create an interest in finding out which cytokines they secrete during leishmaniasis infection in vervet monkeys and hence their possible role in immunity to this infection.

It was interesting to note that cells around the hair follicles in sections from lesion sites were reacting to the anti CD16 antibody, suggesting that they were Natural Killer cells. These cells secrete several lymphokines including IFN-γ and IL-3 upon stimulation. IL-12 is one of the stimulators of these cells (David and Harn, 1993). They were found to have other functions such as regulatory functions in the adoptive immune system and the haemopoetic system and resistance to infection and tumour growth (Trinchieri, 1992). Treatment with IFN-α and IL-2 increases their cytotoxic activity. Their concentration in the peripheral blood of humans is 5 to

10% but fewer in other organs including the skin (Trinchieri, 1992). They were also found to increase in large numbers in the lesions of humans infected with cutaneous leishmaniasis (Ridel *et al.*, 1988) although their possible role in leishmaniasis is less clear. Information to this effect will enable us understand whether their function is cytotoxicity or lymphokine secretion in this infection. Most of all it would be interesting to note whether they play any protective role in restricting the parasites to the lesion sites. In the normal skin sections of vervet monkeys, the percentage of Natural Killer cells was negligible suggesting their virtual absence from this organ as reported earlier that they are restricted to the peripheral blood (Trinchieri, 1992). In sections from lesion sites they penetrated right into the epidermal layer of the skin. This would mean that these cells have some role in restricting cutaneous leishmaniasis lesions. But more studies need to be done to clarify this.

That majority of the cells in the cellular infiltration in lesions were CD^{3+4-8+} implied that they were T cells belonging to the CD8 phenotype. These are cytotoxic/suppressor T cells. This type of cells were found to increase in lesion sites due to cutaneous leishmaniasis infection (Titus *et al.*, 1987). This has been noticed both in humans and in mice and now in vervet monkeys. Titus and co-workers (1987), suggested that these cells could contribute to the immune response against this infection as elimination of these cells during natural *L. major* infection caused a delay in clearance of the parasites. Muller *et al.*, (1991), (reviewed in Rosat *et al.*, 1993), also supported the participation of these cells in immunity to infection with *L. major*. In several other instances, lesion resolution or development of protective immunity had been attributed to a reduction in the population of these cells at the lesion sites in mice while disease exacerbation was attributed to their presence in higher numbers. In this case they had been suspected to perform a particularly suppressor role (Clark and Howell, 1990). This cell

type has also been found to increase in the peripheral blood of vervet monkeys infected with *L. major* (Olobo, unpublished observations) and vervet monkeys experimentally infected with *L. donovani* (Gicheru, unpublished observations). These cells could therefore play some role in protection against this infection, however more studies are required to elucidate the role of these cells in leishmanial infections.

B cells were stained in low numbers in the normal skin of vervet monkeys. These cells had earlier been reported to be virtually absent in the normal skin of humans (Foster *et al.*, 1990).

There was an increase in MHC class 2 antigen expressing cells in the cortex of lymph nodes draining skin lesion sites as compared to those draining control sites. MHC class 2 antigens are normally expressed on the surfaces of immunocompetent cells. The increase in the expression of these antigens by cells of the nodes draining lesion sites could signify an increase in immune competence by cells of these organs.

Macrophages on the other hand appeared to be in higher numbers in the medullary region in the lymph nodes draining lesion sites (Plate 11). This is an exit point for efferent lymph. The cells in this region are expected to be on their way out of the lymph node. These macrophages did not appear to have parasites in them. This feature was not noticed in lymph nodes draining control sites.

Delayed type hypersensitivity response (DTH) to leishmanial antigens has been widely used as

a measure of exposure to leishmanial infection/antigens in humans and mice (Mauel and Behin, 1982b). This is a characteristic skin test reaction that is caused by T cell mediated macrophage activation and hence can be used as a measure of cell mediated immunity (Sell and Hsu, 1993). DTH has been found positive in vervet monkeys during active cutaneous leishmaniasis infection and to remain so even 48 months after self cure (Olobo *et al.*, 1992; Olobo and Reid, 1993). This indicates that vervet monkeys develop cell mediated immune responses that develop during active cutaneous leishmaniasis infection and remains so several months after self cure, probably for the rest of the life of the animal. These responses were more intensive during active infection. This picture was confirmed in my studies where animals with active infection had significantly higher DTH values than the self cured group (Table 3). This information therefore suggests that vervet monkeys just as humans develop protective immune responses to cutaneous leishmaniasis infection that remain so long after self cure (Modabber, 1989). DTH could therefore be used as a measure of exposure to cutaneous leishmanial antigen in these animals long after they had self cured. From my results it was therefore evident that my experimental animals had developed protective immune responses against the experimental infection and this took the same course and had same characteristics as the natural infection. These animals should be better favoured due to this similarity with the situation in humans. Their use under laboratory conditions, which is not possible with humans due to ethical reasons, should further facilitate vaccine development against leishmaniasis.

From data analysis, it became clearer that the antibodies stained different numbers of cells from each other in all the experimental animals. This implied that there was substantial variation in the number of cells that were expressing each of the antigens the antibodies were

staining for in each animal. What this showed was that each cell type was recorded in a proportion that was different from the other type. No two cell phenotypes were recruited at the lesion site in equal numbers and so were those resident in the normal skin. The other deduction was that the cell types stained for in this work were normally found in different proportions even in lymph nodes draining control sites, not to mention those draining skin lesion sites. It also suggested that during cellular infiltration into the lesion site, no two cell types reached equal levels. It could be inferred from these findings that cells that restricted parasites to the lesion site infiltrated into the lesion in proportions that were different from those they exist in under normal skin condition. This outcome exhibited the fact that the staining obtained here was specific as no variations in cell numbers would have emerged if it was non specific.

MHC class 1 antigen was expressed on a high percentage of cells that were stained.. The characteristic of not staining all cells was useful because it showed the specificity of the staining at the same time allowing for analysis changes in numbers stained under different conditions. This finding also showed that MHC class 1 antigens are conserved in humans, another proof of the close phylogeny between humans and vervet monkeys.

The CD4 antigen was the least expressed. In peripheral blood of humans, this antigen is expressed in the ratio of 2:1 to the CD8 antigen (Ashmore *et al.*, 1989, reviewed in Foster *et al.*, 1990). CD4 positive cells have been proved beyond doubt to be central in the development of protective cell mediated immunity to *L. major* infections (Locksley and Scott, 1991), in humans and mice. The same picture has also been suggested in vervet monkeys (Olobo *et al.*,

1992). The failure of this antigen to be expressed in high numbers in all my experimental groups was therefore interesting. There are two possibilities that could explain this. One revolved around the failure of the antibody to cross react with the monkey system, which has been reported by several workers (Olobo, 1991; Su-Ling *et al.*, 1993), suggesting that the monkey CD4 is probably different from the human one. The other could be that the vervet monkey system has a negligible population of CD4 positive cells. Other ways of characterising the vervet monkey CD4 cells are necessary as this is a key cell type in protective responses to *L. major* infection. The leu 3a antibody (anti CD4), stained very few cells as compared to what the other antibodies recorded. The ratio of CD4:CD8 in non human primates is either 1:1 or less (Mills, 1992), suggesting that there is a possibility that there might be a lower ratio of these two cell types in some non human primates.

The percentages of immune associated cells in control sites were lower than from experimental sites. This difference was more distinct in the skin than in lymph nodes. What this meant was that there was a great influx of immune associated cells into the skin that could be attributed to the infection. The influx was more in the skin because most of these cells were fewer in the normal skin.

The lesion sites in both primary and secondary infection groups recorded similar percentages of the same cell type. This showed that the same types of cells were recruited to the lesions and in similar percentages, hence there was no difference in the cells that restricted the parasites to the lesion sites in both the primary and secondary infection groups. From the design of my work, it was not possible to define whether different cell types were recruited into the lesions at the same rate or not. Normally it would be expected for the recruitment to be more rapid in the secondary infection than primary infection lesions on account of the

secondary response expected in the former group. This outcome therefore suggested that the different animals used in this study mounted the same response against the infection with a similar magnitude of cells restricting parasites to the lesion site.

Naive/normal control animal recorded percentages of cells that were similar to those obtained from the control sites in the experimental animals, but lower than those from infected sites. This meant that the control sites in the experimental animals had similar percentages of cells with the naive animal. This finding supported the fact that cutaneous leishmaniasis in these animals is localised to the site of inoculation. *L. major* infection is also localised in human beings but systemic in some mice models such as BALB/c where it visceralizes (Djoko tammou *et al.*, 1981). The fact that the *L. major* infection had no influence on the outcome at the control sites was further supported by the fact that Spearmans rank correlation analysis did not yield a consistent pattern of correlation between the controls and their corresponding experimental sites. That the naive animal had an outcome lower than infected skin sites and their draining lymph nodes was just similar to the picture that emerged within the experimental animals themselves where their control sites recorded lower percentages of cells than the infected sites.

It was evident from data that NK cells were few in the control sites. The percentages were equal to those of cells expressing anti MHC class 2 antigens in infected sites, implying that it had a high level of infiltration into the lesions. This cell type might therefore be playing some role in restriction of the parasites to the lesion sites. The latest realisation that IL-12 stimulates these cells to secrete IFN-γ which is important in the resolution of cutaneous leishmaniasis

resolution, (Scott, 1993, reviewed in David and Harn, 1993), implicated this cell types in protection.

β_2-Microglobulin is a molecule that is normally associated with the MHC class 1 molecules. From my work, cells expressing β_2-microglobulin antigens, stained cells in a pattern similar to that of cells expressing MHC class 1antigens. Cells expressing these antigens stained higher percentages of cells than the other antibodies. It therefore showed that the β_2-microglobulin molecule and the MHC class 1 antigens in general are conserved in evolution (Mills, 1992), and therefore the cross reactivity between anti human antibodies and vervet monkey system.

Cells expressing the CD8 antigen were stained similar percentages to those expressing CD3 and MHC class 2 antibodies. What this signified was that most of the T cells stained by this T cell marker were actually CD8 positive T cells and most of these expressed MHC class 2 antigens. This observation leaves un answered questions as to the particular role of CD8 positive T cells in leishmaniasis.

Cells expressing the Pan T cell antigens were stained in similar percentages to the MHC class 2 and CD8 antigen bearing cells. This suggests that most of the T cells recorded in the lesion sites were CD8 positive. These antigens were stained by FN18, an anti-rhesus monkey CD3 marker are therefore well conserved amongst the rhesus and vervet monkeys (Mills, 1992). There has however been no clear cross reactivity between FN18 antibody with the human system (Mills, 1992).

Cells expressing MHC class 2 antigens had a staining profile that showed that most of them were actually CD8 positive T cells. From the results, it was clear that cells expressing this antigen increased dramatically into the lesion site and similar but less dramatic changes took place in the draining lymph node. In the lymph nodes there was an equally substantial increase in cells expressing these molecules in nodes draining skin lesion sites than those draining control sites in the skin.

Cells expressing MHC class 1 antigens were stained using B9:12:1 antibody. This is the only antibody that stained keratinocytes in the epidermis in the skin. Having stained higher than the other antibodies wherever used, just illustrated that MHC class 1 antigens were the most expressed. The fact that more cells expressing this antigens were stained in the lesion than control sites could be related to the fact that were recruited into the lesion sites. Studies in rhesus macaques (Mills, 1992) could not find the analogous of HLA-C in these primates hence there might be great variation in the distribution of these antigens in primates with particular types probably expressing antigens that B9:12:1 could not recognise.

UCHM1 is an antibody that stains the CD14 antigen that is expressed on macrophages. This is an important cell in leishmanial infection as it is the one that hosts the parasite while at the same time it is expected to defend the body against the same parasites. Staining with this antibody showed that there was an increase in macrophages at the lesion site. There was no evidence of these cells being laden with parasites. This cell type is one of the antigen presenting cell in conjunction with MHC class 2 molecules. It is believed that activated T helper cells secrete IFN-γ that activates the macrophages leading to parasite destruction (Scott and Locksley, 1991). Latest evidence suggests that *L. major* interferes with MHC class 2 molecules loading in infected macrophages leading to failure in proper antigen presentation to

T helper cells in mice (Fruth *et al.*, 1993). It would be interesting to find out if the macrophages in the lesions were activated or not.

The UCHL1 antibody stains the CD45RO antigen that is mainly expressed on the memory T cells. This antibody did not stain intensely in both the control and infected sites. This could imply that majority of the T cells recorded in the skin and in the lesion not to mention their draining lymph nodes were not of the memory phenotype. This might also mean that the infiltration of the T cells into the lesion usually leaves behind a small proportion of memory cells.

During immunofluorescence staining, there was evidence for CD1 positive cells in the tissues (Plate 3), but as stated earlier analysis was difficult due to the difficulty in defining negative cells. Staining of peripheral blood lymphocytes yielded negative results also. This could be explained as Langerhans cells are tissue dwelling and not in peripheral blood. Studies are on going to use different staining methods with anti CD1 antibodies in order to be able to determine the true extent of these cells at the lesion sites and their draining lymph nodes. The latest information (Moll, 1993), is that this cell could be playing an important role in antigenic presentation by probably carrying parasites to the draining lymph nodes and presenting them to immunocompetent cells for initiation of an immune response. It would be of interest to determine in the vervet monkey system if these cells have any such role during this infection.

4.1. Conclusions

Vervet monkeys (*Cercopithecus aethiops*) are old world monkeys susceptible to experimental infection with *L. major*, a protozoan parasite which causes cutaneous leishmaniasis. The infection in these animals was localized to the site of parasite inoculation and produced lesions which self cured.

Vervet monkeys with cutaneous leishmaniasis due to *L. major* developed cell mediated immune responses during active infection and these as was shown by positive DTH responses.

The normal skin of vervet monkeys had resident lymphocytes that were primarily T cells belonging to the CD8 phenotype. Majority of them were in the dermal layer. Resident epidermal $CD8^{+}$T cells were also found and these, had smooth morphology. B cells were virtually absent from the normal skin. A small population of macrophages was observed in the normal skin. Cells expressing CD4 and CD16 phenotypes were rare in all the layers of the normal skin.

In the cutaneous leishmaniasis lesion due to *L. major*, there was a heavy cellular infiltration consisting primarily of mononuclear cells in both primary and secondary infection groups. Most of these expressed both MHC class 1 and 2 and the CD8 antigens. Other cellular phenotypes recruited included CD16, macrophagwes and to a certain extent B cells. Macrophages recruited into the lesion did not appear to be heavily laden with parasites by day 42 of the infection. No proper conclusion could be derived from the staining of the antibody against CD4 antigen expressing cells.

In the lymph nodes, there was an infiltration of mononuclear cells that primarily expressed MHC class 1 and 2 and CD8 antigens. Cells expressing CD16 and CD4 antigens and B cells

were recruited in low percentages. Staining patterns in the naive control animal were similar to those observed in control sites.

However, there were no noticeable differences between cells that restricted parasites at the lesion sites in both primary secondary infections due to *L. major in* vervet monkeys , suggesting to similar cell types being involved.

4.2. Recommendations

My studies were far from exhausting *in situ* characterisation of immune responses in vervet monkeys experimentally infected with *L. major*. I couldn't as well say that the pathology of this infection was exhaustively examined in my work. The studies in skin immune system also left a few questions un answered. I did the best I could in the available time and resources. However, the suggestions given below might give more insight into these studies.

1. It would be important to characterise *in situ* IFN-γ and IL-2 producing cells in the lesions.

2. Experiments need to be done to clarify the ratio of the receptor expression between γδ and αβ by T cells that infiltrate into the lesion and also those resident in the normal skin.

3. Studies are therefore required in order to establish the extent of involvement of Langerhans cells in leishmaniasis in primates. The studies could be better if they took the course of *in situ* characterisation of any changes in Langerhans cells in the lesions and their draining lymph nodes and if these cells are laden with *Leishmania* parasites. It would also be interesting to examine the pattern of cytokines that Langerhans cells secrete which could be attributed to the infection.

4. It is necessary to establish the extent of the CD4 cells involvement in leishmaniasis in vervet monkeys using both *in situ* and biochemical techniques. *In situ* techniques may involve using anti monkey CD4 antibodies which are yet to be made available commercially. This is so because results with the antihuman CD4 in vervet monkeys are questionable. Other techniques which could probably involve digestion of connective tissue in the lesion to release free cells for characterisation might be useful as these are believed to be the key cells in protective responses in cutaneous leishmaniasis.

5. A simple study to determine the ratio of γ^{δ} and αß T cells in the normal skins of both the humans and vervet monkey would add a little information as to the similarities in the immune systems of the 2 hosts.

6. Future workers should check whether keratinocytes above the lesions in vervet monkeys are activated or not. If activated, then what could be their precise role in leishmaniasis?. Is it cytokine production? and if so, which ones are these and what are their possible roles in immunity against leishmaniasis.

7. More work is required to further define the role of CD8 positive cells in leishmaniasis. Do CD8 positive cells realised in lesions perform suppressor, or cytotoxic or lymphokine secretion that directs the immune system in a particular direction?. These factors can be investigated.

8. Future histological analysis of lesions in vervet monkeys could expand to include neutrophils, eosinophils and mast cells which have been mentioned by some workers.

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APPENDIX 1

BUFFERS AND REAGENTS

AZIDE/H_2O_2 SOLUTION FOR BLOCKING ENDOGENOUS PEROXIDASE.

STOCK SOLUTION.

H_2O_2..... 30%

AZIDE..... 1%

PBS..... 10x

To 40 ml of double distilled water(DDW) add 5mls. of PBS, 5mls. of azide and 500µl of H_2O_2. The final dilution will be 0.1% azide, 0.3% H_2O_2. Always prepare fresh from stock solutions.

ACETATE BUFFER 0.05M pH 5.1.

Stock solution A:

CH_3COOH (Acetic acid glacial)

To 500Ml of DDW add 7.5ml. of acetic acid (0.25M)

Store at 4°C

Stock solution B

CH_3COONa (sodium acetate)

Dissolve 17g in 500ml of DDW to give 0.25M solution. Mix 24ml of **A** with 76ml of **B** and dilute 5 times to give 0.05M solution (100ml of mixture diluted with 400ml of DDW).

The pH should adjust automatically to 5.1

Warm this solution to 30°C before use.)

MODIFIED PBS WITH THIOMERSAL (pH 7.2-7.4)

$NaH_2PO_4.1H_2O$	0.01M	0.69g.
K_2HPO_4	0.04M	3.48g.
NaCl	0.12M	3.6g.
Thiomersal	$4.9x10^{-5}M$	9.9mg.

Add 500 ml of DDW.

PBS alone excludes thiomersal.

3-amino-9-ethylcarbazole (AEC).

Freshly prepared, less than 2 minutes before staining and kept at 28°C.

10 mg dissolved in 2.5mls of dimethylformamide add to 50ml acetate buffer. This should be filtered on paper. Add 100µl of 30% H_2O_2, mix and put on the slides.

NaCl GLYCEROL MOUNTANT (pH 9.5).

Glycerol	45ml.
NaCl 0.9%	5 ml.

Tris base 500mg. Mix tris base and NaCl before adding glycerol.

ALSEVER'S SOLUTION

Dextrose	10.25g
NaCL	2.1g
Tri sodium citrate	4.0g
DDH_2O	500ml

Sterilize by filtering through 0.2µm pore size filter.

Store at 4°C

REAGENTS FOR DETERMINATION OF PROTEIN CONCENTRATION

SOLUTION A:

100gm Na_2CO_3 dissolved in 500 ml 1N NaOH and diluted with distilled water to 1 litre. Can be stored at 4°C for at least 6 months.

SOLUTION B:

2g of Potassium sodium tartrate and 1gm $CuSO_4 .5H_2O$ dissolved in 90ml of distilled water and 10ml 1N NaOH is added. Can be stored at 4°C for at least 6 months.

SOLUTION C:

1 volume Folin-ciocalteu reagent diluted with 15 by volume of water. This is always prepared before use and stored at 4°C in the dark. It should not be left standing for too long as it may go cloudy.

APPENDIX 2

OBSERVATION SHEET FOR VERVET MONKEY *L. major* DISEASE DEVELOPMENT.

SECONDARY INFECTION GROUP.

INOCULATION DATE: 20-5-93

INOCULATED STRAIN: *L.major* NLB144.

DOSE OF INOCULUM: 5×10^7

Vervet #	Nodule/Lesion/Ulcer size		General observation	
	Control	Experimental	Control	Expermental
365				
1430				
1484				
1479				

DAYS POST INFECTION:_____

DATE:_____ DAY:_____ TIME:_____

INVESTIGATOR:_____

PRIMARY INFECTION GROUP.

INOCULATION DATE: 30-6-93

INOCULATED PARASITE STRAIN: *L.major* NLB 144

DOSE OF INOCULUM: 5×10^7

Vervet #	Nodule/Lesion/Ulcer size		General observation	
	Control	Experimental	Control	Experimental
1547				
1562				
1566				
1568				

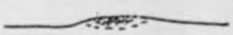
DAYS POST INFECTION:_____

DATE:_____ DAY:_____ TIME:_____

INVESTIGATOR:_____

APPENDIX 3

IPR-KEMRI STANDARDISED STAGES OF ULCERATION IN VERVET MONKEYS FOLLOWING CHALLENGE WITH *LEISHMANIA MAJOR*.

1. Erythmatous nodule: (Induraton) 	Usually tiny reddish swelling(s) and proceeds for a few days soon after challenge (sometimes upto 2 weeks). Rarely measurable.
2. Nodule: 	Appreciable raised skin. Measurable.
3. Open nodule: 	A nodule with an ulcer. Measurements must include: [a] Measurement for ulcer [b] Measurement for nodule [c] combined measurement for ulcer and nodule.
4. Ulcer: 	Progression of an open nodule to an open sore. Prescence of nodule not noticeable.
5. Healing ulcer: 	Ulcer with scab or dried crust forming over an open wound.
6. Healing scar: 	Prescence of avascular fibrous tissue as the end result of healing. Abscence of ulcer or scub. If palpable nodule is present, it should be measured.
7. Healed scar:	Pigmentation of skin with no palpable nodule.

ILL. A.K.K.

Current worldwide distribution of leishmaniases