MECHANISMS OF *LEISHMANIA* UPTAKE AND TRANSMISSION BY PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE)

By

CHRISTOPHER OMUKHANGO ANJILI

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Nairobi

1995

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

Christopher Omukhango Anjili — 9th Nov 1994

Christopher Omukhango Anjili — Date

This thesis has been submitted for examination with our approval as University supervisors:-

(1) _____ 20th Nov. 1996

Prof. Richard W. Mwangi — Date

University of Nairobi

(2) _____ 9 November, 1994

Prof. Davy K. Koech — Date

Kenya Medical Research Institute

DEDICATION

This thesis is dedicated to Dolly, Kate and Bob Anjili

TABLE OF CONTENTS

CHAPTER 2

THE INFLUENCE OF *PHLEBOTOMUS DUBOSCQI* SALIVA ON THE ESTABLISHMENT OF *LEISHMANIA MAJOR* INFECTION

CHAPTER 3

EFFECTS OF DIFFERENT NUMBERS OF MACROPHAGES ON *L. MAJOR* INFECTION AND THE EFFECT OF *P. DUBOSCQI* SALIVA ON HOST COMPLEMENT

CHAPTER 4

EFFECTS OF SAND FLY SALIVA ON THE COURSE OF *LEISHMANIA DONOVANI* INFECTION IN THE SYRIAN GOLDEN HAMSTER

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF PLATES

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BALB/c	*Leishmania major* susceptible inbred mice
BME	Basal Medium Eagle
C3	Third complement component
C3b	Complement component type 3 opsonin
C3bi	Cleaved complement component type 3
C5-C9	Complement membrane attack complex
C57BL/6	*Leishmania* resistant inbred mice
C5a	Complement component type 5a
CCF	Crystal-induced chemotactic factor
CD	Chemotactic differential
CGRP	Calcitonin gene-related peptide
CI	Chemotactic index
CR1(=C3bR)	Complement component type 1 receptor
CR3(=C3biR)	Type 3 complement receptor
DNA	Deoxyribonucleic acid
DPI	Days post-inoculation
DPX	Histological tissue mountant
EC	Enzyme commission marker
EIF	Erythema-inducing factor
gp63	*Leishmania* surface glycoprotease

H&E	Haematoxylin and eosin
HBSS	Hanks balanced salts solution
IFN-γ	Interferon gamma
IL-1	Interleukin 1
IL-2	Interleukin 2
LEF	*Leishmania*-enhancing factor
LPG	Lipophosphoglycan
MEM	Minimum essential medium
NNN	Novy Nicolle McNeal medium
PBS	Phosphate-buffered saline
PKDL	Post kala-azar dermal leishmaniasis
PNA	Peanut agglutinin
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 medium
SGL	Salivary gland lysate
SRBC	Sheep red blood cells
TC-199	Tissue culture medium 199
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
UHU	Alcohol soluble adhesive
VBS	Veronal buffer saline
WBC	White blood cells
WHO	World Health Organization

ACKNOWLEDGEMENTS

I am very grateful to my supervisors Prof. Richard W. Mwangi of Zoology Department, University of Nairobi and Prof. Davy K. Koech, Director, Kenya Medical Research Institute for their guidance, support and constructive criticisms throughout the whole study.

I am indebted to Dr. J.I. Githure, Director, Biomedical Sciences Research Centre, KEMRI who played a big role in training me in field and laboratory based research in leishmaniasis. I would like to thank Mr. P. Mbati and Dr. A. M. Shatry of KEMRI for their suggestions, and Dr. L. Robert of Walter Reed Project for his assistance in providing some of the research material. I would also like to thank Ms N. Rono, T. Ouka, P. Achayo, L. Wangari, A. Ng'ang'a as well as Messrs A. Koske, C. Mwita, S. Miriti, J.M. Kabanga, C. Magiri, K. Mugo, R. Otuoma, R. Lugalia, D. Wahinya, A.K. Sang, D. Siele, P. M. Mwanyumba, S. Odongo, H. Sang, L. Ogutu, S.J. Makasa and J.M. Sagwa all of KEMRI for their technical assistance, and Dr. K. Gathumbi of Pathology Department, Kabete Campus, University of Nairobi.

Last but not least, I would like to to thank Dr. F. Modabber of the World Health Organization, Geneva for his constructive ideas and encouragement. This study was funded by UNDP/WORLD/WHO Special Programme for Research and Training in Tropical Diseases (TDR), and the Kenya Medical Research Institute.

ABSTRACT

Leishmania parasites undergo cyclical development in their vector sand fly through various stages before they can be transmitted by bite. Sand fly saliva aids in blood-feeding by modulating host-immune responses through processes such as prevention of clotting. Sand fly saliva also promotes the invasion and intracellular survival of *Leishmania* in potentially lethal macrophages by suppressing macrophage activation and antigen presenting functions. Sand fly saliva-mediated immunomodulation, together with suppression of the expression of cell surface antigens and phagocytic functions of macrophages ensure successful parasitization of a susceptible host by the *Leishmania* parasite.

To further investigate the role of sand fly saliva in the establishment and uptake of *Leishmania* parasites, various experiments were designed to test: (i) the effect of co-inoculation of *Phlebotomus duboscqi* salivary gland lysates with *Leishmania major* promastigotes on lesion development in BALB/c mice, (ii) to determine the sequence of inflammatory reactions of hamster skins after needle inoculation with *P. duboscqi* salivary gland lysates and after bites of uninfected and *L. major*-infected *P. duboscqi*, (iii) to determine whether *P. duboscqi* saliva is chemotactic to macrophages and its effect on host complement, and lastly, (iv) to determine whether sand fly saliva influences the course of viscerotropic *L. donovani*. Results

obtained from this study showed that saliva of *P. duboscqi* enhances the infectivity of *L. major*. Intradermal inoculation of sand fly saliva either by needle or bites of uninfected or *L. major*-infected *P. duboscqi*, generated an inflammatory reaction that was initially characterised by vascular congestion, neutrophilia and mixed cell infiltrate, and in 48-96 hours the macrophage became the predominant cell type, suggesting that saliva is chemotactic to macrophages *in vivo*.

In vitro, sand fly saliva was shown to be chemotactic to mouse peritoneal macrophages, a factor that may ensure successful parasitization of a host, and partially explain how post kala-azar dermal leishmaniasis develops, and also how *Leishmania* amastigotes are taken up by the sand fly. The sequence of inflammatory reactions was studied using standard histological tissue sectioning and staining with toluidine blue, haematoxylin and eosin, and light microscopy. It was demonstrated that *P. duboscqi* saliva does not prevent lysis of sheep erythrocytes by guinea pig complement. This may also contribute to successful parasitization of a host. Despite the fact that *P. duboscqi* saliva was shown to enhance infectivity of *L. major*, it was demonstrated that it does not influence the course and subsequent uptake of viscerotropic *L. donovani*. This study has demonstrated that like saliva of *Lu. longipalpis* and *P. papatasi*, saliva of *P. duboscqi* contains maxadilan, the *Leishmania* enhancing factor that exacerbates infectivity of *L. major*.

obtained from this study showed that saliva of *P. duboscqi* enhances the infectivity of *L. major*. Intradermal inoculation of sand fly saliva either by needle or bites of uninfected or *L. major*-infected *P. duboscqi*, generated an inflammatory reaction that was initially characterised by vascular congestion, neutrophilia and mixed cell infiltrate, and in 48-96 hours the macrophage became the predominant cell type, suggesting that saliva is chemotactic to macrophages *in vivo*.

In vitro, sand fly saliva was shown to be chemotactic to mouse peritoneal macrophages, a factor that may ensure successful parasitization of a host, and partially explain how post kala-azar dermal leishmaniasis develops, and also how *Leishmania* amastigotes are taken up by the sand fly. The sequence of inflammatory reactions was studied using standard histological tissue sectioning and staining with toluidine blue, haematoxylin and eosin, and light microscopy. It was demonstrated that *P. duboscqi* saliva does not prevent lysis of sheep erythrocytes by guinea pig complement. This may also contribute to successful parasitization of a host. Despite the fact that *P. duboscqi* saliva was shown to enhance infectivity of *L. major*, it was demonstrated that it does not influence the course and subsequent uptake of viscerotropic *L. donovani*. This study has demonstrated that like saliva of *Lu. longipalpis* and *P. papatasi*, saliva of *P. duboscqi* contains maxadilan, the *Leishmania* enhancing factor that exacerbates infectivity of *L. major*.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

The prime economic importance of phlebotomine sand flies (Diptera: Psychodidae) rests on the fact that they are vectors for several forms of leishmaniases. Apart from their vector status, bites of sand flies, particularly *Phlebotomus papatasi* Scopoli also cause a severe skin condition which has been called *Harara* in Israel, Palestine and the surrounding countries. First described by Dostrovsky (1953) in 1925, and later by Theodor (1935), this skin condition unlike cutaneous leishmaniasis caused by *L. major* has become uncommon since the control of mosquitoes with insecticides in areas where they are sympatric with phlebotomine sand flies was started (Alexander, 1984).

Harara, which is actually a form of delayed type hypersensitivity reaction towards sand fly saliva (Dostrovsky, 1953; Theodor, 1935; Stebbings, 1974) is still seen in places where the population of sand flies is large. It is seen in newcomers to the area, neonates and nonimmunized persons sensitized with sand fly saliva, and only exposed areas of the skin are involved (Theodor, 1935; Alexander, 1984). According to Theodor's description, following the bite, itching begins a few days after arriving in the sand fly-infested area and is sufficient to cause insomnia. Painful impetiginised crusted lesions develop from scratching and

sometimes eruptions resembling papular urticaria and clear or haemorrhagic vesicles develop. The areas chiefly affected are the dorsa of the hands, volar aspects of the wrist, dorsal surface of the fore arms and upper arms, feet and the face (Alexander, 1984). These areas that are naturally exposed to bites are the same areas where lesions caused by cutaneous leishmaniasis parasites are usually seen (Giannini, 1986). Both visceral and cutaneous leishmaniases parasites initially develop at the site of the bite of the infected vector sand fly . In the case of *L. donovani*, only a small ulcer may develop at the site of the bite before either the infection progresses and the parasite visceralizes or the infection aborts (Manson-Bahr and Southgate, 1964). *L. major* on the other hand, usually remains confined to a single, yet pronounced, cutaneous lesion at the site of the bite.

The fact that different species of *Leishmania* parasites cause the cutaneous, diffuse cutaneous, and visceral forms of human leishmaniases supports the hypothesis that innate physiological properties of the parasite may delimit disease (Hommel, 1978; Hill, 1988). Following inoculation by the sand fly, *Leishmania* parasites must enter a macrophage to establish a successful infection. It is now known that this successful parasitization of macrophages is aided by chemical components contained in sand fly saliva (Titus and Ribeiro, 1988, 1990: Theodos *et al.*, 1991). Effective control of phlebotomine sand flies has proven to be difficult

because of the inaccessibility of their breeding and resting sites such as termite hills, rock crevices and caves. Control of fatal visceral leishmaniasis has also proven to be as difficult as sand fly control. In the absence of a vaccine and effective vector control measures, chemotherapy is the most commonly used method to control cutaneous and visceral leishmaniases, but chemotherapeutic agents in use are expensive and are known to exert severe toxic reactions in treated individuals (Haidaris and Bonventure, 1983); furthermore, they often do not effect complete cure.

Since it is now known that sand fly saliva determines whether or not the low numbers of *Leishmania* parasites inoculated into a susceptible host by bite of the sand fly will cause a disease, it has been suggested that it is possible to prevent leishmaniases in humans by vaccinating against the *Leishmania* enhancing factor in vector saliva (Theodos *et al.*, 1991; Lerner and Shoemaker, 1992).

This study was therefore an attempt to determine the role played by saliva of *P. (P.) duboscqi* on the establishment of *L. major* and *L. donovani* infections in inbred BALB/c mice and in golden hamsters respectively. An attempt to determine whether saliva of *P. duboscqi* exacerbates infection with *L. major* when the two are co-inoculated was made, as well as a study on to determine the sequence of cellular inflammatory reactions generated by the host at the

site of inoculation of sand fly saliva by uninfected sand flies, by *L. major*-infected sand flies, and by needle inoculation.

Another objective was to determine whether *P. duboscqi* saliva is chemotactic *in vitro* to vertebrate host macrophages, and whether this saliva can inhibit vertebrate host complement-mediated lytic activity and lastly to determine whether sensitization against sand fly saliva influences the course of *L. donovani* and its subsequent uptake by sand flies. Understanding mechanisms by which sand fly saliva aids *Leishmania* parasites to establish an infection is important because this information can be incorporated in leishmaniases transmission-blocking control strategies through immunization against the bioactive substances in sand fly saliva, and also in immunization against the parasite.

1.2. Literature Review

1.2.1. The Genus *Leishmania*

The leishmaniases comprise several diseases caused by various species of unicellular protozoan parasites of the genus *Leishmania* Ross, about ten of which infect humans (WHO, 1987). Prevalence and incidence data are not known precisely and are very difficult to determine. The leishmaniases often occur in remote areas and their epidemiological patterns can change dramatically with population movements associated with natural disasters and social upheavals (WHO, 1987). The worldwide prevalence of leishmaniases is estimated at about 12 million cases (Marinkelle, 1980; WHO, 1987), with an annual incidence of 1.5-2 million cases based upon extrapolations from data available (Walton, 1987). Leishmaniases can be grouped into three broad clinical forms: visceral leishmaniasis or kala-azar, mucocutaneous leishmaniasis or espundia, and cutaneous leishmaniasis including the diffuse forms (WHO, 1984).

Visceral leishmaniasis is caused by *Leishmania donovani* Laveran and Mesnil, particularly *L. donovani archibaldi* Castellani and Chambers in the Aethiopian Zoogeographical Region (Sub-Saharan Africa), *L. donovani*

in the Palaearctic and Neotropical Regions, and *L. donovani* in the Oriental Region, mainly in India and may be endemic, sporadic or epidemic (WHO, 1984). Although the clinical features tend to differ between the three situations, visceral leishmaniasis is generally characterised by hepatosplenomegaly, weight loss, skin darkening, anaemia, irregular fever and lymphadenopathy, and is usually fatal when untreated. Mucocutaneous leishmaniasis is caused by *L. braziliensis* Vianna, *L. panamensis* Lainson and Shaw and *L. guyanensis* Floch in the New World. In the Old World, this disease is caused by *L. donovani sensu lato* in adult males in the Sudan. This disease initially produces lesions that resemble those of cutaneous leishmaniasis, but later, may metastasize in the oronasal and pharyngeal mucosae, causing disfiguring leprosy-like tissue destruction and skin granulomas (WHO, 1987). The third clinical type, cutaneous leishmaniasis, is caused by *L. major* Yarkimoff and Schokhor, *L. tropica* Wright, *L. aethiopica* Bray, Ashford and Bray and sometimes *L. infantum sensu lato* (Gramiccia *et al.*, 1987). In the New World, the disease is caused by *L. braziliensis* Vianna, *L. panamensis, L. guyanensis, L. peruviana* Velez, *L. mexicana* Biagi, *L. amazonensis* Lainson and Shaw and *L. venezuelensis* Bofante-Garrido (WHO, 1984).

New World cutaneous leishmaniasis tends to be more severe and chronic than Old World cutaneous leishmaniasis. Cutaneous leishmaniasis typically produces a skin ulcer

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

Leishmania are parasitic protozoa of the Order Kinetoplastida, Subfamily Trypanosomatidae. These parasites have two main forms in their life cycle: motile uniflagellate promastigotes found in culture and in guts of infected sand fly vectors, and non-motile amastigotes that infect macrophages of their mammalian hosts. Promastigote forms of various species of *Leishmania* are morphologically indistinguishable. This morphological similarity has for many years hampered progress in several aspects of research in leishmaniasis.

Initially, the taxonomy of *Leishmania* was based on geographical distribution, epidemiology and on the clinical picture. In recent years, it has become clear that the genus *Leishmania* is a complex and nomenclature based on geographical distribution, on host, or even on clinico-

pathological changes is of limited value (Schnur *et al.*, 1981). Lumsden (1974) summarised the features by which morphologically similar organisms may be differentiated into intrinsic and extrinsic characters.

Currently, methods used in the identification of *Leishmania* species are based mainly but not exclusively on intrinsic characters (WHO, 1984), that can be identified by biochemical and immunological techniques. In order to standardize identification procedures, WHO (1984) recognized several methods of identification that combine both intrinsic and extrinsic characters. These methods are (1) biological characters (development in sand flies and virulence of clones in rodents; (2) immunological characters (Noguchi-Adler test, excreted factor serotyping, monoclonal antibodies and *in vitro* cross-immunity tests); (3) biochemistry (DNA sequence analysis, DNA (nuclear) buoyant density, DNA-RNA hybridization, restriction endonuclease fragment analysis, isoenzyme characterization, cell membrane structure, fatty acid analysis and radiorespirometry. Isoenzyme characterization is the most commonly used at the moment to identify strains at specific or infraspecific levels.

1.2.2. Vectors of the leishmaniases

The leishmaniases are transmitted by the bite of infected female sand flies (WHO, 1984). Within the subfamily Phlebotominae of the Order Diptera, about 600 species and subspecies of sand flies are known, of which about 70 are proven vectors of leishmaniases (Minter, 1984; Young and Lawyer, 1987). In the Old World, vectors of leishmaniases belong to the genus *Phlebotomus* Rondani, and in the New World to genus *Lutzomyia* Franca. The maintenance of all the known *Leishmania* species depends on an efficient and constant contact between the vertebrate and the sand fly hosts. Although phlebotomine sand flies are considered to be the principal vectors of both Old and New World *Leishmania* species, mechanical transmission of these parasites is also suspected to be a factor in the epidemiology of leishmaniasis (Garnham, 1965; Lightner and Roberts, 1984). Experimental transmission of cutaneous leishmaniasis by contaminated mouthparts of *Stomoxys calcitrans* (L.)(Diptera: Tabanidae) has been demonstrated (Berberian, 1938). Similarly, possible transmission by non-biting insects has been demonstrated (Thomson and Lamborn, 1934). It has also been possible to detect live *L. donovani*, *L. infantum* and *L. tropica* promastigotes in droplets of the excreta of *Musca spectanda* Wied (Diptera: Muscidae) several hours after they had fed (Thomson and Lamborn, 1934).

A study conducted by Lainson and Southgate (1965) using *S. calcitrans*, achieved mechanical transmission of *L. mexicana* between hamsters. Lightner and Roberts (1984) demonstrated that the tsetse fly, *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae) through interrupted feeding is able to transmit *L. major* between mice and hamsters. These authors argued that since *L. major* occurs in foci scattered throughout the Aethiopian zoogeographical region of Africa, many of which are within the ranges of one or more species of tsetse, under favourable conditions, *Glossina* spp. could transmit *Leishmania* mechanically. Despite these observations, Lainson (1982) has argued that it is doubtful if any mode of spread other than the bite of an infected sand fly plays any great role in nature.

Recent studies have indicated that there is a natural limitation of certain species of *Leishmania* to certain sand flies even when all are sympatric. This is particularly so in the Old World. Limitation of spread of a particular species of *Leishmania* to man or other animals will clearly depend on the host preference shown by the vector (Lainson, 1982), and the suceptibility of a particular sand fly vector to infection with a specific strain of *Leishmania*. The taxonomic relationships of vectors of Old World *Leishmania* suggest that co-evolution of parasite and sand fly is a common phenomenon resulting in a co-adaptation which

restricts the range of sand flies in which a parasite can readily develop (Killick-Kendrick, 1985).

As with mosquitoes in malaria or filariasis, it is thought that the susceptibility of a sand fly to a particular *Leishmania* species may be genetically controlled (Killick-Kendrick, 1985). This notion has been further strengthened by the recognition of mating barriers between *Lutzomyia longipalpis* Lutz and Neiva species (Ward *et al.*, 1983). In the Old World, the apparent restriction of transmission of species of *Leishmania* to closely related sand flies strongly supports the genetic control of susceptibility. Examples of this restriction are seen in the transmission of *L. major*, which is only transmitted by sand flies of the subgenus *Phlebotomus*, and *L. donovani* which is transmitted by sand flies of the subgenus *Synphlebotomus* (Killick-Kendrick, 1985, 1990). The *Leishmania* species are primarily parasites of wild animals among which they are transmitted by certain species of phlebotominae sand flies and for which they exibit little pathogenicity. Most leishmanial infections are therefore considered zoonoses, and man is almost always an accidental host, when he intrudes into the natural habitat of the wild animal hosts (Lainson, 1982).

1.2.3. Development of *Leishmania* in the sand fly vector

Phlebotomine sand flies are pool feeders with short biting mouthparts (less than 0.5 mm) and are unable to penetrate beyond the superficial layers of the skin (Lewis, 1987). Sand flies feed by lacerating the capillary loops and ingesting the blood pooling into the resulting haematomas (Lewis, 1987; Lainson, 1982). If the vertebrate host is infected with *Leishmania*, amastigotes liberated from macrophages and intracellular amastigotes may be taken up with the blood meal. Further development of the parasite is then determined by the physical and physiological suitability and sufficient longevity of the vector for the completion of the extrinsic cycle (Molyneux and Ashford, 1983). In the first moments of infection, the blood and parasites are exposed to the anticoagulant saliva, to considerable pressure changes in the proboscis, to mechanical action of the cibarial and pharyngeal teeth in some species and, usually to decreasing temperature (Molyneux and Ashford, 1983). Within the bloodmeal in the midgut, *Leishmania* amastigotes divide one or more times (WHO, 1984; Warburg *et al.*, 1986).

During the first 72 hours, and before the sand fly defecates, the amastigotes elongate and the rudimentary flagellum grows from the flagellar pocket. The resulting promastigotes (nectomonads) may be seen within the bloodmeal, confined to the posterior part of the midgut,

within a diffuse peritrophic membrane wall secreted by midgut epithelium. They attach to the midgut wall by inserting their flagellae between microvilli (Lainson, 1982; Warburg *et al.*,1986). These highly motile nectomonads divide either in the abdominal midgut or hindgut (WHO, 1984) depending on the species. Subsequent development of the infection in the sand fly varies in different *Leishmania* species, principally in the position of attachment of the parasites throughout the gut, and in the time required for the production of the infective metacyclic forms. Based on the mode of development in the sand fly host, Lainson and Shaw (1979) subdivided the genus *Leishmania* into 3 sections (with no taxonomic status): hypopylaria, peripylaria and suprapylaria. Except for a few species of *Leishmania*, most of these parasites fall within these 3 sections. When this classification was suggested, *Leishmania*-like parasites of reptilian origin were classified as *Leishmania*. These parasites are now grouped in a separate genus, *Sauroleishmania* Ranque (Killick-Kendrick *et al.*, 1986), with *Sauroleishmania tarentolae* as the type species.

Sections Hypopylaria consists of reptilan *Leishmania*-like parasitic flagellates that migrate to the pylorus, ileum and rectum of the sand fly. In these parts, the parasites attach to the cuticular lining of the rectum and rectal ampullae where they become rounded and immotile. It is assumed that hypopylarial *Leishmania*-like parasites are

transmitted to the reptile when the infected sand fly is eaten (Lainson, 1982). *Leishmania* species within this section can occur as promastigote and amastigote forms in blood of the lizard host. An example of such species is *Leishmania agamae* David.

Section Peripylaria comprises species whose multiplication occurs in the pylorus and ileum followed by an anterior migration to the thoracic midgut, stomodeal valve and foregut. In the hindgut, the principal form is the paramastigote which attaches to the cuticular intima by distal expansion of the flagellar tip to form a sticky hemidesmosome (Lainson, 1982; Molyneux and Ashford, 1983). Transmission occurs through the bite of a sand fly. *L. adleri* Heisch, *L. tarentolae* Wenyon (parasites of Old World lizards) and *L. braziliensis* complex are included in this section. Through isoenzyme characterization, *S. tarentolae* has been shown to be *Trypanosoma platydactyli* Catouillard (Wallbanks *et al.*, 1985).

Section Suprapylaria consists of *Leishmania* parasites whose development in the sand fly is confined to the midgut and foregut. Included in this section are *L. mexicana* and *L. hertigi* Herrer complexes, *L. aethiopica, L. tropica, L. major, L. gerbilli* Wang, Qu and Guan (Old World) and the *L. donovani* complex. Classification of leishmanial parasites according to Lainson and Shaw (1979) is shown in Figure 1.

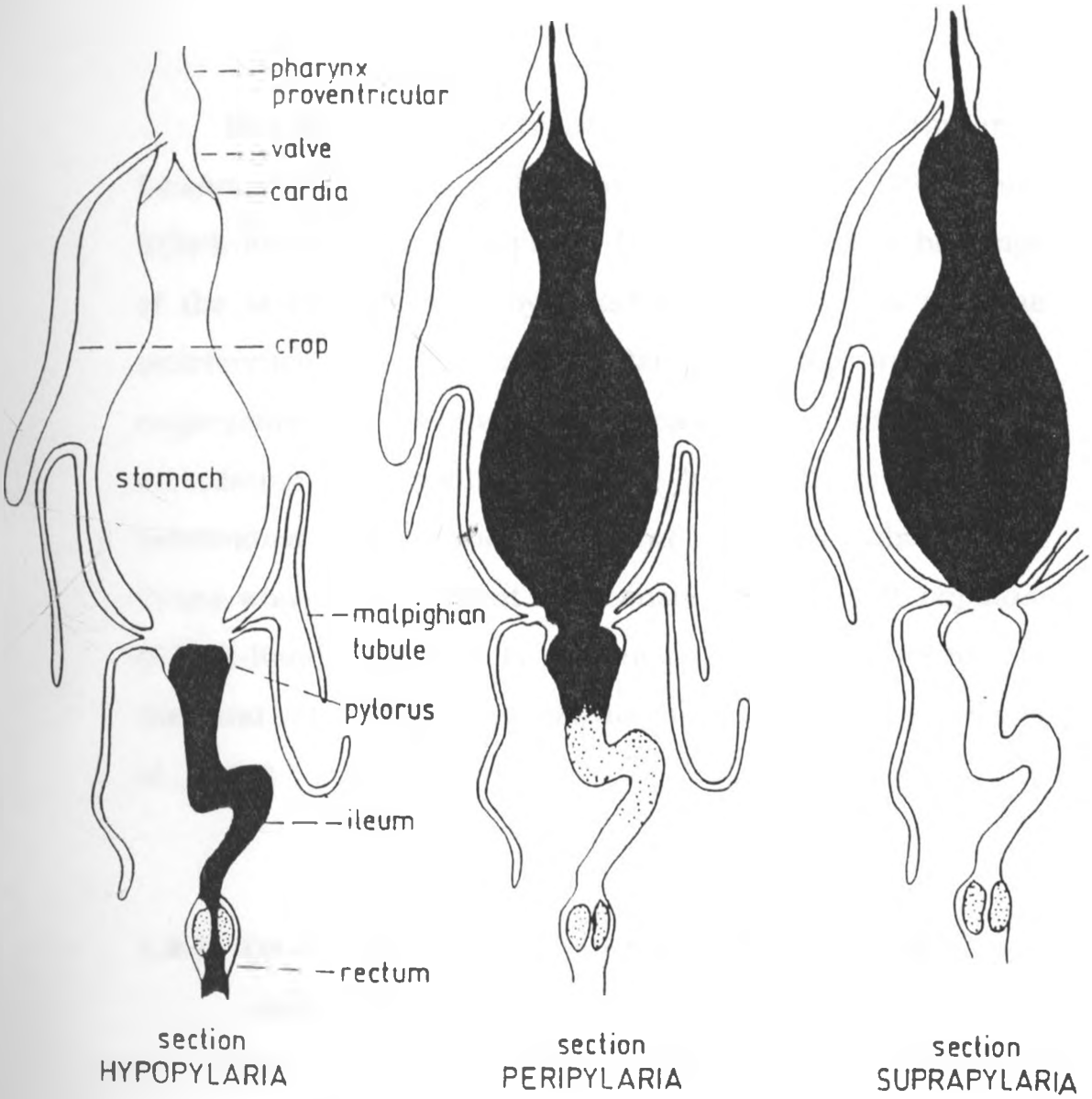

Figure 1. Classification of *Leishmania* according to their development in the sand fly vectors. Shaded parts are the areas of development. (Adapted from Lainson, 1982).

Mechanisms of transmission of Suprapylarial *Leishmania* are not well known. Ryan *et al.* , (1987) and Killick-Kendrick and Molyneux (1981) believe that blockage of the stomodael valve by *Leishmania* parasites and the interference of mouthpart sensillae controlling probing and engorgement respectively, may cause regurgitation and inoculation of parasites, into the host. There are some *Leishmania* species such as *L. arabica* Peters, Elbihari and Evans whose development in its suspected vector *P. papatasi* (Killick-Kendrick, 1990) is not known. This parasite infects the sand rat (*Psamommys obesus*) in Saudi Arabia (Peters *et al.*, 1986).

1.2.4. Transmission of *Leishmania* by the bite of the sand fly

The extrinsic life-cycle of most species of peripylarial and suprapylarial *Leishmania* are completed within a single ovarian cycle of the sand fly, and the parasites may therefore be transmitted at the first bloodmeal after an infective feed (Killick-Kendrick, 1978). In some species like *L. donovani* and *L. infantum*, the life cycle is not complete until after the second bloodmeal, hence transmission does not take place until the third feed (Killick-Kendrick, 1978; WHO, 1984). It is believed that the infective forms of *Leishmania* first

arise in the midgut of an infected sand fly 3 or more days after an infective feed (Sacks and Perkins, 1984, 1985; Lawyer *et al.*, 1987; Walters *et al.*, 1987). These stages are the only parasite forms seen in the proboscis of the sand fly and are deposited in the skin when the sand fly bites.

Transmission by bite is highly efficient and the infectivity of metacyclic promastigotes in the sand fly appears to be high. The efficiency of transmission by bite of the sand fly has been demonstrated by Beach *et al.* (1984), who found that probing of *Phlebotomus duboscqi* Neveu-Lemaire infected with *L. major*, was able to produce infection in five different mice. Killick-Kendrick *et al.* (1985) recorded 11 lesions on the arm of a volunteer where a sand fly (*P. papatasi*) infected with *L. major* had probed 26 times. In a more recent study, Lawyer *et al.* (1990), using *P. duboscqi* infected with *L. major*, were able to demonstrate that sand fly bites were more efficient in parasite transmission to nonhuman primates than needle infections, and as in the other studies cited above, this study showed that probing alone without imbibing blood is sufficient for *Leishmania* transmission.

Multiple probing is a common feature with *Leishmania*-infected sand flies of both the genera *Lutzomyia* and *Phlebotomus*. The exact physiological basis for such changes in behaviour have not been fully elucidated. The only hypothesis that has been put forward to explain this

observation is that the presence of parasites in the pharynx and cibarium directly interferes with the normal functioning of chemoreceptor and/or mechanoreceptor sensillae which are presumed to control engorgement (Killick-Kendrick and Molyneux, 1981; Beach *et al.*, 1985). The probing and inability to engorge may therefore be due to this interference with receptor function, or by the perception of a reduced blood flow by the mechanoreceptors due to intense pharnygeal infections or a combination of both. The feeding behaviour modification caused by *Leishmania* enhances the chances of the parasite being transmitted to the vertebrate host (Molyneux and Jefferies, 1986)

Studies on *in vitro* growth and the development of *Leishmania* within the sand fly midgut have shown that there is sequential development of promastigotes from a noninfective to an infective stage. By directly comparing the infectivity of promastigotes as they developed temporally within the sand fly, it was demonstrated conclusively that midgut promastigotes are not uniform with respect to infectivity. In these studies (Sacks and Perkins,1985), clones of *L. mexicana* and *L. major* that were recovered from midguts of *Lu. longipalpis* and *P. papatasi* respectively, 3 days after sand fly infection were relatively avirulent for BALB/c mice, whereas midgut promastigotes that were recovered on days 4-7 after infection were progressively more virulent.

Infective promastigote populations (metacyclics) appeared shortly after bloodmeal passage, coinciding with the time at which another meal is sought by the sand fly. Metacyclics appear in culture during the stationary phase in axenic cultures. These metacyclic forms are the ones that are inoculated by the sand flies. While the development of infective promastigote forms (metacyclogenesis) is not accompanied by any obvious morphological change, *L. major* metacyclics can be distinguished and even purified because they are not agglutinated by the lectin peanut agglutinin (PNA) at concentrations which agglutinate all noninfective (procyclic) promastigotes (Sacks *et al.*, 1985). A similar change in surface carbohydrates has now been defined for *L. donovani* metacyclic promastigotes (Howard *et al.*, 1987).

Metacyclogenesis is accompanied by the expression of a number of developmentally regulated molecules. Enhanced expression of the *Leishmania* surface protease gp63 a glycosylphosphatidylinositol-anchored zinc metalloproteinase (Bordier, 1987) has been described for *L. braziliensis* stationary-phase promastigotes (Kweider, *et al.*, 1987). The other surface molecule that increases during metacyclogenesis is the glycoconjugate lipophosphoglycan (LPG). This is a tripartite molecule in structure, consisting of repeating phosphorylated saccharide units linked via a phosphocarbohydrate core to a *lyso*-1-o alkylphosphatidylinositol lipid anchor (Orlandi and Turco,

1987; Turco, 1988; Turco and Sacks, 1991; Sacks *et al.*, 1990).

The phosphorylated repeating units are species-specific. In the LPG of *L. donovani* these units are [PO_4→6Gal(β1,4)Manα1]. There is an average of 16 of these units linked together in a linear array by α-glycosidic linkages (Turco, 1988). The units of *L. major* are slightly more complex, consisting of mainly tetrasaccharide units, with lesser amounts of penta-, tri- and disaccharide units containing galactose, mannose, glucose and arabinose (Orlandi and Turco, 1987). A similar glycoconjugate from *L. adleri* also contains high proportions of mannose and galactose (Turco, 1988). It is now clear that during metacyclogenesis, the LPG on *L. major* promastigotes undergoes considerable modification. These include a change in the proportion of tri-, and disaccharides expressed, and a doubling of the number of subunits expressed (Sacks and da Silva, 1987).

Metacyclic promastigotes have been shown to be free-swimming, highly active with a short cell body and a long flagellum (Howard *et al.*, 1987), and are non-dividing (Davies *et al.*, 1990). These parasite forms have been observed in the cibaria and pharynx of infected *P. papatasi* (Killick-Kendrick *et al.*, 1988). The identification of metacyclic forms both in culture and in the sand fly support the idea put forward earlier by Shortt *et al.* (1931) and Adler and Ber

(1941) that there is one particular form of promastigote in the sand fly which is adapted for life in the vertebrate host.

1.2.5. Interactions between *Leishmania* promastigotes and host macrophage surfaces

To establish an infection in a susceptible mammalian host, the metacyclic promastigote deposited in the skin by the bite of a phlebotomine sand fly must find, or be found by a cell of the mononuclear phagocyte series. The parasite must attach to, or be attached by the cell, then invade or be taken up by the cell. An important role of the macrophage is to identify and eliminate foreign matter, including parasites. Certain macrophages are capable of killing *Leishmania* and other parasites directly, while others must be stimulated to do so. Only unstimulated resident macrophages are suitable for the establishment of *Leishmania* infection (Molyneux and Ashford, 1983).

Entry of promastigotes into host macrophages occurs through the interaction between promastigote ligands and macrophage receptors according to the "zipper hypothesis" described by Silverstein (1977). Macrophage receptors that have been shown to be important in the binding of *Leishmania* parasites are the mannose/fucose receptor (MFR) and the type 3 complement receptor (CR3) for the cleaved C3 complement component (Blackwell *et al.*, 1985;

Wozencraft *et al.*, 1986). *Leishmania* promastigote surface molecules that act as ligands for macrophage receptors have been identified.

The 63 kDa (gp63), a surface zinc metalloproteinase (Schneider *et al.*, 1992), and the 9 kDa major surface glycoconjugate LPG are the two best-characterized molecules on the surface of promastigotes of *Leishmania*. Both molecules mediate the uptake of promastigotes by macrophages (Turco, 1988; Blackwell, 1985). Various activities and functions have been demonstrated or suggested for LPG. Several are believed to involve attachment and entry of the parasite into the host cell, while others are thought to be crucial in enabling the parasite to survive within the host's phagolysosome. Regarding parasite attachment onto the host macrophage, because of the role of LPG in complement activation and resulting C3 deposition on the surface of the parasite, it promotes attachment of promastigotes to macrophages via C3 receptors (Puentes *et al.*, 1988).

Resistance of metacyclics to complement-mediated lysis in the face of efficient complement activation might seem paradoxical, but deposition of C3 on the parasite surface facilitates attachment to macrophages and even promotes intramacrophage survival (Blackwell, *et al.*, 1985; Mosser and Edelson, 1987). A natural infection with *Leishmania* parasites occurs in the presence, within the

infected tissue, of serum that includes components of the complement system. Metacyclic *L. major* attach to human macrophages only after serum complement activation and opsonization with C3.

The predominant form of C3 found on the parasite surface is either C3b for *L. major*, or C3bi, for *L. donovani* (Puentes *et al.*, 1988). These forms of C3 are then bound covalently to gp63 on the parasite surface. In this form, gp63 and LPG function either directly or indirectly as ligands for various macrophage receptors, among which the complement receptors CR1 (= C3bR) and CR3 (= C3biR) appear to be the most important (Blackwell, 1985; Mosser and Edelson, 1987; Wozencraft *et al.*, 1986). Macrophage receptors for fixed C3 are poor at triggering the respiratory burst (Wright and Silverstein, 1983). By entering host macrophage through the CR1 and CR3 complement receptors and the MFR, the parasites are able to avoid the generation of oxidative metabolites (hydrogen peroxide, hydroxyl radical, singlet oxygen and superoxide anion) by the macrophage (Mosser and Edelson, 1987; Wozencraft *et al.*, 1986). Once inside the macrophage, the promastigote transforms into the amastigote form that is capable of staying alive in the phagolysosomes.

Upon entry into the phagolysosomes of macrophages, the parasite is open to attack from the cytocidal activities of the oxidative burst and action of degradative enzymes.

Erythrocytes coated with LPG have been shown to be protected from lysis by macrophages (Eilam *et al.*, 1985). LPG has also been shown to be capable of inhibiting hydrolytic activity of lysosomal enzyme ß-galactosidase (El-On *et al.*, 1980). It is not known how LPG achieves the inhibitory activity, but it has been suggested that LPG may act as a cell surface barrier preventing hydrolases from attacking the parasite. The high anionic nature of LPG may afford protection for the parasite against enzymatic attack (Turco, 1988). The LPG has been shown to inhibit protein kinase C, one of the key enzymes responsible for activating the normally dormant oxidative killing events in phagocytic cells.

The phagolysosome of *Leishmania* has an acidic pH. The gp63 molecule has protective functions in the phagolysosome. Its protease activity, with an optimum pH 4, inactivates proteolytic enzymes of the macrophage and thus protects parasite proteins from phagolysosomal degradation (Kweider *et al.*, 1987). *Leishmania* are able to maintain a neutral pH despite the acidity inside the phagolysosome (Gottlieb and Dwyer, 1981). Under these conditions, the uptake of essential nutrients like glucose, proline and pyrimidine bases is dependent on the co-transport of protons (H^+) and is therefore optimal at pH 4.0-5.5 (Mukkada *et al.*, 1985; Glaser *et al.*, 1988). Neutralization or elimination of reactive oxygen metabolites is another

method by which *Leishmania* amastigotes are able to survive within the macrophage.

Amastigotes show a high activity of enzymes such as glutathione peroxidase, superoxide dismutase and catalase. These enzymes are known to degrade toxic macrophage products. It is by this form of scavenging that amastigotes as opposed to promastigotes are able to survive intracellularly (Murray, 1981; Murray, 1982).

Transforming growth factor-ß (TGF-ß) is a multipotential cytokine with diverse effects on cells of the immune system, including down-regulation of certain macrophage functions. This 24 kDa protein is produced by B and T lymphocytes and activated macrophages. It is usually secreted as a latent precursor, that requires enzymatic cleavage of carbohydrate groups or transient acidification to release the active cytokine (Kehrl *et al.*, 1986). Among the inhibitory effects of TGF-ß on the immune system are decreases in interleukin 2 (IL-2) receptor induction, interferon gamma (IFN-γ)-induced Class II expression, cytotoxic and lymphokine-activated killer cell production.

Leishmanial infections induce TGF-ß production by host macrophages both *in vivo* and *in vitro*. TGF-ß also blocks IFN-γ-induced macrophage activation, which diminishes their oxidative responses (Ding *et al.*, 1990). *L. braziliensis* causes cutaneous leishmaniasis in humans but

does not cause disease in mice. BALB/c mice that are not normally susceptible to infection with *L. braziliensis* develop lesions when they are treated with TGF-ß. Similarly, this cytokine has been shown to reverse the genetic controlled resistance to leishmaniasis in C57BL/6 mice (Barral-Neto *et al.*, 1992).

1.2.6. Modulation of host-immune responses by arthropod saliva and parasite transmission

Saliva of tick vectors of livestock viruses and that of arthropod vectors of leishmaniases, onchocerciasis, and malaria has been shown to contain different pharmacologically active molecules which enable them to obtain blood meals by compromising host defenses such as haemostasis and inflammation. Saliva of the mosquitoes *Anopheles freeboni, An. stephensi* Liston and *Aedes aegypti* Linnaeus contain enzyme apyrase that prevents blood from clotting hence aiding the mosquito in blood vessel location, and the feeding process (Ribeiro *et al.*, 1984, 1985). More recently, salivary gland extracts of female *Ae. aegypti* have been shown to inhibit tumour necrosis factor alpha (TNFα) release from rat mast cells, but does not inhibit antigen-induced histamine secretion. This selective inhibition reduces immediate proinflammatory effects of TNFα at the

feeding site and may facilitate completion of the blood meal (Bissonnette *et al.*, 1993).

Ixodid ticks (the best studied in immunomodulation) have substances in their saliva that are highly immunosuppressive to vertebrate hosts they infest. T lymphocytes obtained from animals exposed to ticks exhibit a significant reduction in their responsiveness to mitogens, after both primary and secondary infestations (Wikel, 1982b). Salivary gland factors of partially engorged, uninfected, female ixodid ticks, *Rhipicephalus appendiculatus* Neumann have been shown to enhance transmission of Thogoto virus to uninfected ticks 10-fold when feeding on a host without a detectable viraemia (Jones *et al.*, 1992).

Ramachandra and (1992) demonstrated that salivary gland extracts collected from days 0-5 of engorgement from the ixodid tick *Dermacentor andersoni* Stiles suppress interleukin 1 (IL-1) production by macrophages. Similarly, these workers showed that saliva of *D. andersoni* suppresses levels of TNFα, production of interleukin 2 (IL-2) and interferon gamma. Significant suppression of IL-1 production in the presence of salivary gland extracts from days 0 through 5 of engorgement correlates with the time the tick-exposed host would be mounting an immune response to infestation, and this is usually the time when the tick is attempting to initiate feeding and transmit pathogens

(Wikel, 1984). Attacking the first link in the host immune response network is an effective strategy to enhance the likelihood of obtaining a blood meal and successfully passing infectious agents to a host.

Immunization of mice against saliva of the black fly *Simulium vittatum* Zetterstedt (Diptera: Simuliidae) by multiple injections of salivary gland extracts or fly bites has been shown to prevent mice from producing antibodies against any form of the known bioactive components of black fly saliva (Cross *et al.*, 1993a). When administered intradermally, saliva extracts of *S. vittatum* suppresss Ia expression by splenic leukocytes, and *in vitro*, it suppresses proliferation of T and B cells when these cells are stimulated by concanavalin A and the B cell mitogen *Salmonella typhosa* lipopolysaccharide respectively (Cross *et al.*, 1993b).

Some of the immunomodulatory properties described for mosquito, tick and black fly saliva have also been demonstrated in the salivary apparatus of phlebotomine sand flies described below. The earliest description of the salivary apparatus of phlebotomine sand flies was made by Newstead (1911). Description of *Phlebotomus papatasi* salivary apparatus was made by Adler and Theodor (1926).

The salivary apparatus of *P. papatasi* and other phlebotomine sand flies consists of a pair of salivary glands, salivary ducts, salivary pump and the salivary channel through the hypopharynx. Salivary glands consist of two

broadly dilated or lobe-like acinous glands, lying one upon either side of the uppermost ventral part of the thorax. They are hollow, almost spherical organs lined with a single layer of secretory columnar epithelium which rests on a basal membrane (Newstead, 1911; Adler and Theodor, 1926). A fully-distended salivary gland may reach the size of 180μ long by 140μ wide. The salivary ducts are two annulated tubes with thin chitinous walls 140μ long and a lumen which is 7.5μ wide.

The ducts leading from the acini unite near the mid-region of the head to form the common salivary duct that is 190μ long and 11μ wide and has the same structure as the salivary ducts (Adler and Theodor, 1926), which then passes in the middle of the head underneath the inferior ganglion of the brain and opens into the salivary pump. The inferior wall of the common salivary duct shortly before its entrance into the salivary pump joins the inferior lamina of hypopharynx (Newstead, 1911; Adler and Theodor, 1926). The position of the entire salivary apparatus of *P. duboscqi* is shown in Figure 2.

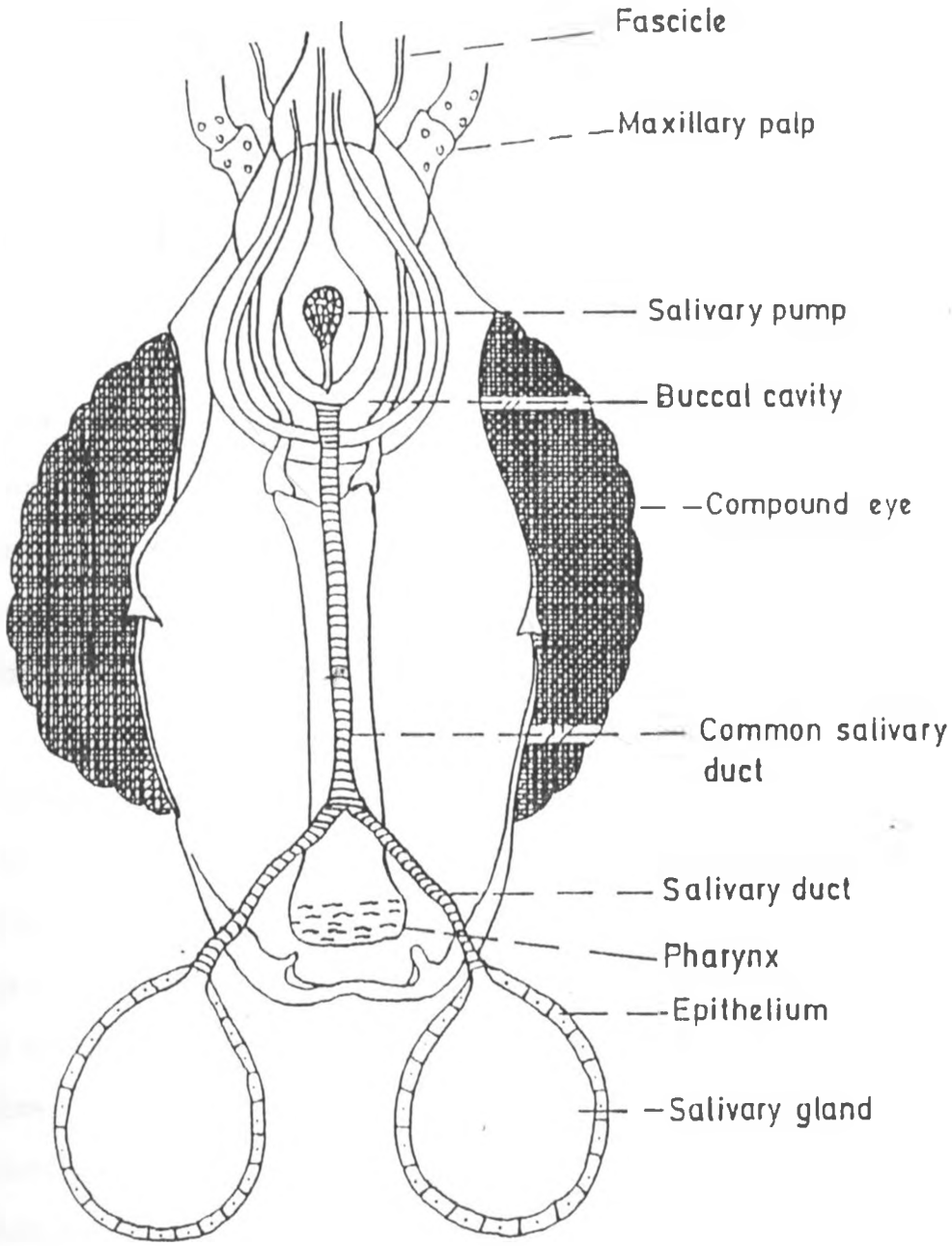

Figure 2. The salivary apparatus of *P. duboscqi* (Adapted from Adler and Theodor, 1926).

The salivary pump is elliptical, 100μ long by 45μ wide. Its walls are formed of thick chitin and are traversed interiorly by strong circular ridges. A little infront of the entrance of the common salivary duct, the floor of the salivary pump contains a small yellow elevation from which a number of minute teeth project into the lumen of the pump. Anteriorly the lumen of the salivary pump is chitinous with the salivary canal which pierces the hypopharynx (Adler and Theodor, 1926).

Apart from studies of the morphology of *P. papatasi* salivary apparatus, Adler and Theodor (1926) also studied the functions of sand fly saliva. They found that saliva of unfed *P. papatasi* is faintly alkaline, and that when salivary gland lysates prepared from this sand fly were mixed with an equal amount of human blood, coagulation was delayed for fifteen minutes. They concluded that saliva of *P. duboscqi* contains an anticoagulant that may aid the sand fly in haematophagy. It is now known that sand fly saliva plays a role in the establishment of *Leishmania* infections. The amount of protein in a pair of sand fly salivary glands has been estimated to be 1μg (Theodos *et al.*, 1991). It is not known how much of the bioactive component contained in the saliva is inoculated into the host during haematophagy.

1.2.7. The role of sand fly saliva in the establishment of *Leishmania* infection

During extrinsic and intrinsic phases in their life cycle *Leishmania* parasites encounter three different micro-habitats. These are (i) the sand fly gut (an enviroment where only the promastigote form is adapted to survive); (ii) the intercellular spaces of the vertebrate host; and (iii) the phagolysosomes of vertebrate macrophages, in which only the amastigote form is able to survive. Not much is known about the events occuring between the time of inoculation of promastigotes and infection of macrophages. It is during this period that the promastigotes are exposed to the potentially lethal effects of serum complement.

Three main mechanisms have been suggested to explain how promastigotes inoculated by the sand fly into the vertebrate host evade lysis by complement. These are: (i) rapid uptake of promastigotes by macrophages (Pearson *et al.*, 1983); (ii) inactivation of vertebrate host complement by sand fly saliva (Pearson and Steigbigel, 1980); and (iii) differentiation of promastigotes in the sand fly into complement resistant infective metacyclic forms (Franke *et al.*, 1985). Whereas there is no evidence in the literature to refute or support the first two theories, a lot of evidence is available in support of the third mechanism. More studies are therefore needed to examine the first two theories listed above.

Recent studies on feeding habits of phlebotomine sand flies and experimental inoculations of parasites into suitable animal models, suggest that sand fly saliva may influence the establishment of *Leishmania* infections in the host. In their studies on the blood-feeding behaviour of adult female *Lu. longipalpis*, Ribeiro *et al.*, (1986) showed that saliva of this sand fly inhibits platelet aggregation and it is induced by ADP and collagen. This inhibition of platelet aggregation was attributed to the presence of an antiplatelet substance, an apyrase enzyme (an adenosine 5'-triphosphatase and an adenosine 5'-diphosphatase, E.C. 3.6.1.5) commonly known as ATP diphosphohydrolase. In addition to these two substances, an erythema-inducing factor (EIF) has been identified in saliva of *Lu. longipalpis* (Ribeiro *et al.*, 1989). They suggested that apyrase and EIF together enhance the formation of haematomas and increase the blood flow to the superficial capillaries which in turn reduces the amount of time the sand fly spends on the host.

Citing an unpublished study, Ribeiro(1987) reported that saliva of the Old World sand fly *P. papatasi* contains antiplatelet and apyrase activities, but lacks EIF. This finding suggests that the saliva of different sand flies may differ in chemical composition and pharmacological activities and is therefore an area that needs to be studied further. Titus and Ribeiro (1988) demonstrated that salivary gland lysates of the New World sand fly *Lu. longipalpis*

enhances the growth of *L. major* (the causative agent of cutaneous leishmaniasis in the Old World), when co-injected with parasites into BALB/c mice footpads. In their study, as little as 5% of salivary gland homogenate co-injected with 10 parasites was the difference between having or not having an established infection after 3 weeks. The presence of saliva also induced an increase in the number of parasites by several magnitudes when compared with controls. Despite the fact that this study was performed using salivary gland lysates from a sand fly that does not naturally transmit the *Leishmania* strain used, it demonstrated that saliva plays an impotant role in enhancing parasite transmission. It is not known whether saliva of sand fly vectors of cutaneous and visceral leishmaniases in Kenya also exacerbate infection when co-inoculated with the parasites they naturally transmit.

CHAPTER 2

THE INFLUENCE OF *PHLEBOTOMUS DUBOSCQI* SALIVA ON THE ESTABLISHMENT OF *LEISHMANIA MAJOR* INFECTION

2.1. The effect of *P. duboscqi* saliva on the course of *L. major* infection in BALB/c mice

2.1.1. Introduction

Medically important haematophagous arthropods are known to salivate at the site of the wound created by the bite when probing for a blood meal in the vertebrate host. Phlebotomine sand flies have been shown to exhibit this behaviour (Adler and Theodor, 1926). Various functions, some of which have a direct effect on haematophagy have been ascribed to the saliva of haematophagous dipterans. Among these are: Inhibition of platelet aggregation (Ribeiro *et al.*, 1986), agglutination of erythrocytes (Yorke and Macfie, 1924), anticoagulant activity (Adler and Theodor, 1926; Yorke and Macfie, 1924) and more recently, enhancement of infectivity of parasites (Titus and Ribeiro, 1988).

Most studies on haematophagy in arthropods have shown that saliva of these arthropods is in most cases highly antigenic. The antigens in saliva have other properties, and there may be other substances present which have pharmacological or irritant properties, and can thus alter the immunological reactions to the bite (Mann and Bates, 1960). Such substances contained in salivary secretions of different insects are usually hyaluronidase, proteases,

peptidases or phospholipase A; peptides such as kinins, histamine or heparin-releasing agents, and biogenic amines like histamines, 5-hydroxytryptamine, or acetylcholine (Alexander, 1984; Wirtz, 1988). The general effects vary according to the concentrations or total amount inoculated into the victim. The listed functions are not always evident in saliva of any one blood-feeding arthropod. Interspecific and intergeneric differences have been demonstrated in properties of saliva from different phlebotomine sand flies.

Saliva of the sand fly *Lu. longipalpis* has been shown to enhance infectivity of *L. major* culture promastigotes when the two are co-inoculated. Saliva of mosquitoes (*Aedes aegypti*), ticks (*Ixodes dammini*) and kissing bugs (*Rhodnius prolixus*) proved to be ineffective (Titus and Ribeiro, 1988). In their study, Titus and Ribeiro did not use a proper *Leishmania*-vector combination. They used *L. major* an Old World parasite that causes zoonotic cutaneous leishmaniasis, and salivary gland lysates prepared from *Lu. longipalpis*, a New World sand fly. *L. major* is also known to suppress phagocytic and lytic lysozyme activities in heavily infected macrophages (Al Mofleh, 1987).

Where *L. major* is endemic in the Old world, it is transmitted by sand flies of the genus *Phlebotomus* Rondani and Berte, particularly sand flies in the subgenus *Phlebotomus*, *Paraphlebotomus* Theodor and *Synphlebotomus* Theodor. Sand fly species incriminated as

vectors of *L. major* in subgenus *Phlebotomus* are *P. (P.) duboscqi*, Neveu-Lemaire, *P. (P.) papatasi* Scopoli and *P. (P.) salehi* Mesghali. Those in subgenus *Paraphlebotomus* are *P. (Pa.) sergenti* Parrot, *P. (Pa.) caucasicus* Marzinowsky, *P. (Pa.) andrejevi* Shakirzyanova, *P. (Pa.) mongolensis* Sinton, and *P. (Pa.) alexandri* Sinton. In subgenus *Synphlebotomus*, *P. (Syn.) ansarii* Lewis, is the only sand fly species reported from Iran that is known to transmit *L. major* (Bray, 1972; Lewis and Ward, 1987; Killick-Kendrick, 1990).

In Kenya, *L. major* is endemic in Baringo District of the Rift Valley Province. In this focus, it is transmitted by *P. duboscqi* (Beach *et al.*, 1984). This sand fly is phylogenetically related to *P. papatasi*, a sand fly whose saliva was recently shown to enhance infection with *L. major* in C57BL/6 mice (Theodos *et al.*, 1991). Such data is not available for *P. duboscqi* in the literature. This part of the study was carried out to determine whether the infectivity of an indigenous *L. major* strain can be enhanced by saliva of its local natural vector *P. duboscqi*, and to determine what dose of *L. major* culture promastigotes is comparable to that inoculated by infected *P. duboscqi*.

2.1.2. Materials and methods

2.1.2(a). Preparation of parasite inoculum

L. major (strain IDUB/KE/83=NLB-144) isolated from a *P. duboscqi* female collected in Marigat, Baringo District, Rift Valley Province, Kenya, an endemic focus for *L. major* (Beach *et al.*, 1984) was used. This strain has always been routinely maintained by serial passage in BALB/c mice to maintain its infectivity (Katakura and Kobayashi, 1985).

A saline aspirate was taken from a nasal lesion of an infected BALB/c mouse. Some of the aspirated material was cultured in $25cm^2$ culture flask containing NNN medium overlayed with Schneider's *Drosophila* medium supplemented with 20% heat-inactivated (56^0C for 30 minutes) foetal bovine serum, 250 Unitsml^{-1} penicillin, 250µg ml^{-1} streptomycin (Hendricks and Wright, 1979) and 500µg ml^{-1} 5-fluorocytosine (Kimber *et al.*, 1981). To ascertain the presence of amastigotes in the cultured material, a smear prepared using some of the aspirate was stained with Giemsa. Amastigotes that were seen are shown in Plate 1.

Promastigotes were grown to stationary phase to generate infective metacyclic forms (Plate 2) and then counted. The concentration of promastigotes was adjusted to $1x10^6ml^{-1}$. Every 1ml of the parasite suspension was centrifugally washed twice in sterile phosphate-buffered

saline at 3000 revolutions per minute (rpm) to remove as much culture media as possible. The final inoculum that was used to inoculate the footpad of each mouse contained $1x10^6$ parasites/40µl PBS.

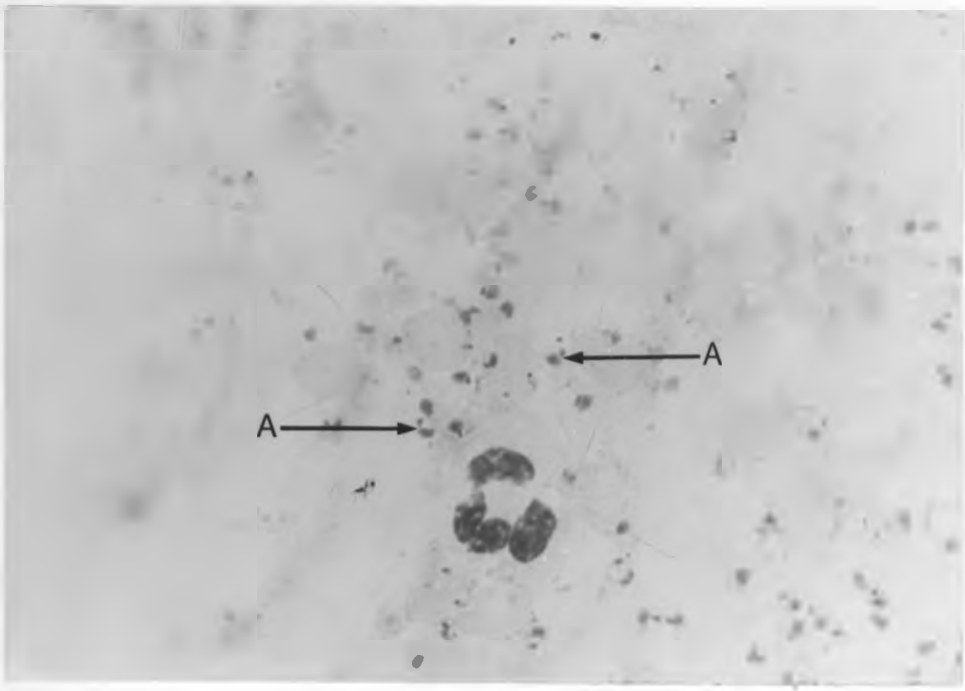

Plate 1. ***Leishmania major*** **amastigotes (A) from a mouse nasal lesion** (Giemsa x1000).

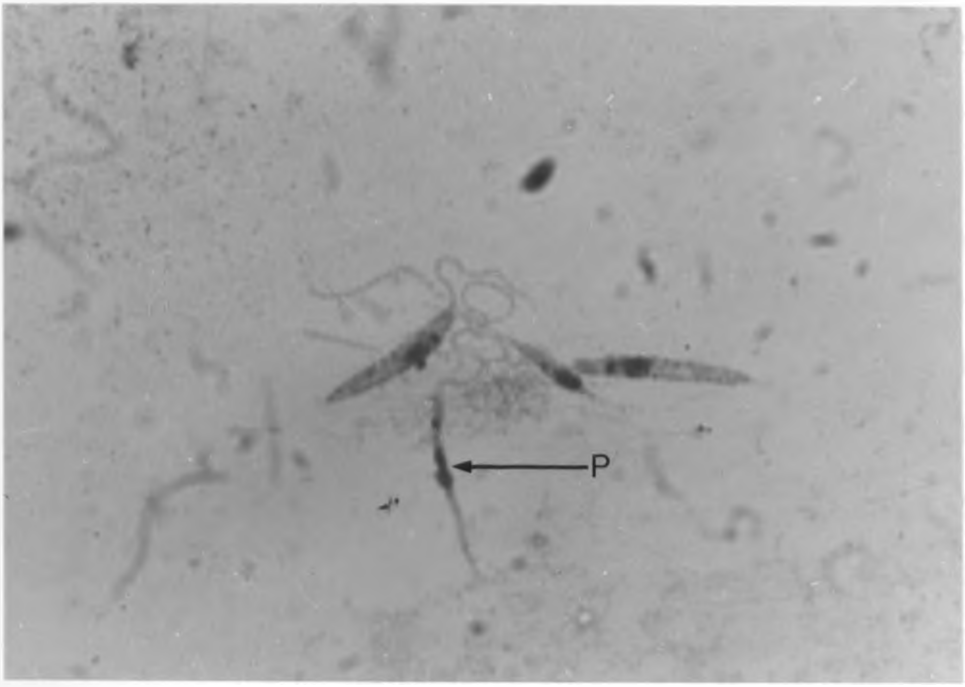

Plate 2. ***Leishmania major*** **culture-derived metacyclic promastigotes (P) that were used for mouse inoculation**(Giemsa x1000).

2.1.2(b). Preparation of *P. duboscqi* salivary gland lysates

One hundred and fifty laboratory bred 3-5 day old adult female unfed *P. duboscqi* were ether-anesthetized. Heads of individual females were dissected in a drop of ice-cold filter-sterilized 0.15M sodium chloride solution. Sets of five pairs of salivary glands were transfered to sterile nunc tubes containing 20µl of ice-cold phosphate-buffered saline. The glands were stored at -81°C until use. Salivary glands of *P. duboscqi* as they appear soon after dissection are shown in Plate 3.

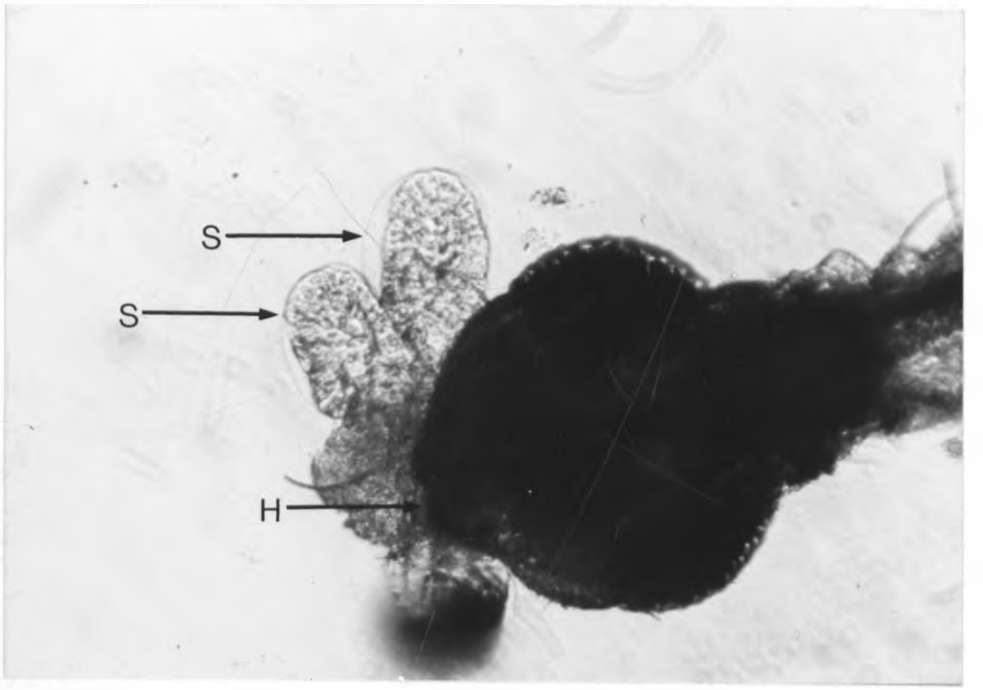

Plate 3. Salivary glands (S) of *Phlebotomus duboscqi*. attached to the head (H). (x400).

2.1.2(c). Infection of *P. duboscqi* with *L. major*

Three hundred 3-day old female *P. duboscqi* were fed on a *L. major* lesion on the nasal dorsum of a BALB/c mouse that had been anesthetized with pentobarbital sodium (Sagatal^{R}). The mouse was placed within a cloth envelop with a hole in it, ensuring that feeding took place only on the exposed lesion (Plate 4). Engorged females were held in the colony cage in the insectary at 25 ± 1^{o}C and RH 90% for 10 days post-infective feed to ensure development of mature infections. Fresh apple slices were placed in the cage each day to serve as an energy source for the putatively infected female sand flies. Sand fly infections were carried out 2 days before aspirates of *L. major* were taken for promastigote culture as outlined earlier. This was done to ensure that both sand flies and cultures had large numbers of metacyclics at the time of mice inoculation.

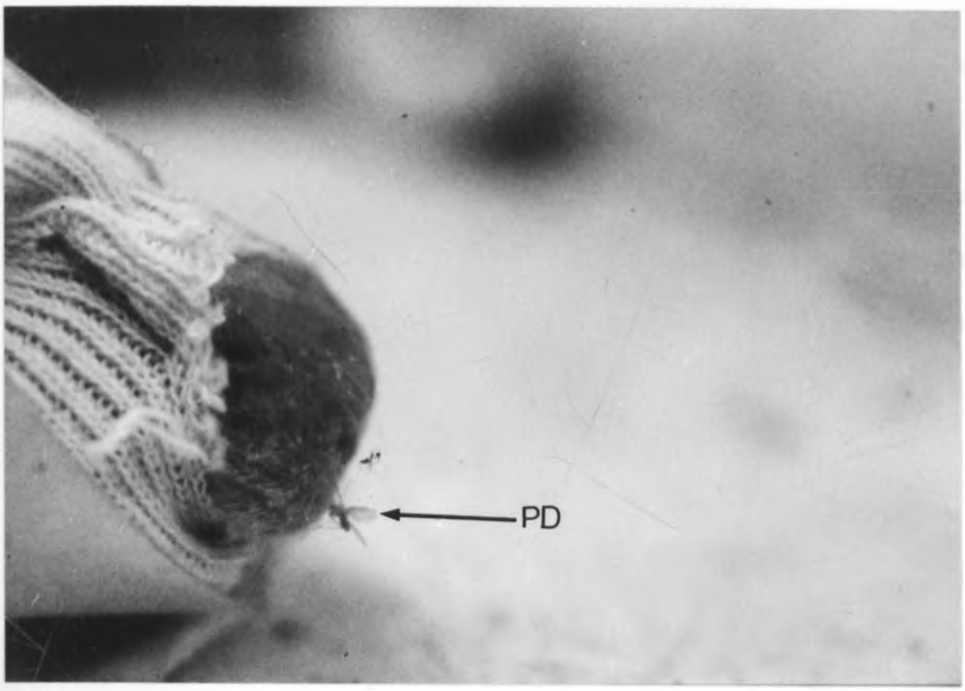

Plate 4. Infection of *P. duboscqi* (PD) by feeding them on a BALB/c mouse nasal lesion.

2.1.2(d). Infection of BALB/c mice using *L. major*-infected *P. duboscqi* and culture promastigotes

Two groups of 30 adult inbred weanling BALB/c mice were individually marked for easy identification. Hind footpads of all the mice were measured using direct reading vernier calipers. The two groups of mice were treated as follows: The first group was inoculated with $1x10^6$ stationary phase culture promastigotes mixed with *P. duboscqi* salivary gland lysates (SGL). The $1x10^6$ stationary phase culture promastigotes inoculum in 40µl PBS was gently mixed with the 20µl PBS containing 5 pairs of salivary glands (equivalent to 5µg of salivary protein) and then thoroughly mixed for about 30 seconds. Each mouse in this group recieved 60µl of the above inoculum containing $1x10^6$ parasites mixed with SGL. The parasites were inoculated intradermally in the left hind footpad of each mouse. The contralateral right hind footpad was left to serve as the uninfected control. Thicknesses of infected and uninfected hind footpads were measured every week for 12 weeks.

The second group of mice (control) was inoculated in the left hind footpad with $1x10^6$ stationary phase culture promastigotes in 60µl PBS. Footpad thicknesses were measured as explained above. In order to compare sand fly bite and needle inoculation of culture promastigotes, two other groups of 30 mice each were marked for easy identification and both hind footpads measured. One group

of 30 mice was inoculated with $1x10^3$ stationary phase culture promastigotes of *L. major* in 60µl PBS in the left hind footpad. The other control group of mice was subjected to bites of *P. duboscqi* with a 10-day old putative *L. major* infection.

Individual mice were first anaesthetized by intraperitoneal inoculation with 50µl pentobarbitone sodium. Each left hind footpad was exposed to bites of 10 putatively infected sand flies. During the feeding, 30 groups of 10 sand flies were placed in 30ml feeding caps fitted with fabric-screen lids (12 holes per linear cm). The outer surface of the screen lid of each feeding cup was pressed directly on the sole of the mouse. To prevent movement, footpads were taped onto the feeding cup. Sand flies were allowed to feed uninterrupted for 1 hour. Immediately after feeding, putatively infected sand flies were anesthetized with anesthetic ether, cleaned in 2% detergent saline for 2 minutes and dissected in a drop of normal saline on a slide.

Guts of all sand flies were examined microscopically using 400x magnification. Presence or absence of parasites, parasite positions and forms of parasites were recorded. The number of infected re-fed sand flies were determined. Thicknesses of hind footpads were measured as previously described in this text. Using a direct reading vernier caliper, footpad lesions at the site of inoculation for all the three groups of mice were measured as the thickness of the

infected footpad minus the thickness of the uninfected footpad, with the difference being reported as the lesion size (Nolan and Farrell, 1987). All mice were sacrificed at week 12. At necropsy, smears of liver and spleen and cultures of the uninfected control right hind footpad were made in NNN medium overlayed with Schneider's *Drosophila* medium. Culturing of the control footpads was done to detect metastatic spread of *L. major*. All cultures were examined for 14 days before they were discarded.

2.1.3. Results

Table 1 and Figure 3 are results summarising lesion sizes for the two groups of mice. Standard errors shown in the Figure 3 are deviations of mean lesion sizes for every week during which the mice were sampled. The results show that throughout the 12 weeks of observation, mice inoculated with $1x10^6$ *L. major* mixed with *P. duboscqi* salivary gland lysates (SGL) exhibited larger lesions than the control group inoculated with $1x10^6$ *L. major* only. The difference between lesion sizes in these two groups of mice was highly significant ($P < 0.01$, t-test). At week 12, when the study was terminated, most of the mice inoculated with *L. major* mixed with SGL had large lesions that had started losing necrotic tissue. This was also observed in few mice

inoculated with *L. major* only. In this group, a noticeable decline in lesion size was evident from weeks 8 to 11.

When sacrificed, none of the mice in the two groups had visceral infections in the liver and spleen as seen in Giemsa-stained impression smears of these organs. Cultures of the control uninfected footpads were all negative for parasites, an indication that at this time parasites had not metastasized to this site. Mice subjected to bites of *L. major*-infected *P. duboscqi* initially developed larger lesions than mice inoculated with $1x10^3$ *L. major*. Lesions were persistently larger from week 1 to week 7, at which time the lesion size stabilised and then started to decline steadily. Very few of these lesions ulcerated. In contrast, mice inoculated with $1x10^3$ parasites initially had lower lesion sizes from week 1 to 7.

After week 7 this group developed very large lesions that remained big in size even during the time of ulceration at week 12, when the study was terminated. The difference between the lesion sizes in the two groups was statistically not significant ($P > 0.01$, t-test), particularly in weeks 6,7 and 8 post-infection. Figure 4 is a graphic representation of the pattern of lesion development in the two groups. Table 2 is a summary of details of sand fly infection rates. All infected sand flies that refed were found to have stomodael valve infections, and parasite forms seen were nectomonads, haptomonads and metacyclic promastigotes.

Table 1. A summary of lesion sizes in two groups of 30 BALB/c mice each inoculated with *L. major* mixed with *P. duboscqi* salivary gland lysates (SGL), and control mice inoculated with *L. major* only.

Time (weeks)	MEAN LESION SIZES ($mm^2 \pm SE$)	
	L. major with SGL	*L. major* only
0	0	0
1	0.1 ± 0.03	0.05 ± 0.02
2	0.4 ± 0.05	0.3 ± 0.40
3	1.0 ± 0.10	0.6 ± 0.04
4	1.5 ± 0.20	1.0 ± 0.10
5	2.0 ± 0.20	1.3 ± 0.10
6	2.5 ± 0.30	1.8 ± 0.20
7	2.6 ± 0.30	2.0 ± 0.20
8	2.7 ± 0.30	2.4 ± 0.20
9	2.7 ± 0.30	2.2 ± 0.20
10	2.9 ± 0.30	2.0 ± 0.20
11	2.7 ± 0.30	1.8 ± 0.20
12	2.7 ± 0.30	1.9 ± 0.20

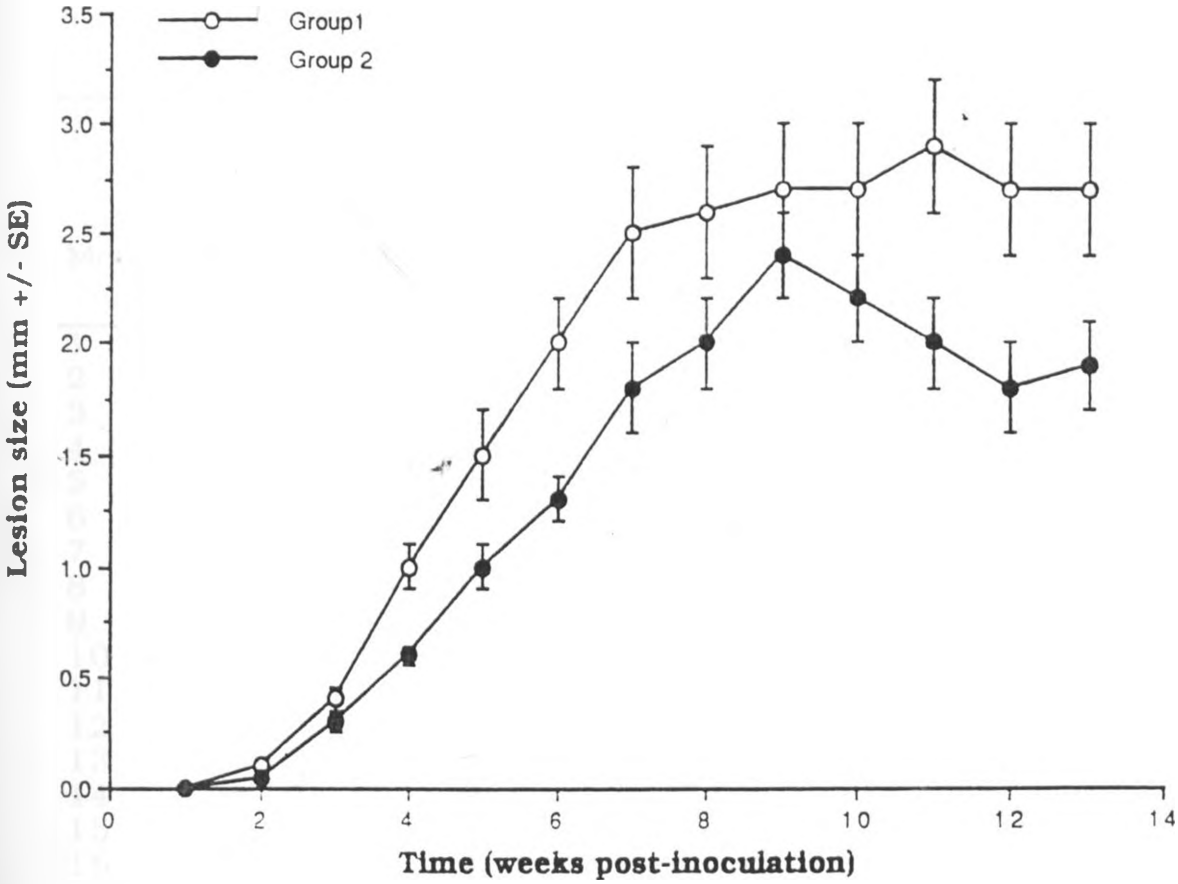

Figure 3. Results of lesion development in BALB/c mice inoculated with *L. major* stationary phase culture promastigotes mixed with *P. duboscqi* salivary gland lysates (Group 1) and control mice (Group 2) inoculated with promastigotes only.

Table 2. Results of post-exposure dissections of *L. major* -infected *P. duboscqi* fed on BALB/c mice footpads.

SAND FLY DISSECTION RESULTS

Mouse No.	No. flies exposed	No. flies infected	No. of infected flies that refed
1	10	7	2
2	10	8	1
3	10	5	0
4	10	4	0
5	10	3	1
6	10	4	0
7	10	5	1
8	10	6	2
9	10	1	0
10	10	8	2
11	10	9	3
12	10	7	5
13	10	6	3
14	10	9	6
15	10	2	1
16	10	4	2
17	10	4	3
18	10	7	2
19	10	8	1
20	10	6	1
21	10	10	4
22	10	6	2
23	10	5	2
24	10	3	2
25	10	3	0
26	10	7	2
27	10	8	3
28	10	4	1
29	10	5	0
30	10	7	3

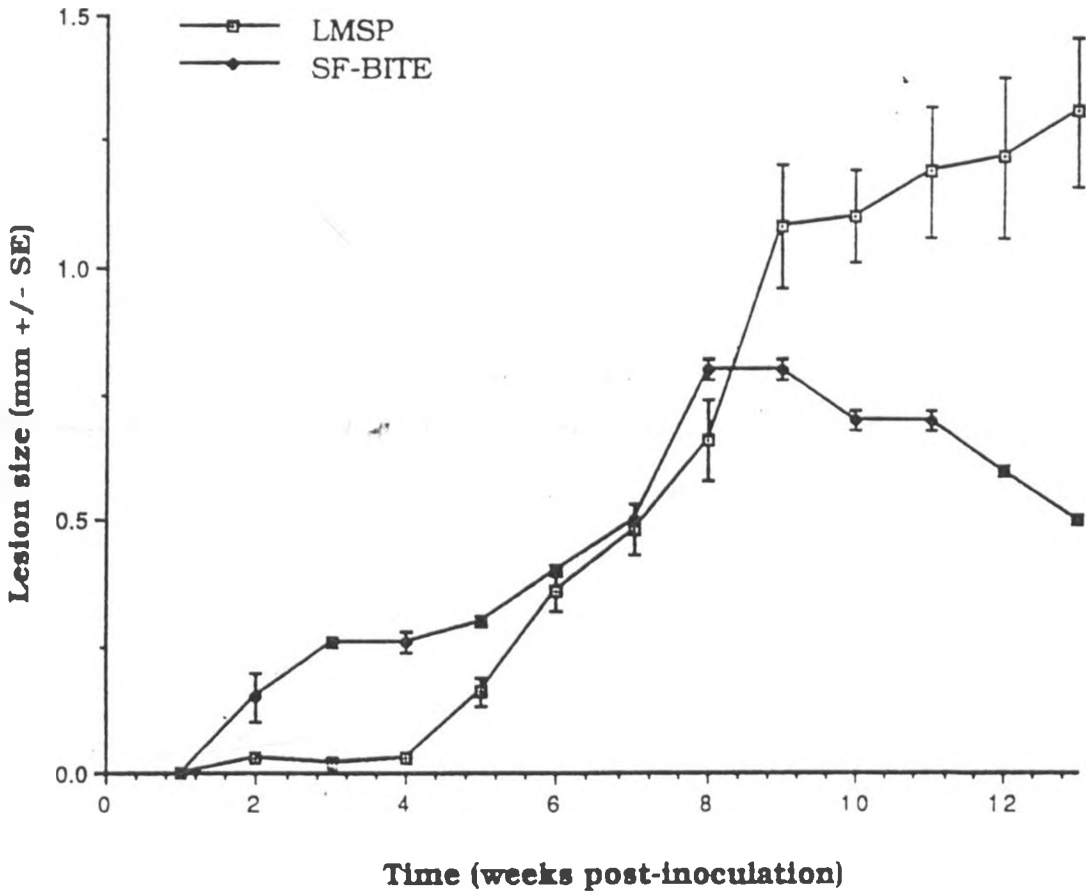

Figure 4. Results of lesion development in BALB/c mice inoculated with 1x10^3 *L. major* stationary phase culture promastigotes (LMSP) **and mice subjected to bites of *L. major*-infected *P. duboscqi*** (SF-BITE).

2.1.4. Discussion

Enhancement of *L. major* infection by saliva of *Lu. longipalpis* has been attributed to the erythema inducing factor (EIF). This component of sand fly saliva has also been referred to as the *Leishmania*-enhancing factor (LEF) by Titus and Ribeiro (1990), and more recently as maxadilan (Lerner *et al.*, 1991). It is the most potent vasodilator known so far and has a molecular weight of 3900 Daltons (Ribeiro, 1989; Titus and Ribeiro, 1990).

Maxadilan induces long lasting erythema in both human and rabbit skins, and has been found to act on the host rather than the parasite. Maxadilan has been shown to act on the macrophage directly by significantly inhibiting production of hydrogen peroxide in response to stimulation by gamma interferon (IFN-γ), and also by preventing antigen presentation (Nong *et al.*, 1989). These immunosuppressive effects have been shown to be present four days after injection with *Lu. longipalpis* saliva (Theodos and Titus, 1991), and are likely to play a role in enhancing *L. major* infection.

Erythemas are observed following bites of *Lu. longipalpis*, *Lu. gomesi* Nitzulescu and *Lu. whitmani* Antunes and Coutinho (New World sand flies), but are not observed following bites of *P. papatasi*, *P. (Euphlebotomus) argentipes* Annandale and Brunetti and *P. (Larroussius) perniciosus*

Newstead which are Old World sand flies (Ribeiro, 1989). This observation at one time led Ribeiro (1989) into concluding that enhancement of *Leishmania* transmission by saliva of Old World sand flies should not be expected. In later studies, Titus and Ribeiro (1990) and Theodos *et al.* (1991) reported that saliva of *P. papatasi* also exacerbates infection with *L. major*. In this study, it has been clearly demonstrated that mice co-injected with *L. major* stationary phase culture promastigotes and salivary gland lysates of *P. duboscqi* develop larger lesions than controls inoculated with *L. major* alone ($P < 0.01$, t-test). The mechanism by which enhancement of *L. major* infection in mice is achieved by *P. duboscqi* saliva is not known but this experiment suggests that *P. duboscqi* saliva also contains maxadilan in amounts that are able to enhance infectivity of *L. major*.

Sand flies of the genus *Phlebotomus* have five to ten times more salivary apyrase (ATP diphosphohydrolase) than those of the genus *Lutzomyia* (Ribeiro, 1989). During the biting act, sand flies inoculate apyrase into the wound of the host. As a result of tissue injury, platelets come into contact with collagen in subendothelial structures where injured cells release adenosine triphosphate (ATP) and adenosine diphosphate (ADP) (Vargaftig *et al.*, 1981). Acting as a major signal of cell lesion, ADP is a mediator of platelet aggregation in most vertebrates (Vargaftig *et al.*, 1981). Salivary apyrase converts ATP and ADP to AMP (adenosine monophosphate)

and orthophosphates. By so doing, it destroys the signal for platelet aggregation thus impairing platelet function by preventing blood from clotting. This process may be important in establishing *Leishmania* infection. Continuous bleeding of the host caused by enzyme apyrase may bring to the site of the bite more macrophages for parasitization by *Leishmania* promastigotes.

The large lesions observed during the first 7 weeks post-inoculation in mice subjected to bites of *L. major*-infected *P. duboscqi* may be attributed to persistence of oedema as a result of apyrase activity, maxadilan, and other sand fly salivary components. Sand fly saliva may be important in ensuring the slow development of a lesion thus slowing destructive pathology. Mice inoculated with $1x10^3$ parasites in the absence of sand fly saliva develop lesions at a steady rate and eventually, most of the lesions ulcerate resulting in the loss of necrotic tissue. This observation contrasts that of Bretscher *et al* (1992) who reported that mice inoculated with $1x10^3$ *L. major* promastigotes do not develop lesions but instead develop immunity to a second challenge with a homologous parasite strain. That there was no difference between lesion sizes in mice either inoculated with $1x10^3$ parasites or bites of 10 infected sand flies suggests that these two methods of parasite administration can supplement one another. Another possible mechanism by which enhancement of *L. major* infection in mice may be

achieved by saliva of *P. duboscqi* saliva may be by recruitment of macrophages to the site of inoculation by a chemotactic factor. This may also avail macrophages for parasitization in the absence of maxadilan. This possibility has been examined in another part of this study.

2.2. Inflammatory reactions of hamster skins towards *Phlebotomus duboscqi* saliva

2.2.1. Introduction

During the biting act, mandibles of the sand fly cut the host's skin in a combination of scissor-like and sawing movements. The maxilla-blade or lacinia, which lies outside of the fascicle is armed with teeth which engage the sides of the wound and hold the mouthparts in it during penetration (Lewis, 1975).

The salivary duct opens at the tip of the blade-shaped hypopharynx which may participate in making the wound. The lacinia, mandible, epipharynx and hypopharynx all act as piercing stylets (Adler and Theodor, 1926; Lewis, 1975). Lewis (1987) observed that the biting mouthparts of phlebotomine sand flies are short (less than 0.5 mm) and are unable to penetrate beyond the superficial layers of the skin. The sand flies being pool feeders, feed by lacerating

the capillary loops and ingesting blood pooling into the resulting heamatomas (Lewis, 1987; Lainson, 1982).

While studying sections of mouse skins with stylets of female *Phlebotomus argentipes* embedded in them, Short and Swaminath (1928) found that the fascicle (the bundle of stylets in the proboscis) was thrust into the skin for about 0.6 of its length (0.15 mm) and, steadied by the labium might extend into the dermis for six times the thickness of the epidermis thus reaching the smaller blood vessels. Adler and Theodor (1926) observed that in several Old World sand fly species, an interval of 15 seconds to 6 minutes occurs between biting and ingestion of blood. This observation led them to suggest that penetration of the mammalian skin by sand fly mouthparts could be difficult. Later studies on the feeding behaviour of uninfected and *L. major*-infected *P. duboscqi* (Beach *et al.*, 1985), and *L. major*-infected *P. papatasi* (Killick-Kendrick *et al.*, 1985) showed that uninfected sand flies locate blood more easily than *Leishmania*-infected sand flies, which tend to probe severally. Very little work has been done on the host's reaction towards the bites of sand flies.

The earliest account of skin reactions of people not previously exposed to sand fly bites was carried out by Theodor (1935) who used *P. papatasi*. He observed that there was little or no immediate reaction to first bites except the sharp pain, and further noted that 8-14 days

after the bite, a papule or a small blister appears which may persist for several weeks. In later bites on the same person, the papules appeared sooner, were more inflammed, larger and irritating. After several biting experiments the whole area around the bite sometimes became oedematous and the person showed a rise in temperature. At this stage, old bites which had disappeared were reactivated and the condition resembled urticaria. In still later biting experiments, the reactions were weaker except that the papules appeared earlier and sometimes immediatley after the bite. The wheal reaction persisted for a long time after which there was no skin reaction following the bite.

Although the cells at the site of infiltration and inflammation were not examined in Theodor's study, it suggests that the reactions taking place are those of type 1 hypersensitivity. It is well documented that when probing the host skin, sand flies eject saliva into the host (Adler and Theodor, 1926). It is possible that sand fly saliva could be an allergen responsible for the skin reactions, and that these reactions may have biological significance either in uptake of *Leishmania* by sand flies or the establishment of infection in the host. In order to test this hypothesis, this part of the work was designed to investigate the sequence of cellular inflammatory reactions of hamster skins following needle inoculation with salivary gland lysates, and after bites of uninfected and *L. major*-infected *P. duboscqi*.

2.2.2. Materials and methods

2.2.2(a). Inflammatory reactions of hamster skins to bites of uninfected *P. duboscqi*

Nine adult naive male hamsters with a mean weight of 140 ± 3.0 grams each and not previously exposed to bites of phlebotomine sand flies were used. Each hamster was anesthetized with 0.15ml pentobarbitone sodium (Sagatal^{R}). The belly of each hamster was shaved and each of these animals covered with a cloth envelop with a hole in it around the shaven belly to ensure that only the belly was exposed to the sand flies. The nine hamsters were separately introduced into feeding cages containing 20 laboratory-bred 3-day old uninfected female *P. duboscqi* that had been maintained on apple slices and kept at 25 ± 1°C and 90% relative humidity. Sand flies were allowed to feed on the hamsters for 10 minutes.

The external skin reactions to the bites were observed carefully, 15 minutes after the bite. The area around the bite spots was marked with Indian ink for accurate excision. Zones of redness for every ten bite spots were measured using a direct reading vernier caliper. Skin sections measuring 2x2mm with the bite spot in a central position were carefully excised from the hamsters at 15, 45, 60 minutes, 2, 4, 6, 24, 48 and 96 hours after sand fly bites. Nine animals were used for each biting interval, giving a

total of 9 replicates. For each replicate, a hamster inoculated with sterile PBS and not exposed to sand fly bites was sacrificed and used as a control. At each of the sampling times, skin portions that had been marked were excised from bellies (bite sites) of the experimental hamsters. Skin portions were similarly taken from control hamsters. The skin portions were fixed in 10% neutral buffered formalin (pH 7) prepared by weighing 4g of sodium dihydrogen phosphate (monohydrate), 6.5g anhydrous disodium hydrogen phosphate, and measuring 100ml formalin. These ingredients were all dissolved in sterile distilled water to make 1 litre.

2.2.2(b). Processing of histopathology skin tissue

Each skin portion was fixed in 10% neutral buffered formalin for 24 hours. These were then dehydrated in increasing concentrations of ethanol, starting with 70%, 80%, 90% and absolute analytical grade ethanol using the Automatic Tissue Processor (VRX-23). The dehydrated skin portions were transfered into chloroform (3 changes, each lasting 2 hours) and then impregnated with parafin wax (4 changes, lasting 2 hours). The impregnation was carried out manually and the tissues were then embedded in molten wax and stored at room temperature to dry. The skin tissue blocks were incubated at 20°C overnight to harden the wax.

These were transfered to 4°C for storage. Skin sectioning was done with the aid of the rotary microtome (Erma Optical Works Ltd, Tokyo, Japan). Each skin portion was cut giving a thickness of 5 microns. The sections were then transfered to a waterbath heated to 56°C to melt the wax and straighten the tissues. The tissues were picked up using a camel brush and mounted on slides. For every tissue, duplicate or triplicate sections were made depending on the type of staining to be carried out.

Skin tissue on microscope slides were dipped in xylene for 6 minutes to dissolve the wax. Sections that were to be examined for mast cells were hydrated in decreasing concentrations of ethanol, starting with analytical grade absolute ethanol, 90% and 70% ethanol for 3, 3 and 5 minutes respectively. The sections were rinsed in tap water for 5 minutes and then stained with 0.25% Toluidine blue stain for 10 seconds. The sections were rinsed in tap water and dried. At each site of the bite, ten 0.02mm^2 areas of the sections were examined for connective tissue mast cells. Similar areas were examined in sections of unbitten control hamsters. The number of connective tissue mast cells in each section were counted and recorded.

Sections to be stained with Myers haematoxylin and eosin were de-waxed in xylene, intermediate xylene and alcohol xylene. To remove xylene from tissue sections, the tissues were serially hydrated in two changes each of

absolute analytical grade ethanol, 95%, 90%, 80% and 70% ethanol. The sections were stained in haematoxylin for 20 minutes and then examined microscopically to determine whether the degree of staining was adequate. Sections found to have stained insufficiently were restained and those that had stained sufficiently were differentiated in 0.5% hydrochloric acid in 70% ethanol for 5 seconds to remove excess stain.

The sections were washed for 5 minutes in alkaline running tap water to stop the decolourization and then examined microscopically to determine the degree of decolourization. Sections seen to have decolourized sufficiently were stained in 1% aqueous eosin for 3 minutes. Surplus stain was washed off with tap water and sections let to dry after which they were examined microscopically to determine the degree of staining. Stained sections were dehydrated in increasing grades of ethanol as outlined above and cleared in xylene. Sections were air-dried and mounted in DPX. From each sand fly feeding site, ten 0.02mm fields were examined. The size of the field was determined by using the ocular micrometer. The numbers of connective tissue mast cells, eosinophils, neutrophils, lymphocytes and macrophages from each area were counted and recorded. The counts were compared with those from control hamsters.

2.2.2(c). Inflammatory reactions of hamster skins subjected to repeated bites of uninfected *P. duboscqi*

In order to determine the inflammatory reactions of hamster skins to repeated bites of uninfected *P. duboscqi*, 6 naive hamsters (138 ± 2.0g each) were shaven in the belly regions as previously described and exposed twice a week for 5 weeks to bites of 20 laboratory-bred 3-day old uninfected female *P. duboscqi*. The exposure duration was 10 minutes during which time 10 bite spots were measured with direct reading vernier calipers. The amount of time taken by individual sand flies to take a full bloodmeal was determined. During week 6, all the 6 hamsters and 2 unbitten controls were anesthetized with sodium pentobarbitone and sacrificed. Skin sections measuring 2x2mm were excised from bellies of the unbitten control and bitten experimental hamsters. These were fixed in 10% neutral buffered formalin and processed prior to staining as described in the section 2.2.2(b). The sections were examined as previously described for connective tissue mast cells, neutrophils, eosinophils, lymphocytes and macrophages.

2.2.2(d). Needle inoculation of hamsters with salivary gland lysates and sampling procedure

To serve as controls for the natural saliva inoculation by bites of *P. duboscqi*, the sand fly bite was substituted with needle inoculation. Salivary glands from 50 laboratory-bred female *P. duboscqi* were dissected as earlier described. Every 5 pairs of glands were transfered to 5µl of ice-cold phosphate-buffered saline and kept in ice until ready for use. Eighteen naive hamsters were anesthetized with pentobarbital sodium. The bellies were shaved as previously described and each of these animals was inoculated with 5µl of *P. duboscqi* salivary gland lysates in phosphate buffered saline containing 5 pairs of glands. To minimize tissue destruction and trauma caused during inoculation, animal inoculations were carried out using a 100µl capacity glass Hamilton syringe fitted with a fine 27 gauge needle. Nine hamsters were used as controls and were inoculated with a similar amount of phosphate-buffered saline.

Any reaction seen at the site of inoculation and any spots were measured with a direct reading vernier caliper. Two hamsters from the experimental group and one control at a time were sacrificed and sampled at 15, 45, 60, 120 minutes and at 4, 6, 24, 48 and 96 hours post-inoculation. Skins excised from the site of inoculation were processed, stained with 0.25% toluidine blue, and haematoxylin and

eosin (H&E). Skin sections were examined for connective tissue mast cells, eosinophils, neutrophils, lymphocytes and macrophages as previously described.

2.2.2(e). Inflammatory reactions of hamster skins exposed to bites of *L. major*-infected *P. duboscqi*

One hundred and eighty 3-day old female *P. duboscqi* were fed on *L. major* lesions on a nose of an anaesthetized BALB/c mouse placed in the colony cage. The mouse was placed within a cloth envelop with a hole in it through which the infected nose could protrude, ensuring that feeding took place only on the exposed lesion. Engorged sand flies were held in the colony cage in an insectary at 25 ± 1°C and 90% relative humidity for 9 days post feeding to ensure development of mature (metacyclic promastigotes) infections. Fresh apple slices were placed in the cage each day to serve as an energy source for the putatively infected sand flies.

On the tenth day, nine groups of 20 *L. major* infected *P. duboscqi* were placed in 30ml feeding cups fitted with fabric-screen lids (12 holes per linear cm). The outer surface of the screen lid of each feeding cup was pressed directly on the shaved belly skins of the anesthetized hamster. The sand flies were allowed to feed for 20 minutes. Hamsters fed on by sand flies were sacrificed and

skin portion at the bite sites biopsied at 15, 45, 60 minutes, 2, 4, 6, 24, 48 and 96 hours post infection. To ensure that the hamsters received infective doses of the parasites, 2 hamsters were kept and monitored for lesion development for 6 weeks. Sand flies were dissected within 24 hours after refeeding (11 days post-infection) to determine whether they had imbibed blood and if they were infected at the time of refeeding. Dissections were performed according to the technique of Johnson *et al.* (1963). Parasite forms and their positions in the sand fly gut were examined. Skin sections were examined as described previously for connective tissue mast cells, neutrophils, eosinophils, lymphocytes and macrophages.

2.2.3. Results

2.2.3(a). Histopathology of naive hamster skins subjected to bites of uninfected *P. duboscqi*

In all instances, sand fly-feeding on naive hamsters induced the formation of very tiny red spots measuring 0.7 to 1mm in diameter. These spots appeared 5-10 minutes at the site of the bite and disappeared after 1 to 2 hours. No other macroscopic manifestations were evident. Most sand flies took 5-10 minutes to take a full bloodmeal. Some sand flies were fully engorged after only 3 minutes.

A normal hamster skin before inoculation of sand fly saliva either by bite or artificially is shown in Plate 5(a). Plate 5(b) is a control normal hamster skin showing a constricted capillary. Both plates show few cells that are characteristic of the normal skin. Histological reactions in skin tissue prepared from hamsters dissected 15 minutes after the bites of sand flies were characterized by loose areolar fibrous connective tissue, with blood capillaries that were congested with red blood cells (Plate 6). The main inflammatory cells seen were neutrophils that were intravascularly located in the dilated blood vessels. Some of the neutrophils in the capillaries were seen marginating towards the vascular endothelium (Plate 7). Occasionally, some neutrophils could be seen in the connective tissue. Other inflammatory cells were rare. Few lymphocytes and eosinophils per $0.02mm^2$ areas could be seen. In tissues prepared 45, 60 minutes and 2 hours after the bite, vascular congestion with accompanying oedema was still the main pathological reaction. The reaction tended to diminish with time and was less pronounced after 2 hours. Peak neutrophilia was observed in biopsies prepared at 45 and 60 minutes after the bite. In these skin sections, there were a few sites with extravascular neutrophils but the majority were intravascularly located. Tissue macrophages were increasing in number.

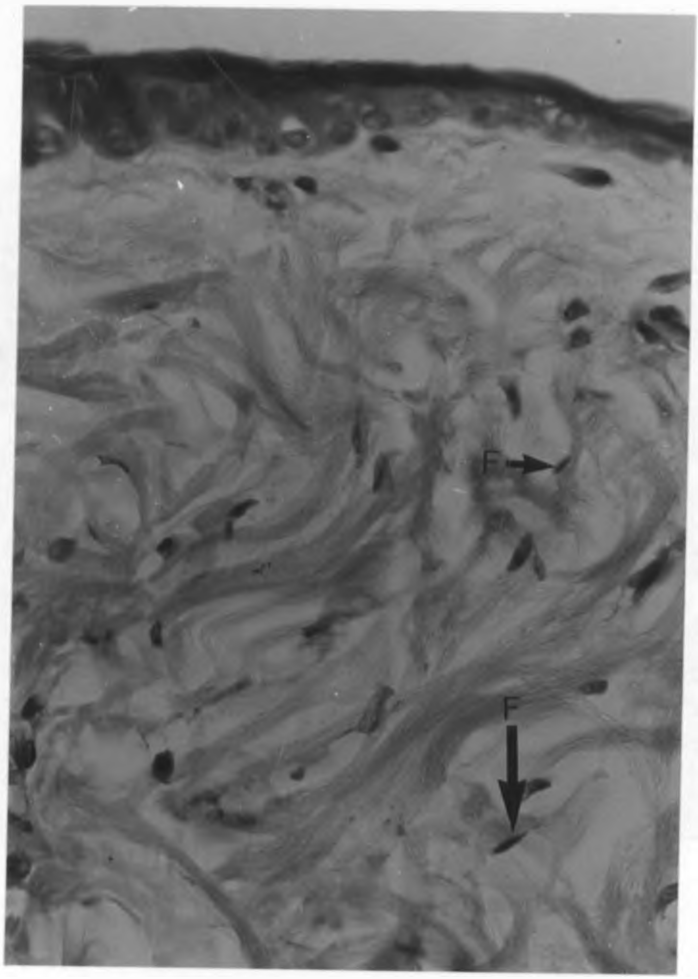

Plate 5(a). A normal control hamster skin before inoculation of *P. duboscqi* salivary gland lysates. F - fibroblasts, and no inflammatory cells (H&E x400).

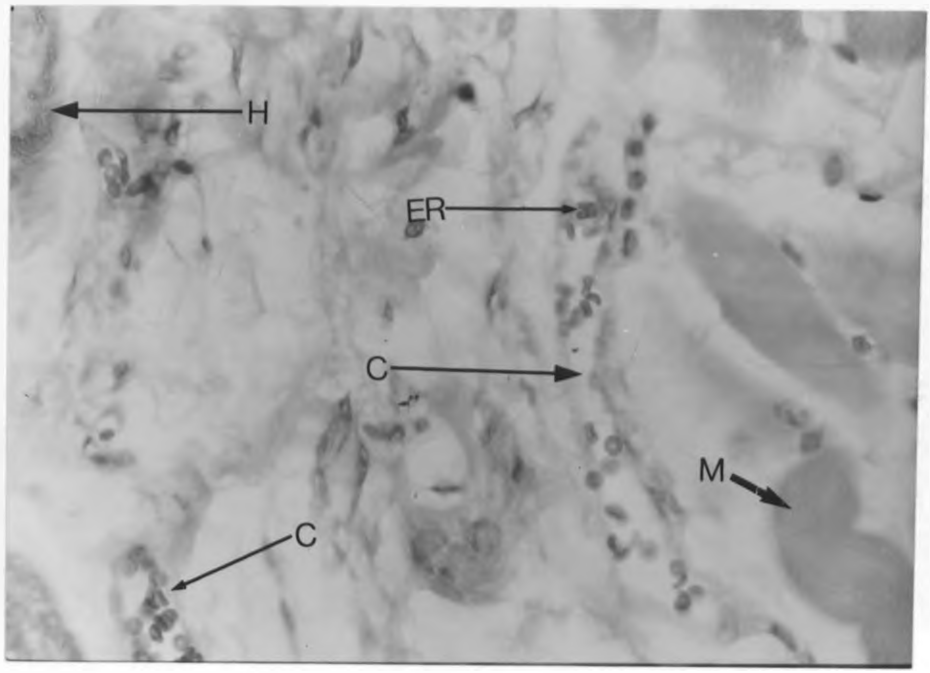

Plate 5(b). A normal hamster skin showing a capillary with few erythrocytes in the lumen. C - capillary, ER - erythrocytes, H - hair root and M - muscle (H&E x400).

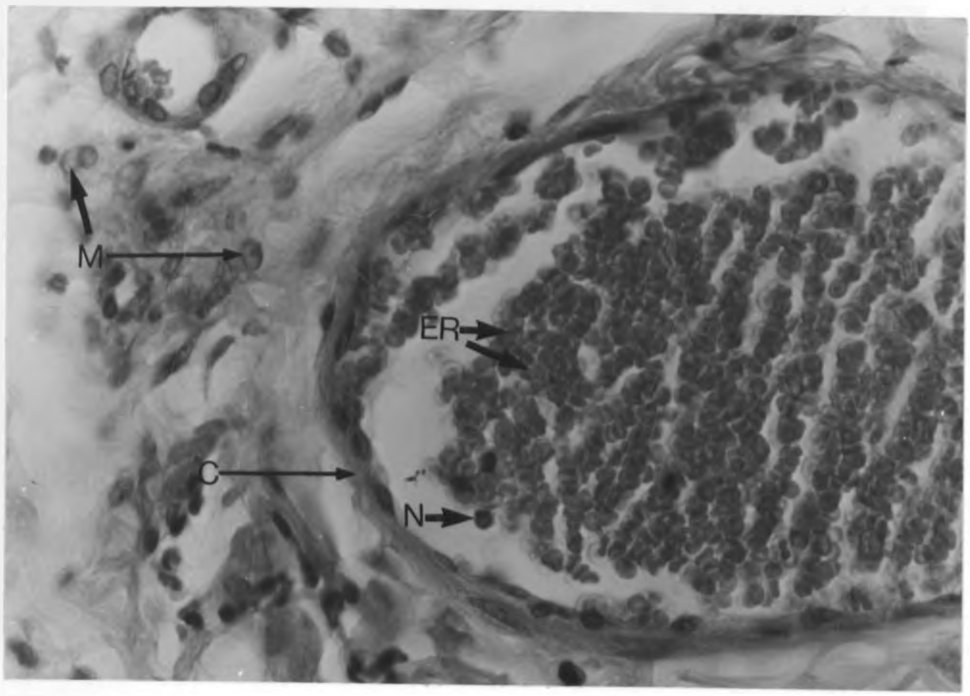

Plate 6. Capillary showing congestion with erythrocytes 15 minutes after bites of uninfected *P. duboscqi* . C - capillary, N - neutrophils, ER - erythrocytes and M - macrophages (H&E x400). See Plates 5(a) & 5(b) for controls.

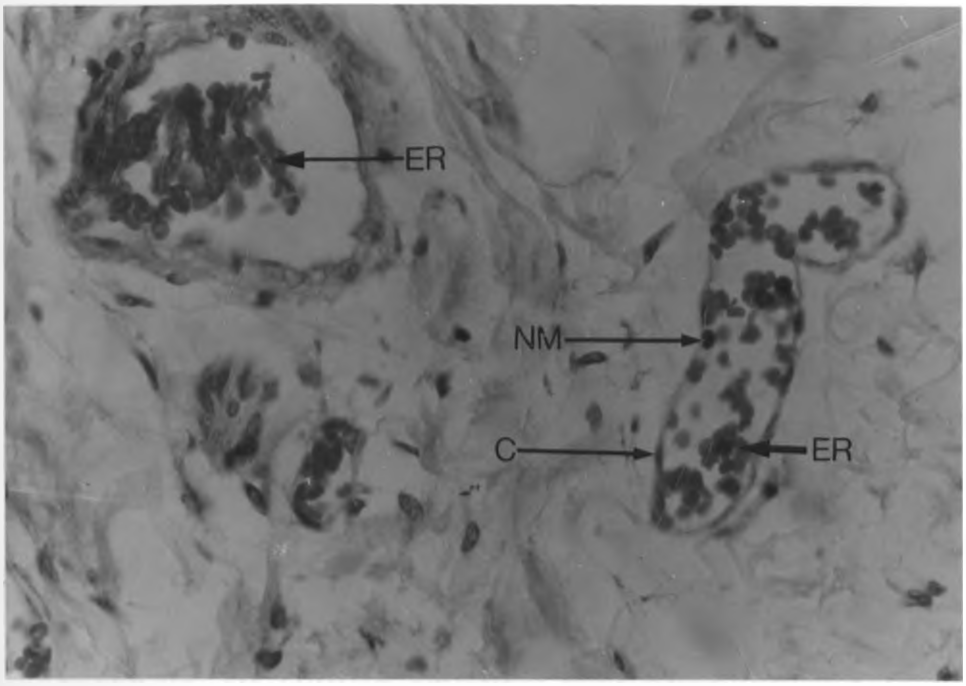

Plate 7. Capillary showing neutrophil margination and another showing congestion with erythrocytes 15 minutes after bites of uninfected *P. duboscqi*. C - capillary, NM - marginating neutrophil and ER - erythrocytes (H&E x400). See Plates 5(a) and 5(b) for controls.

In the 2-hour biopsies, oedema was strongly marked. Sites of extravasated red blood cells (a feature denoting the presence of haemorrhages) were seen. A few lymphocytes, eosinophils and macrophages could be seen occasionally. This type of reaction was also observed in 4 and 6-hour skin biopsies but in these sections, vascular congestion was unremarkable (Plate 8). Biopsies taken 24 hours post-sand fly bite showed a spread of inflammation and mild oedema in most of the dermal tissues. Inflammatory cells were predominantly neutrophils, macrophages and eosinophils. Vascular congestion had subsided to a level compared to that observed in control biopsies and inflammatory cells were spread in most of the epidermal tissue. In biopsies taken 48 hours after the bite, the predominant inflammatory cell type was the macrophage (Plate 9). Neutrophils and eosinophils were also present in lower numbers. No vascular distension was evident, but lymphocytes had slightly increased in number. This type of inflammatory reaction did not change drastically in biopsies taken 96 hours after the bite. There was however an increase in macrophages, fibroplasia and an accompanying increase in fibrous tissue and epidermal hyperplasia (epidermis was 4-6 cells thick). Most of the inflammatory cells were concentrated at the site of the bite. The inflammatory reaction in 24, 48 and 96-hour biopsies was composed of mixed cell inflitrate with macrophages as the main cells.

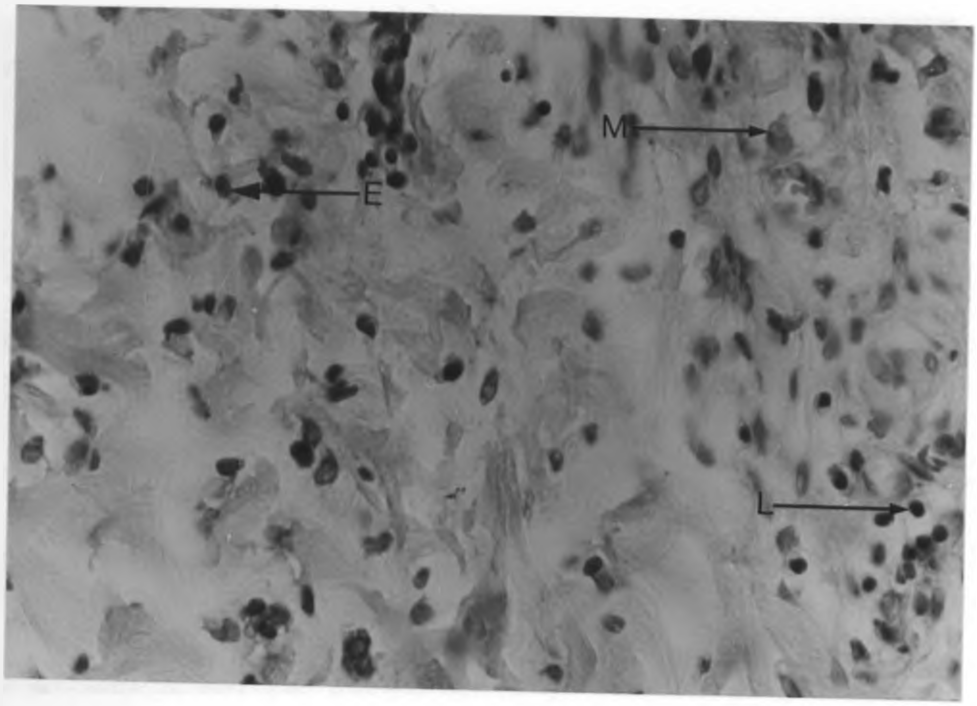

Plate 8. Skin biopsy taken 6 hours after bites of uninfected *P. duboscqi* showing a mixed cell infiltrate. E-eosinophil, L-lymphocyte and M-macrophage (H&E x400). See Plate 5(a) for the control.

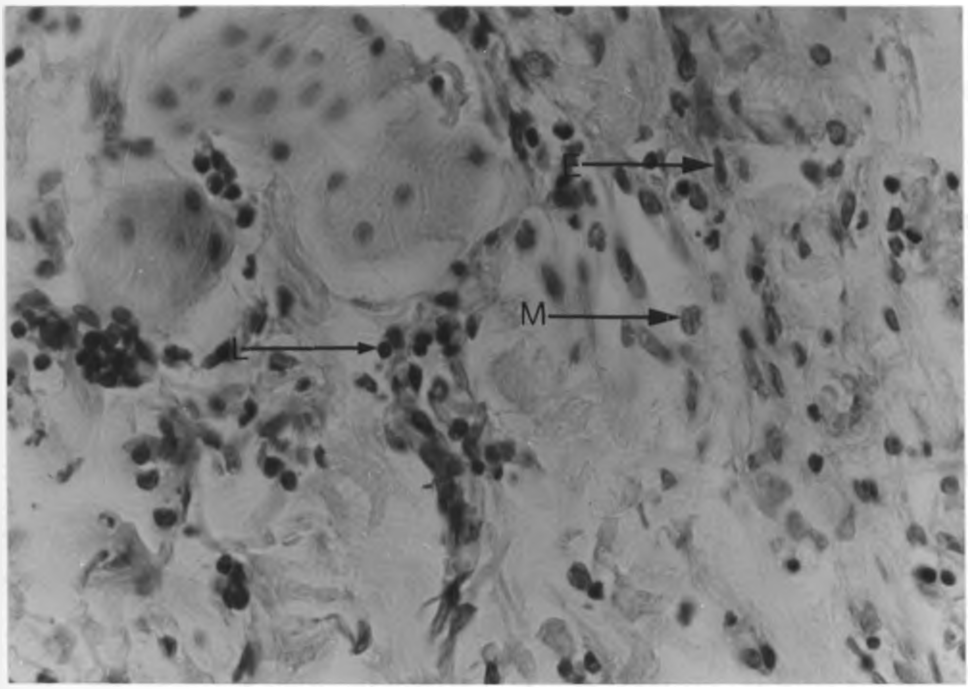

Plate 9. Skin biopsy taken 48 hours after the bite of uninfected *P. duboscqi* showing a mixed cell infiltrate. E-eosinophil, M-macrophage and L-lymphocyte (H&E x400). See Plate 5(a) for the control.

In some sections, within the superficial dermis, neutrophils, eosinophils and occasional lymphocytes could be seen. Surrounding these areas were numerous macrophages. One or two eosinophils could be seen in the epidermis. The entire cellular infiltrate was morderately wedge shaped. These areas are the actual bite sites. Away from the bite, the number of cells tended to decrease. In all biopsies examined there was no noticeable difference in the numbers of dermal mast cells when compared with the controls. Tables 3, 4, 5, 6, and 7, are summaries of cell numbers counted per $0.02mm^2$ skin biopsy sections respectively. In most of the sections, the number of neutrophils and macrophages was higher than those observed in skin biopsies from control hamsters.

Table 3. Summary of mean numbers of tissue mast cells in skin biopsies of hamsters.

Biopsy time	Mean number of skin mast cells ± standard error			
	Uninfected fly-bite skin	Infected fly-bite skin	Needle-inoculated skin	N
Control (0 min)	7.7 ± 0.6	7.7 ± 0.6	7.7 ± 0.6	9
15 min	8.0 ± 0.6	10.0 ± 2.5	7.2 ± 1.6	9
45 min	8.4 ± 0.7	7.1 ± 1.2	5.6 ± 0.7	9
60 min	8.7 ± 0.8	11.7 ± 1.4	5.3 ± 0.6	9
2 hr	9.6 ± 1.0	7.0 ± 0.7	4.0 ± 0.5	9
4 hr	10.0 ± 1.0	11.4 ± 2.0	7.1 ± 1.0	9
6 hr	10.0 ± 1.1	6.5 ± 1.4	7.7 ± 1.2	9
24 hr	8.0 ± 1.4	7.0 ± 1.8	8.0 ± 1.6	9
48 hr	6.0 ± 1.0	8.8 ± 1.0	3.5 ± 0.7	9
96 hr	9.0 ± 1.0	7.6 ± 1.7	1.2 ± 0.4	9

Note: Repeated bite skin mast cell mean count was 20 ± 3.0

Table 4. Summary of mean numbers of neutrophils in skin biopsies of hamsters.

Biopsy time	Mean number of neutrophils ± standard error			
	Uninfected fly-bite skin	Infected fly-bite skin	Needle-inoculated skin	N
Control (0 min)	14.0 ± 0.4	14.0 ± 0.4	14.0 ± 0.4	9
15 min	5.1 ± 1.3	5.5 ± 1.0	2.0 ± 0.6	9
45 min	15.4 ± 1.3	8.0 ± 3.9	2.3 ± 2.0	9
60 min	24.8 ± 6.0	8.0 ± 2.3	3.0 ± 1.0	9
2 hr	6.0 ± 1.1	9.7 ± 3.0	9.0 ± 1.6	9
4 hr	6.2 ± 2.1	8.0 ± 3.7	10.0 ± 3.0	9
6 hr	5.0 ± 1.3	10.4 ± 2.9	14.0 ± 9.2	9
24 hr	6.0 ± 2.1	5.0 ± 1.9	2.5 ± 0.6	9
48 hr	6.3 ± 1.1	2.6 ± 0.5	4.2 ± 1.4	9
96 hr	3.3 ± 1.5	2.4 ± 0.2	9.0 ± 2.0	9

Note: Repeated bite skin neutrophil mean was 18.7 ± 6.2.

Table 5. Summary of mean numbers of macrophages in skin biopsies of hamsters.

Biopsy time	Mean number of macrophages ± standard error			
	Uninfected fly-bite skin	Infected fly-bite skin	Needle-inoculated skin	N
Control (0 min)	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	9
15 min	1.2 ± 0.5	2.3 ± 0.5	1.5 ± 0.3	9
45 min	1.0 ± 0.4	3.8 ± 0.8	1.0 ± 0.5	9
60 min	1.0 ± 0.4	8.5 ± 1.4	1.0 ± 0.3	9
2 hr	2.0 ± 1.1	13.0 ± 1.6	1.5 ± 0.6	9
4 hr	2.5 ± 1.5	13.0 ± 0.9	3.4 ± 0.5	9
6 hr	2.5 ± 1.0	14.0 ± 1.5	5.8 ± 1.2	9
24 hr	4.0 ± 0.5	18.0 ± 2.6	10.0 ± 0.7	9
48 hr	6.2 ± 1.8	23.0 ± 3.0	8.0 ± 2.1	9
96 hr	16.8 ± 2.6	19.0 ± 1.2	20.0 ± 3.8	9

Note: Repeated bite skin macrophage mean was 18.0 ± 1.7

Table 6. Summary of mean numbers of eosinophils in skin biopsies of hamsters.

Biopsy time	Means number of eosinophils ± standard error			
	Uninfected fly-bite skin	Infected fly-bite skin	Needle-inoculated skin	N
Control (0 min)	0	0	0	9
15 min	8.0 ± 0.6	10.7 ± 2.5	7.2 ± 1.6	9
45 min	8.4 ± 0.7	7.1 ± 1.2	5.6 ± 0.7	9
60 min	8.7 ± 0.8	10.7 ± 1.4	5.3 ± 0.6	9
2 hr	9.6 ± 1.0	8.0 ± 0.7	6.0 ± 0.5	9
4 hr	10.0 ± 1.0	11.4 ± 2.2	7.1 ± 1.0	9
6 hr	10.0 ± 1.1	6.4 ± 1.4	7.7 ± 1.2	9
24 hr	8.0 ± 1.4	7.0 ± 1.8	8.0 ± 1.6	9
48 hr	6.0 ± 1.0	8.8 ± 1.0	3.5 ± 0.7	9
96 hr	9.0 ± 1.0	7.6 ± 1.7	1.2 ± 0.4	9

Note: Repeated bite skin eosinophil mean was 3.0 ± 0.5.

Table 7. Summary of mean numbers of lymphocytes in skin biopsies of hamsters.

Biopsy time	Mean number of lymphocytes ± standard error			
	Uninfected fly-bite skin	Infected fly-bite skin	Needle-inoculated skin	N
Control (0 min)	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	9
15 min	8.0 ± 0.6	6.7 ± 2.5	7.2 ± 1.6	9
45 min	8.4 ± 0.7	7.1 ± 1.2	5.6 ± 0.7	9
60 min	8.7 ± 0.8	11.0 ± 1.4	5.3 ± 0.6	9
2 hr	9.6 ± 1.0	9.0 ± 0.7	5.0 ± 0.5	9
4 hr	10.0 ± 1.0	11.0 ± 2.2	7.1 ± 1.0	9
6 hr	10.0 ± 1.1	7.4 ± 1.4	7.7 ± 1.2	9
24 hr	8.0 ± 1.4	7.0 ± 1.8	8.0 ± 1.6	9
48 hr	7.0 ± 1.0	8.8 ± 1.0	3.5 ± 0.7	9
96 hr	9.0 ± 1.0	7.6 ± 1.7	1.2 ± 0.4	9

Note: Repeated bite skin lymphocyte mean was 4.5 ± 1.2

2.2.3(b). Inflammatory reactions of hamster skins to repeated bites of uninfected *P. duboscqi*

On recovering from anesthesia, hamsters exhibited intense grooming after the first bite. The grooming intensity declined with subsequent sand fly feeding and was barely noticeable after the eighth feeding. A week later, after the first and second sand fly feeding, the site of the bite sometimes developed a scaly appearance. This was not seen to happen after the sixth bite. A tiny red spot at the site of the bite was the only macroscopic reaction observed at the site of the bite. The diameter of the red spots determined by taking an average of 10 bite spots was 0.7mm. As observed in naive hamsters, the tiny erythema disappeared after 1-2 hours.

Microscopic reactions towards repeated sand fly bites included epidermal hyperplasia that was very marked with 3-4 cell layers. The bite site had a moderate population of inflammatory cells most of which were located in dermal tissue. The predominant cells were macrophages and neutrophils with mean values of 18.0 $\pm$ 1.7 and 18.7 $\pm$ 6.2 per 0.02mm^2 area respectively (Plate 10). Fibroblast proliferation was remarkable in areas with mixed inflammatory cells particularly macrophages. These biopsy tissues exhibited a large number of mast cells per unit area with a mean of 20.2 $\pm$ 3.0 compared to the control mean of 5.4 $\pm$ 0.3 cells per 0.02mm^2 area.

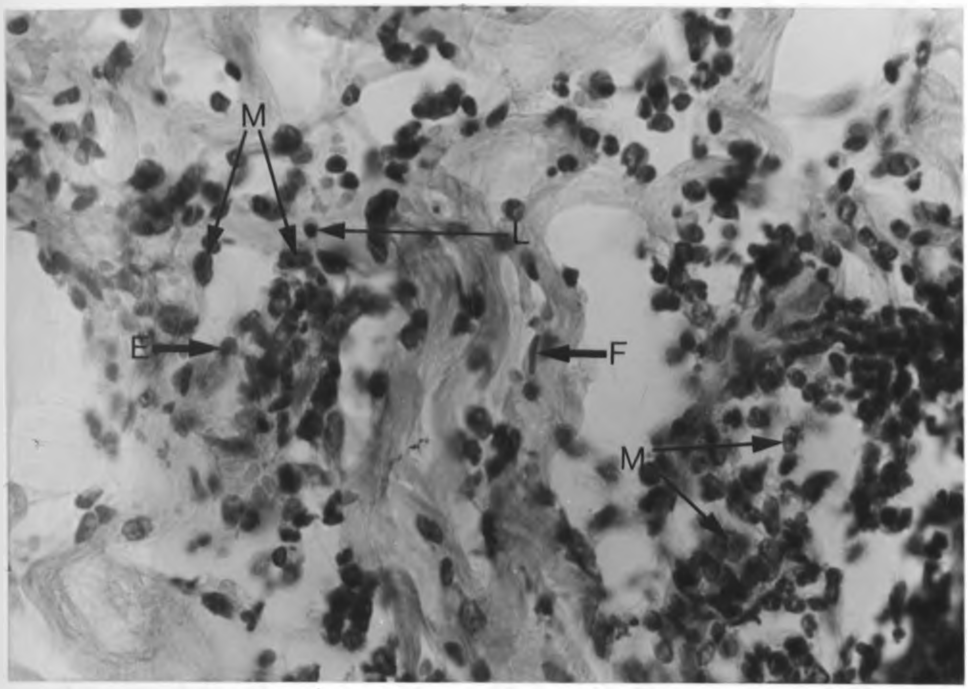

Plate 10. Skin biopsy taken from hamsters subjected to repeated bites of uninfected *P. duboscqi* showing many macrophages and cellular infiltrate. M-macrophage, F-fibroblast, E-eosinophil and L-lymphocyte (H&E x400). See Plate 5(a) for the control.

2.2.3(c). Inflammatory reactions of hamster skins after needle inoculation with *P. duboscqi* salivary gland lysates

Unlike hamsters bitten by uninfected sand flies, all control hamsters inoculated with 5µl *P. duboscqi* salivary gland lysates in phosphate buffered saline intradermally, did not display any erythema at the site of inoculation. Inflammatory reactions to needle inoculation with salivary gland lysates (SGL) after 15 minutes were very marked. At the site of the bite, a mixed cell infiltrate consisting of neutrophils, lymphocytes, eosinophils and macrophages was observed (Plate 11). The mean numbers of eosinophils observed in these sections (7.2 ± 1.6) was not different from that of biopsies from hamsters fed on by uninfected *P. duboscqi* which was 8.0 ± 0.6 (Table 6.). Mild distension of capillaries and areas of extravasated erythrocytes and oedema were seen. One to two macrophages and neutrophils were seen per 0.02mm^2 area. This reaction did not change much after 45, 60 minutes and 2 hours. In biopsies taken at 4 hours and 6 hours post-needle inoculation, the number of neutrophils and macrophages were seen to increase several times above those of controls (Table 4 and 5). Capillary distension and congestion was not apparent.

At 24, 48 and 96 hours post-needle inoculation, the inflammation was composed of a mixed cell infiltrate, with

macrophages replacing neutrophils and eosinophils (Plates 12a and 12b). This was highly evident in the 48 and 96 hour biopsies, respectively. Marked hyperkeratosis, intracellular oedema in epidermal cells, focal haemorrhages with extravasated erythrocytes and focal early granulomas with lymphoplasmacytic cells and macrophages were seen in the 96 hour biopsies skin sections. Other areas were characterized by multifocal to diffuse areas of cellular infiltration by lymphoplasmacytic cells admixed with numerous macrophages. In all SGL needle inoculated biopsies, the total numbers of dermal mast cells were lower than those of controls and those from hamsters subjected to repeated bites of *P. duboscqi*.

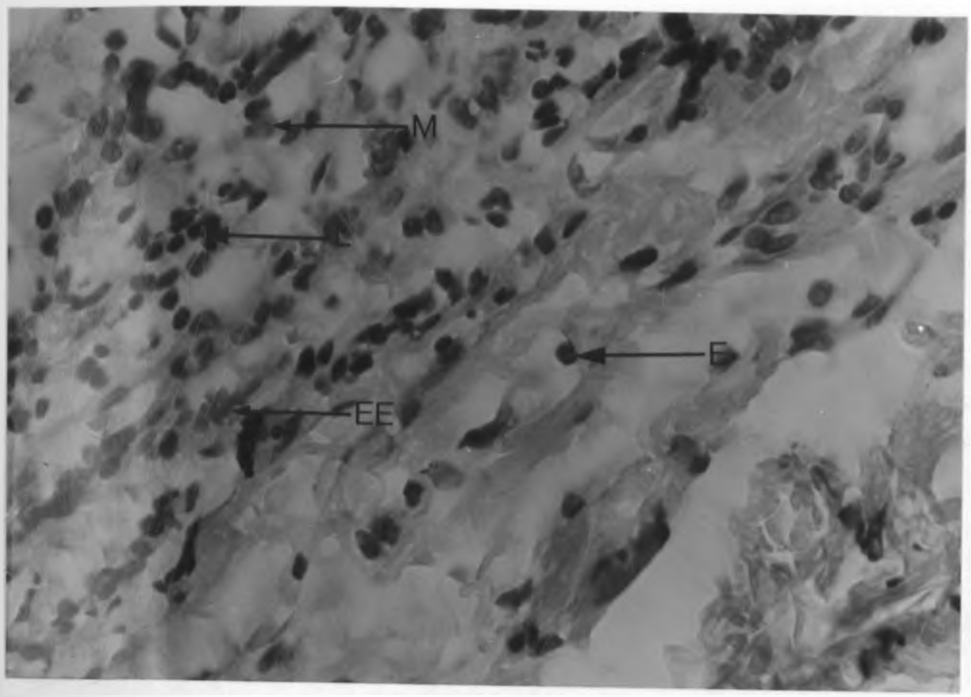

Plate 11. Skin biopsy taken 15 minutes after needle inoculation of *P. duboscqi* salivary gland lysates showing a mixed cell infiltrate. E - eosinophil, M - macrophage, EE - extravasated erythrocytes and L - lymphocyte (H&E x400). See Plate 5(a) for the control.

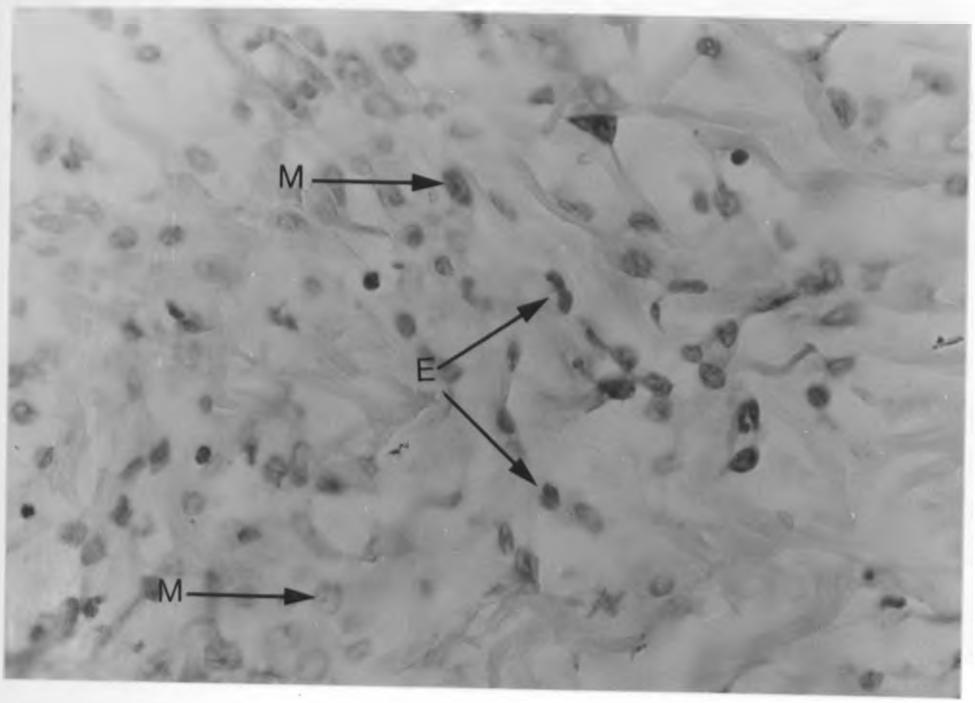

Plate 12(a). Skin biopsy taken 24 hours after needle inoculation of *P. duboscqi* showing a mixed cell infiltrate with macrophages. E-eosinophil and M-macrophage (H&E x400). See Plate 5(a) for the control.

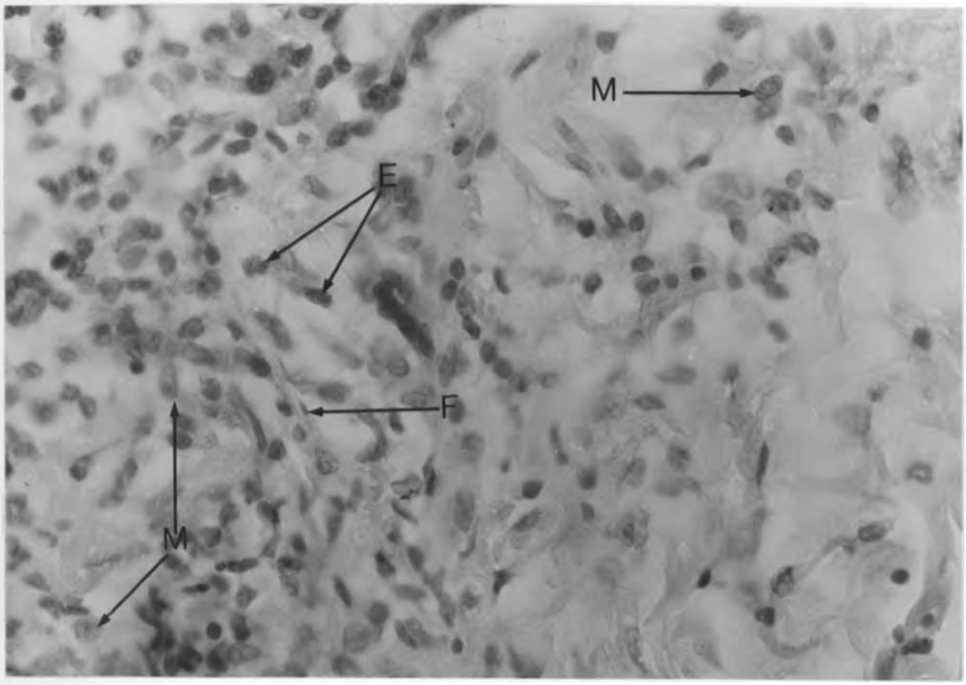

Plate 12(b). Skin biopsy taken 96 hours after needle inoculation with *P. duboscqi* salivary gland lysates showing a mixed cell infiltrate with many macrophages. E - eosinophil, M - macrophage and F - fibroblast (H&E x400). See Plate 5(a) for the control.

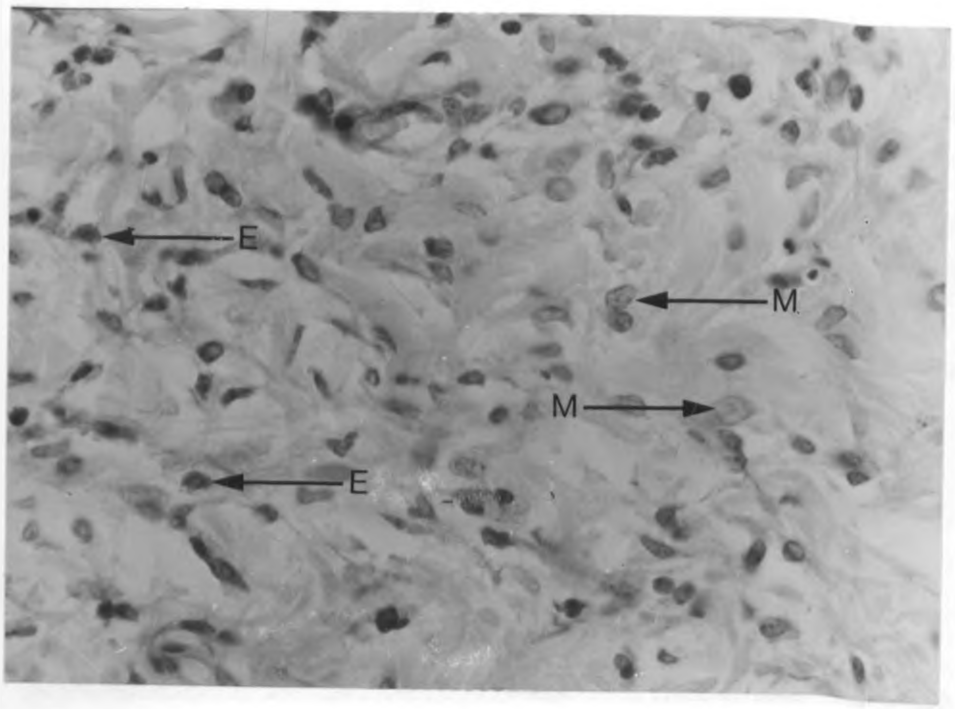

Plate 13. Skin biopsy taken 48 hours after the bite of *L. major*-infected *P. duboscqi* showing a mixed cell infiltrate. E - eosinophil and M - macrophage (H&E x400). See Plate 5(a) for the control

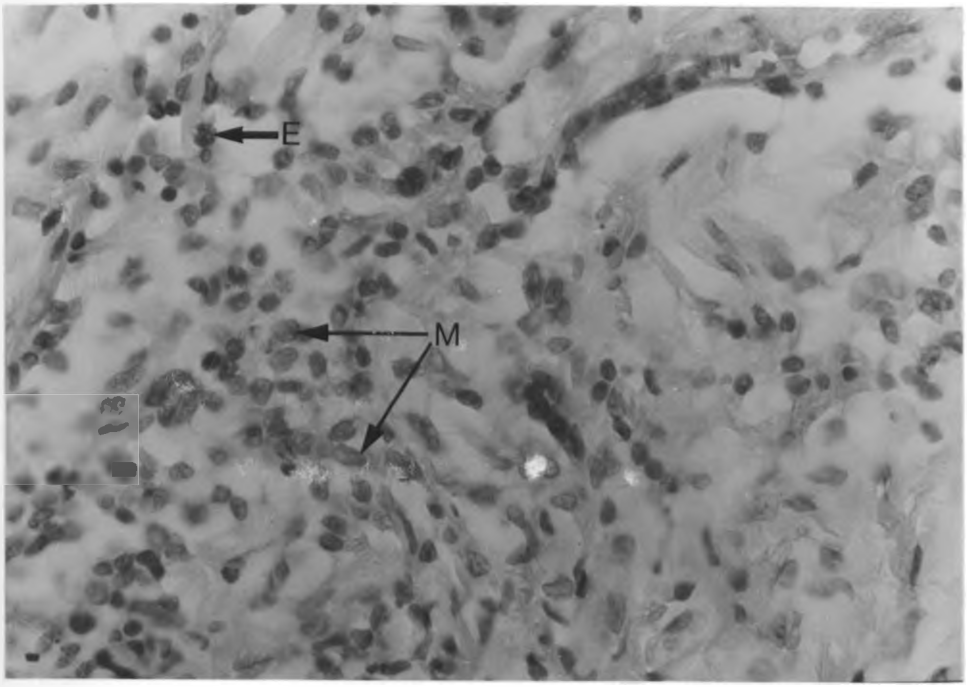

Plate 14. Skin biopsy taken 96 hours after bite of *L. major*-infected *P. duboscqi* showing a mixed cell infiltrate with numerous macrophages. E - eosinophil and M - macrophage (H&E x400). See Plate 5(a) for control.

2.2.3(d). Inflammatory reactions of naive hamsters to bites of *L. major*- infected *P. duboscqi*

All sand flies that were used had 10 day old infections of *L. major*. Examination of the sand flies after dissection revealed the presence of nectomonad, haptomonad, paramastigote and metacyclic promastigotes. At the start of the biting experiment, 20 putatively infected *P. duboscqi* were used per hamster. This number was maintained for the 15, 45, 60 minutes, 2 and 4-hour biopsy groups of hamsters, but due to high mortality of the putatively infected sand flies during oviposition, the number of putatively infected sand flies that were used in biting the 6, 24, 48, and 96-hour biopsy groups of hamsters was increased to 45 per hamster.

During the feeding exercise, most of the uninfected sand flies refed and took 5-10 minutes to take a full bloodmeal. Some heavily infected sand flies that were not able to feed had blood anterior to the heavily infected stomodael valve and none in the midguts. In these sand flies, metacyclics were seen swimming in the pharynx, anterior to the blood clot. Like in earlier observations where uninfected *P. duboscqi* were used, bites of *L. major*-infected *P. duboscqi* induced the formation of tiny red spots in hamster skins. Biopsy sections taken 15 minutes after the bite were similar to those previously described and were

characterized by vascular congestion. Intravascular neutrophilia and marked oedema were seen. Margination of neutrophils was evident. Few lymphocytes, eosinophils and macrophages were occasionally seen.

The histological reactions observed in skin sections taken at 45, 60 minutes, 2, 4 and 6 hours after the bite changed from the predominantly neutrophilic reaction seen in the 15 minutes biopsy section to a mixed cell infiltrate. In these sections, neutrophilia and vascular congestion declined and the cellular infiltrate was composed of eosinophils, macrophages and lympocytes which were seen to increase in number with time. In the 6 hour biopsies, oedema could still be seen. Notable in these sections was the tremendous increase in the number of dermal macrophages in the mixed cell infiltrate. Occasionally, nucleated red blood cells (acidophilic normoblasts were seen).

In biopsy sections taken at 24, 48 and 96 hours after the bite, the inflammatory reaction was composed of a mixed cell infiltrate with macrophages as the predominant cells (Plates 13 and 14). Eosinophils, most of which had degranulated, lymphocytes, tissue neutrophils and acidophilic normoblasts were also seen. In the 96 hour biopsies, macrophages, neutrophils and eosinophils could be seen in blood vessels and in the adipose layer. In all the sections, the number of macrophages and neutrophils was

higher than in controls (Tables 4 and 5). Apart from the 15 minutes biopsy section, there was no noticeable difference between the number of mast cells in the other sections and the control.

The pattern of increase in macrophages from time 0 to 96 hours was not different in skins subjected to bites of uninfected *P. duboscqi*, *L. major*-infected *P. duboscqi* and skins inoculated with salivary gland lysates using a needle. When analysed using the Analysis of variance test (ANOVA) and t-test, means of macrophage counts in skins subjected to bites of uninfected *P. duboscqi* and skins inoculated with salivary gland lysates using a needle were not significantly different ($P > 0.05$), but were significantly different from the mean count of skins of hamsters bitten by *L. major*-infected *P. duboscqi* ($P < 0.05$). This difference could have been caused by the presence of promastigotes at the site of the bite. Using ANOVA, the means of macrophages in the 3 groups of hamster skins were found to be significantly different ($F_{0.05\ [2,24]} = 5.081$, $P < 0.05$).

There was no significant difference in the numbers of eosinophils, lymphocytes and neutrophils in skins of hamsters bitten by uninfected *P. duboscqi* and in skins bitten by *L. major*-infected *P. duboscqi*. Significant differences in numbers of eosinophils, mast cells and lymphocytes but not neutrophils were seen between these skins and skins inoculated with salivary gland lysates using a

needle. Throughout the study, the difference in neutrophil counts was not significant between and within the three groups ($F_{0.05,[2,24]} = 0.629$, $P > 0.05$). The difference in numbers of mast cells between hamster skins bitten by uninfected *P. duboscqi* and those bitten by *L. major*-infected *P. duboscqi* was not significant (t-test $P > 0.05$). The difference between these two groups and skins inoculated with salivary gland lysates using a needle was significant ($P < 0.05$).

2.2.4. Discussion

It is apparent that at all sampling time intervals, skins of hamsters bitten by *L. major*-infected *P. duboscqi* had higher macrophage mean counts than skins bitten by uninfected sand flies and those inoculated with salivary lysates using a needle. This difference suggests that the presence of promastigotes must have also attracted more macrophages at the site of the bite.

The inflammatory reactions of hamster skins after needle inoculation with sand fly salivary gland lysates, and after exposure to bites of uninfected and *L. major*-infected *P. duboscqi* were histologically studied. The sequence of inflammatory reactions towards sand fly saliva inoculated either by needle or by bite of uninfected or infected sand

flies was generally similar, but later intensified in skins bitten by sand flies. The reactions towards the bite can be classified as either macroscopic or microscopic.

Macroscopic reactions involved the formation of tiny red bite spots with diameters of 0.7-1.0mm. These spots formed 10-15 minutes post bite and disappeared after 1-2 hours. Needle inoculation did not generate this type of reaction. The reason for this difference may be that needle size causes a rather deeper and diffuse distribution of the salivary lysate, hence the reaction is generally diffuse. However, the sand fly bite is usually much smaller and the reaction more defined. But it may be due to formation of a haematoma. The small size of the erythema that forms at the bite spot, and the fast rate at which it disappears, makes it not easily noticeable. This has led most scientists to erronously conclude that saliva of sand flies of the genus *Phlebotomus* does not cause erythema. The difference between erythema caused by bites of sand flies of this genus, and that caused by sand flies of the genus *Lutzomyia* is that the latter is of a higher intensity and it tends to persist unlike the former.

During the first 15-120 minutes after either needle inoculation or sand fly bite (uninfected and infected), microscopic reactions initially involved vasodilation accompanied by vascular congestion with erythrocytes and neutrophils. Neutrophils were the predominant cell type.

This reaction was later accompanied by infiltration by eosinophils and lymphocytes. This kind of reaction has been reported in skins of patients bitten by uninfected *Aedes aepyti* and *Anopheles quadrimaculatus* Say (Goldman *et al.*, 1952). After 2-6 hours, sites of extravasated erythrocytes (haemorrhages) were evident. The presence of haemorrhages is clear histological evidence that sand flies are pool feeders and not solenophagous. Epidermal hyperplasia which was very pronounced in the early phases of inflammation has been reported before in several cases of arthropod bites including those by mosquitoes (Goldman *et al.*, 1952), mice lice (Nelson *et al.*, 1972), chiggers and ticks (Allen, 1948). The inflammatory reaction after 6 hours changes to a mixed cell infiltrate consisting of tissue neutrophils, eosinophils, lymphocytes and macrophages. Oedema and vascular congestion with erythrocytes and neutrophils decline. After 24-48 hours, the numbers of neutrophils, eosinophils and lymphocytes decline sharply, and macrophages are seen to increase. By 96 hours, the macrophage is the predominant inflammatory cell type.

Histological sections of the skins that had been subjected to repeated bites of uninfected *P. duboscqi* had the highest number of macrophages. Some sections had clusters of these cells within the superficial dermis accompanied by mild hyperplasia. In most foci, macrophages admixed with fewer neutrophils, eosinophils,

and lymphocytes were seen. These sections had the highest number of mast cells. Whether mast cells are involved in host defense against sand fly bites is yet to be established. Nelson *et al.* (1972) reported a similar increase in mast cells in RML white mice subjected to bites of the louse *Polyplax serrata* (Anoplura: Haematopinidae).

Notable in this study is the finding that inoculation of sand fly saliva into host skin either artificially, or naturally by bite leads to an inflammatory reaction that culminates in an increase in the number of macrophages. In skins subjected to repeated bites, granulomas of macrophage origin may form. A recent study by Laurenti *et al.* (1992) that centred on microscopic examination of the inflammatory response of hamsters at subcutaneous sites at which *L. chagasi* mixed with *Lu. longipalpis* salivary gland lysates were inoculated gave results similar to those reported in this experiment.

Establishment of *Leishmania* infection in a host is dependent upon successful parasitization of macrophages. Any factor that increases the number of macrophages in the skin will therefore increase the chances of successful parasitization, by availing large numbers of macrophages to the *Leishmania* deposited by the feeding infected sand fly. Allen (1948) reported that insect bite reactions may persist for 2 or more years in the form of active dermal eosinophilic granulomas. These granulomas consist of a dense dermal infiltrate characterised by large numbers of eosinophilic

leucocytes, plasma cells and macrophages. This reaction is accompanied by epidermal hyperplasia. The author concluded that the stimulating agent of the arthropod somehow persists actively in the focus of these lesions for a long time.

These observations were made in histological studies of reactions to bites of ticks, chiggers, mosquitoes and other arthropods, but the agent responsible for the persistent cutaneous reaction remains to be established. Experiments carried out by Theodor (1935) on bites of *P. papatasi* showed that at least the macroscopic reactions which are determined by microscopic reactions can be reactivated in subsequent bites. In this study, a person who had been bitten by *P. papatasi* 20 years ago in Mesopotamia gave an immediate wheal reaction at the previous bite site. This reaction occured following a second bite by the same sand fly species at a different site. In Theodor's study, the histological nature of the reaction was not studied. It remains to be established whether or not the increase in macrophages observed in this study persists in host skin following sand fly bite.

Considering that skins of hamsters subjected to repeated bites of *P. duboscqi* display a large number of macrophages, it may be that people in endemic foci who are subjected to many sand fly bites may display this phenomenon. If this is true, it may explain how post-kala-

azar dermal leishmaniasis, whereby after successful chemotherapy for visceral disease, clinically cured patients from the endemic zones usually develop huge skin granulomas of macrophage origin that are full of *Leishmania* amastigotes. An increase in skin macrophages may influence the course and later the outcome of infections with *L. donovani* and *L. major*. This part of the study has shown that sand fly saliva *in vivo* attracts macrophages and may therefore be chemotactic to these cells. This possibility has been examined *in vitro* in the next part of the study.

2.3. *In vitro* testing of chemotactic effects of *Phlebotomus duboscqi* saliva on mouse peritoneal macrophages

2.3.1. Introduction

Experimental attempts to infect laboratory bred *Phlebotomus martini* and *P. duboscqi* by feeding them on *L. donovani*-infected patients and laboratory animals have so far been unsuccessful. Preliminary results of a xenodiagnosis study carried out using these sand fly species showed that about 90% of the kala-azar patients subjected to bites of these uninfected sand flies develop papulonodular lesions similar to those seen in post kala-azar dermal leishmaniasis (PKDL), at sites of the bites 1-2 weeks after an experimental

feed (Lawyer *et al.*, 1989). In this study, all the sand flies used were found not to harbour any promastigotes on dissection. When biopsied, the papulonodular lesions were found to be full of amastigotes, suggesting that the inflammatory reaction caused by bites of sand flies attract infected circulating macrophages.

Histological studies carried out on hamster skins subjected to bites of uninfected and *L. major*-infected *P. duboscqi* have demonstrated that a sequence of inflammatory reactions is initiated that culminates in recruitment of macrophages at the site of the bite. This kind of reaction has also been shown to occur when hamsters are inoculated with sand fly saliva using a needle. These observations and the xenodiagnosis results suggest that post kala-azar dermal leishmaniasis may probably occur due to sand fly bites and the introduction of components of sand fly saliva. In order to determine whether sand fly saliva is able to recruit macrophages independent of the normal inflammatory reactions that result from tissue injury either during the biting act, or as a result of needle inoculation and trauma, an *in vitro* assay to test for putative chemotactic properties of sand fly saliva to mouse peritoneal macrophages was designed.

This part of the study was designed in order to supplement the earlier histopathological results, and to shed light on the possible mechanisms of *Leishmania* uptake and

transmission by the sand fly, the development of post kala-azar dermal leishmaniasis, and to determine whether macrophage recruitment at the bite site may be one of the reasons for disease exacerbation by sand fly saliva.

2.3.2. Materials and methods

2.3.2(a). Preparation of *P. duboscqi* salivary gland lysates

Phlebotomus duboscqi salivary glands were dissected from 3-day old unfed adult female sand flies. Sand flies were anesthetized with anesthetic diethyl ether and the glands dissected out in a drop of ice-cold 0.15M sodium chloride on a cold slide. Pairs of 50 glands were transfered to 100µl ice-cold filter-sterilized phosphate-bufered saline and vortex-mixed before use. All glands were used soon after homogenization.

2.3.2(b). Preparation of agarose chemotactic assay slides

Unused clean microscope slides were made grease-free by dipping them in a cleaning solution composed of 3M hydrochloric acid-90% Ethyl alcohol (1:1). These were immediately rinsed in deionized distilled water and then pretreated by dipping in hot freshly prepared 0.5% gelatin. Slides were then rinsed in clean deionized water and left to

air-dry in a vertical position (Chenoweth *et al.*,1979). Wiping of the slides with lens tissue or other forms of manipulation of the slides that might enhance the negative surface charge that is normally found on glass were assidously avoided.

Agarose (Indubiose A37, Gallard-Schlesinger Chemical Manufacturing Corporation, Long Island N.Y.) was weighed and 750mg dissolved in 50ml sterile doubly distilled water by heating in a boiling-water bath for 10-15 minutes. The solution was maintained at 48°C in a water bath (Nelson, *et al.*, 1975). A 10x concentrated solution of tissue culture Medium 199 (TC-199) was prepared. To 10ml of this solution, 10ml of heat-inactivated foetal bovine serum, 1ml of 1% L-glutamine, 1ml of 1% penicillin/streptomycin, 0.009g of sodium bicarbonate, and 28 ml of doubly distilled water were added. The mixture was warmed to 48°C and then added to the agarose media prepared as described above. The resultant mixture was thoroughly mixed with an automatic shaker in the 48°C water bath for 5 minutes. Using a levelling board and a spirit level, 5ml of the mixture was dispensed onto each pretreated microscope slide and allowed to cool at room temperature for 10 minutes. After the media had hardened, the slides were transfered to a refrigerator for 60 minutes to facilitate cutting of the wells in the slide gels. Series of three wells, 2.4mm in diameter and spaced 2.4mm apart were cut in each slide in a straight

line using a plexiglass template and a stainless steel gel punch (Cutler, 1974; Nelson *et al.*, 1975). Agarose plugs were plucked out using a fine pastuer pipette.

2.3.2(c). Preparation of mouse peritoneal macrophages

Weanling 6-8 week old inbred male BALB/c mice weighing 20-30g were sacrificed by cervical dislocation. A total of 10 mice were used at each time. Bellies of the mice were surface-sterilized with 70% ethanol. Skins were dissected with fine scissors and forceps to expose the peritoneum. To obtain peritoneal macrophages, a sterile 10cc syringe fitted with a 21G needle was used to introduce 10ml of chilled ice-cold sterile incomplete RPMI 1640 medium with antibiotics (250 units ml^{-1} Penicillin and 25µg ml^{-1} Streptomycin sulfate) into the unstimulated peritoneum. The abdomen was massaged for 2 minutes to circulate the RPMI. The medium containing peritoneal macrophages was then withdrawn.

The volume of the cell suspension was topped upto 10ml with more RPMI in a sterile universal tube. The cell suspension was centrifuged at 1500 rpm for 15 minutes at 4°C in a refrigerated centrifuge. The pellet was resuspended in RPMI and washed twice. Where few erythrocytes were seen among the monocytes, they were lysed with sterile deionized ice-cold water for 30 seconds.

Fresh sterile ice-cold RPMI was then used to resuspend the cells which were then centrifuged for the last time. The media was discarded leaving only 1ml in which all the cell-pellet was resuspended. Cells were counted using a haemocytometer and then later stained with Giemsa to determine the number of macrophages. Where necessary, these cells were adjusted to give a concentration of $1x10^7$ macrophages per 10ml of RPMI.

Viability of peritoneal macrophages was determined using the trypan blue dye exclusion method. Briefly, a 5% (w/v) stock solution of trypan blue (Evans dye) was dissolved in sterile deionized water and filtered. A 0.5% dilution of this solution was made in Hanks Balanced Salt Solution (HBSS). Macrophages were then diluted (1:10) in 0.5% trypan blue and left for 5 minutes to allow uptake by damaged cells. An aliquote of this mixture was counted using the haemocytometer. A total cell number count and viable cell number count were made. Calculation of the viability of the cells in the suspension was carried out using the following formula:

Viable cell count = Total cell count x % viability

Viable cells were determined by their ability to exclude trypan blue, whereas dead or damaged cells sucked up the dye. Only cell suspensions showing a viability above 85% were used in these assays.

2.3.2(d). Preparation of the positive chemotactic control agent

Two grams of casein were dissolved in 180ml of sterile deionized water and the pH adjusted to 11 with sodium hydroxide solution. To the above solution, 20ml of 10x concentrated TC-199 (DIFCO) solution was added. The pH was adjusted to 7.2 with phosphoric acid. The final stock solution containing 10mg of casein per millilitre of TC-199 was dispensed into 5ml aliquots and stored at -40°C. Before use in the chemotaxis assay, the positive control chemotactic stock casein was diluted in 1x TC-199 to give a final concentration of 1mg ml^{-1}.

2.3.2(e). Chemotactic assay using *P. duboscqi* salivary gland lysates

Before dispensing into the agarose wells for the chemotaxis assay, macrophages were concentrated to a final count of $1x10^7$ cells per 10µl of RPMI. Into the central well of each of the three well series in the chemotaxis assay

slides, $1x10^7$ mouse peritoneal macrophage suspension was added; one of the outer wells received 10µl of the test *P. duboscqi* salivary gland lysate. The second outer well received the negative control non-chemotactic minimum essential medium (MEM). In another replicate, the central well received $1x10^7$ macrophages, one outer well received 10µl MEM and the second outer well 10µl casein. This control was included in order to compare the distances of migration between the positive chemotactic control (casein) and the test reagent (sand fly saliva). For each combination, five replicates were performed. The chemotaxis slide is shown in Plate 15.

The agarose slides were incubated at 37°C in a 5% CO_2 humidified incubator for 18 hours. After the incubation period, cells were fixed with agarose in place by carefully adding 3ml of absolute methanol to the dish for 30 minutes. The methanol was drained out and the cells were then further fixed with 3 ml of 47% buffered formalin for another 30 minutes (Nelson *et al.*, 1975). When time was not available to complete fixation and staining, the agarose slides were left in absolute methanol at 4°C overnight. The agarose gel was carefully removed, the slides with cells were dipped in absolute methanol to remove all non-adherent cells. The slides were left to air-dry on a flat surface before staining for the determination of migration distances.

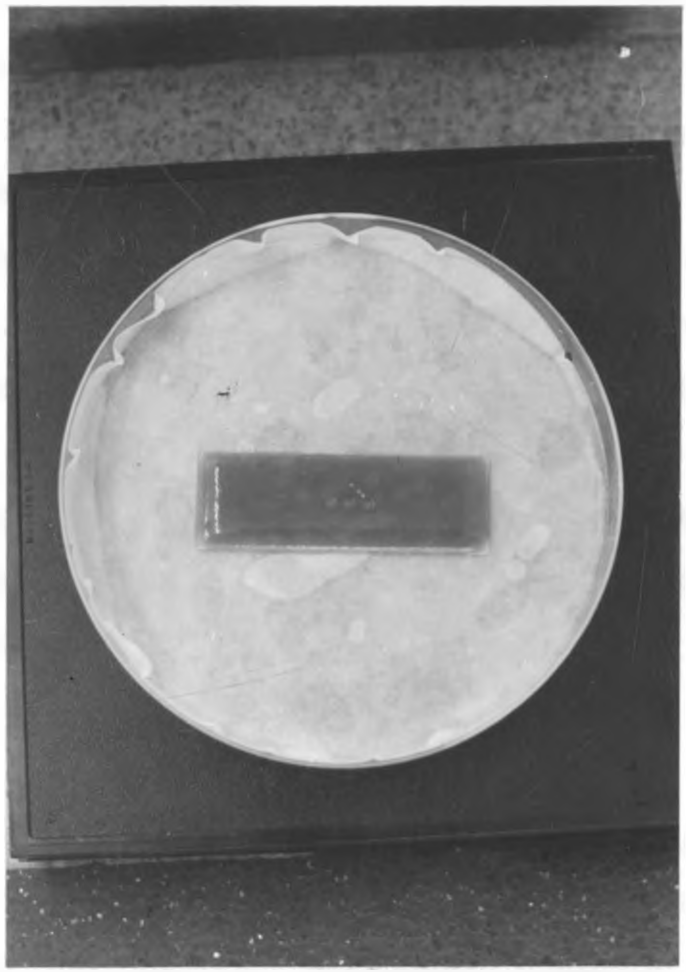

Plate 15. Agarose-coated slide with a series of 3 wells that were used to determine chemotaxis of mouse peritoneal macrophages towards *P. duboscqi* saliva.

2.3.2(f). Staining of chemotaxis assay slides

Wright's staining solution was prepared by dissolving 0.3g of the powder form in 100ml absolute analytical grade methyl alcohol and adding 3ml of glycerine in a stoppered bottle. The resultant mixture was thoroughly mixed on a rocker for 24 hours at room temperature. The undiluted stain was slowly poured onto the slides and left for 5 minutes. Phosphate-buffered distilled water was then poured onto the surface of the slide until a metallic stint formed. These were left to stain for 20 minutes and then washed gently in running distilled water to differentiate the cytoplasm from the nucleus. Slides were air-dried and then examined for enumeration of migration distances.

2.3.2(g). Enumeration of macrophage migration distances

A compound microscope was calibrated using ocular and stage micrometers. Macrophage migration distances were quantitated using 40x magnification. The respective migratory distances of macrophages towards the well with the test chemotactic factor (distance A: chemotaxis or directed migration) or towards the negative nonchemotactic control MEM (distance B: chemokinesis or spontaneous nondirected migration) were defined as the distance from the edge of the central well to the furthest point that 3 cells were seen to be aligned in the same plane and parallel to the

margin of the central well as described by Nelson *et al.* (1975) and Chenoweth *et al.* (1979). Results were expressed as chemotactic index (CI) which was obtained by dividing chemotactic distance of migration (A) by the chemokinetic migration distance (B). In this case, where A was found to be equal to B, CI was 1.0, an indication that the test substance was not chemotactic. Where A was greater than B, CI was greater than 1.0 and the test substance was considered chemotactic to mouse peritoneal macrophages (Nelson *et al.*,1975; Chenoweth *et al.* 1979; Territo, 1981). These results were also expressed as the chemotactic differential (CD). This differential was obtained by subtracting distance B from distance A (Nelson *et al.*, 1975; Chenoweth *et al.*, 1979). For the final analysis, means of A, B, A/B and A-B were calculated. A similar calculation was performed for the positive control (casein) so as to compare the distances of migration of macrophages towards a known chemotaxin with that of a putative test chemotaxin.

2.3.3. Results

Enumeration of migration distances of mouse peritoneal macrophages *in vitro* towards *P. duboscqi* salivary gland lysates showed that sand fly saliva contains a substance that is mildly chemotactic to mouse peritoneal macrophages.

The mean chemotactic index of migration calculated from five replicates as recommended was 1.70µm for cells migrating towards *P. duboscqi* saliva. This index was lower than that calculated for cells migrating towards the positive chemotaxin control (casein). In this positive control, the mean chemotactic index was 2.90µm. Macrophages did not migrate towards the MEM negative control. The chemotactic index in this case was 1.01µm, an indication that no oriented movement had occured. Attempts made to establish a dose-dependent relationship using 4, 6, 8, 10 and 20 *P. duboscqi* salivary gland lysates did not give any difference in the migration distances. Chemotaxis could not be initiated with less than 4 pairs of salivary glands. Results of migration distances are summarised in Table 8. The difference between the chemotactic index of cells migrating towards saliva and the casein positive control was 1.19µm whereas the difference between the index of migration towards saliva and the negative control was 0.71µm, which means that mouse peritoneal macrophages only moved 0.71µm towards *P. duboscqi* saliva.

Table 8. Migration of mouse peritoneal macrophages under agarose towards *P. duboscqi* salivary gland lysates after 18 hours of incubation.

Material in chemotaxis well	Chemotaxis distance (A)	Chemokinesis distance (B)	Chemotactic Index (A/B)	Chemotactic Differential (A-B)
SGL	510.0 ± 3.0µm	298.0 ± 2.7µm	1.71µm	212.0µm
Casein	900.0 ± 2.1µm	310.0 ± 0.5µm	2.90µm	590.0µm
MEM	309.0 ± 0.2µm	307.0 ± 0.4µm	1.01µm	2.0µm

SGL - *P. duboscqi* salivary gland lysate (Test chemotaxin)

MEM - minimum essential medium (Negative control)

Casein - (positive control chemotaxin)

These results demonstrate that sand fly saliva is chemotactic to mouse peritoneal macrophages.

2.3.4. Discussion

Polymorphonuclear leucocytes and mononuclear cells have been shown to exhibit two forms of movements that can be classified as either random or chemotactic. Studies carried out on these cells have shown that in the absence of any stimulus, these cells exhibit random locomotion. This type of locomotion is multidirectional (the tendency of the cell to go in one direction is equal to the tendency to go in any other direction). This chemically enhanced nondirectional locomotion has been refered to as chemokinesis (Wilkinson, 1974). The other kind of locomotion is chemotaxis, and is oriented, unidirectional movement of cells in response to a concentration gradient of a chemical attractant (Wilkinson, 1974, Territo, 1981). Chemotaxis of cells can be initiated by a variety of chemical stimuli or chemoattractants. These substances are referred to as cytotaxins. It can also be initiated by substances that lead to the generation of chemotactic factors in serum. These substances are known as cytotaxigens (Territo, 1981).

Several methods have over the years been developed for the measurement of cellular random locomotion and

chemotaxis *in vitro*. These techniques all involve systems in which leucocytes are physically separated from the chemoattractant. The oldest of these methods was developed by Boyden (1962). The Boyden chamber consists essentially of two compartments divided by a micropore (millipore) filter. Using this method, material to be tested is placed in a chamber below a 3µm millipore filter and leucocytes are placed in the upper chamber in a suitable medium such as Hanks Balanced Salts Solution; after a suitable period of incubation (usually 37°C for 3 hours) in a humidified atmosphere, the filters are removed, fixed in xylene and stained with Giemsa. Counts are made of the number of cells that have traversed the filter and are on the bottom side. An increase on this number is taken as an indication of a chemotactic influence. This method of testing for chemotaxis is the predominant one in use today.

In practice, the standard Boyden chamber technique has some shortcomings and is expensive as well as tedious. Results obtained using this technique may also be affected by factors such as adhesiveness of cells to the filter material, tortousity and size of the pore channels, and detachment of cells from the bottom surface (Keller and Sorkin, 1967; Keller *et al.*, 1972; Zigmond and Hirsch, 1973; Cutler, 1974).

To enable better distinction between influences on rate of chemotactic migration of polymorphonuclear

leucocytes, Zigmond and Hirsch (1973) modified the millipore chamber system by direct microscopic observation of populations of cells migrating into a millipore filter. This modification of the millipore filter method measures the ability of leucocytes to migrate towards a chemotactic stimulant. The leucocyte suspension is separated from the chemotactic agent by a filter through which neutrophils and monocytes can migrate. Either the distance of migration into the filter (leading front method) or the number of cells migrating to the outside of the filter (lower surface counting methods) are enumerated to determine chemotaxis. The method utilizes the Wilkinson Chamber, a chemotaxis chamber made from sawn-off upper parts of 1ml syringe barrels covered with Sartorius membrane (0.8μm pore-size) filters. To ease removal, fixing and staining, the filter is usually glued to the sawn-off syringe barrel using UHU alcohol-soluble adhesive (Wilkinson, 1974; Zigmond and Hirsch, 1973).

Apart from these methods that utilize filters, other more sensitive methods that do not utilize filter membranes have been developed and are all based upon migration of cells under agarose gels as described by Cutler (1974), Nelson *et al.* (1975) and Chenoweth *et al.* (1979). These methods have been variously modified to increase their sensitivity.

The method of Cutler (1974) is the oldest of these non-filter membrane assays. In this method, the phenomenon of leucocyte migration on the surface of a plastic petri dish was used to develop a method for *in vitro* studies of chemotaxis. The technique allows for direct observation of migrating cells; it is technically simpler and possibly more sensitive than the Boyden Chamber method. Basically, molten 0.75% agarose in medium 199 with foetal bovine serum are dispensed into 35x10mm plastic petri dishes. Three wells are then punched in the agarose. The diameter of the wells is 2.4mm and the distance between wells is also 2.4mm. Agarose plugs are removed and then in the central well, cells are added. The putative chemotactic test reagent is added into one of the outer wells, and in the last outer well, either a negative control reagent or a positive control is added. The agarose gels are then incubated at 37^0C in a humidified atmosphere of 5% CO_2 for 2-3 hours for neutrophils. After incubation the cells are permanently fixed *in situ* by treating with absolute methanol overnight followed by removal of agarose and staining with Giemsa. The distances of migration are measured using 40x magnification.

Nelson *et al.* (1975) modified the method for studying chemotaxis under agarose in a manner that it would be used to study chemotaxis and spontaneous migration in both monocytes and human polymorphonuclear leucocytes. In

this method, monocytes are incubated at 37°C in 5% CO_2 for 18 hours and polymorphonuclear leucocytes are incubated for 2 hours because they migrate much faster. The migration medium incorporates pooled human serum or foetal bovine serum, medium 199, L-glutamine and antibiotics. This method was modified further by Chenoweth *et al.* (1979) who instead of using serum or albumin used agarose-gelatin solution mixed with Basal Medium Eagle (BME).-

In an attempt to establish whether *P. duboscqi* saliva is a cytotaxin able to display chemotactic activity to mouse peritoneal macrophages, the method of Nelson *et al.* (1975) was employed. By calculating the chemotactic indices and differentials, sand fly saliva was shown to be chemotactic to mouse peritoneal macrophages *in vitro*. The importance of this finding is that by attracting macrophages at the site of bite by a *Leishmania*-infected sand fly, saliva avails more cells for parasitization by metacyclic promastigotes. This may explain why the few parasites inoculated by an infected sand fly are able to cause an infection.

The chemotaxis shown by *P. duboscqi* saliva can be described as mild because the migration distance of macrophages was only slightly longer than chemokinesis. This is probably due to loss during dissection or denaturation of most of the salivary component that is chemotactic. Another possibility is that the chemotactic

component in saliva occurs in very tiny amounts so that the dilution caused by the phosphate-buffered saline in which the salivary glands are stored, is below the threshold level. It may also be that sand fly saliva alone is not very chemotactic when used directly on the cells *in vitro* but can possibly act as a very powerful chemotactic agent *in vivo* by inducing the generation of chemotactic factors such as C5a in serum. In this case, sand fly saliva may act as a cytotaxigen while at the same time acting as a mild cytotaxin. These modes of actions are inferences, but the actual mechanism of chemotactic activity is not clear, but it may be due to binding on cell membrane sites that detect chemotactic gradients such as those that have been described for neutrophils.

Surface receptor sites for the glycoprotein C5a, and for the glycoprotein urate crystal-induced chemotactic factor (CCF) have been described (Chenoweth and Hugli, 1978; Spelberg and Mehta, 1979). Following membrane recognition, there are electrolyte shifts with calcium and sodium influx and membrane depolarization (Gallin and Gallin, 1977; Maccache *et al* 1977; Gallin and Rosenthal, 1974; Bouchek and Snyderman, 1976). Following the sensing of the chemotactin, neutrophil movement probably involves mechanisms similar to muscle contraction. The actin and myosin-like microfilament proteins in the neutrophil participate as the contractile elements in the

control of cellular shape changes, phagocytosis, secretion and movement (Oliver, 1978). The exact mechanisms linking recognition of the chemotactic factor with the oriented motion are still unclear. It is likely that calcium influx or the release of bound calcium from the intracellular compartment induces reorganization and orientation of the cytoskeleton and activates the contractile process leading to polarized contraction (Oliver, 1978). This process may probably occur in mouse peritoneal macrophages that were used while testing the chemotactic effect of *P. duboscqi* saliva. The monocyte, or macrophage, ranks second only to the neutrophils as the best-studied leucocyte in chemotaxis.

In as much as virtually all nonlymphoid mononuclear cells in peritoneal exudates derive from the circulating monocytes (Cohen and Ward, 1971). It has been shown by Ward (1968) and Snyderman *et al.* (1971) that chemotactically, the responses of the blood monocyte and the peritoneal macrophage cannot be differentiated. In the study reported here, mouse peritoneal macrophages were used and they therefore may reflect what happens at the skin site of saliva inoculation when the sand fly bites and inoculates saliva during the probing phase. The attraction of macrophages to the site of inoculation of the chemotactic saliva may therefore be one of the factors that lead to enhancement of *L. major* infection when the parasite is co-inoculated with *P. duboscqi*, *P. papatasi* and *Lu. longipalpis*

salivary gland lysates. More studies are required to isolate the chemotactic component in sand fly saliva, to determine the mode of action of this component and to identify the receptor on macrophages that perceive this cytotaxin. How the attraction of macrophages by chemotactic sand fly saliva directly influences the course of *Leishmania* infection *in vivo* is not known. In the next part of this study, the possible implications have been investigated.

CHAPTER 3

EFFECTS OF DIFFERENT NUMBERS OF MACROPHAGES ON *LEISHMANIA MAJOR* INFECTION AND THE EFFECT OF *PHLEBOTOMUS DUBOSCQI* SALIVA ON HOST COMPLEMENT

3.1. The effect of co-inoculation of *Leishmania major* with macrophages on the development of lesions

3.1.1. Introduction

In an earlier study (Chapter 2), it was demonstrated that BALB/c mice inoculated with *L. major* culture-derived stationary phase promastigotes mixed with *P. duboscqi* saliva develop larger lesions than control mice inoculated with promastigotes only. In another part of the study, it was shown that despite the high success of lesion development in BALB/c mice infected through the bite of *L. major*-infected *P. duboscqi*, lesion development is normally slow, and that mice do not develop the destructive pathology seen in mice inoculated artificially with culture-derived promastigotes.

Histopathological studies of hamster skins inoculated with sand fly saliva either artificially by needle, or naturally by bite of the sand fly showed that an inflammatory reaction is generated that slowly leads to accumulation of macrophages at the site of the bite or saliva inoculation. The accumulation of macrophages may be a reaction that ensures successful parasitization of these cells by *Leishmania*. Of particular importance was the observation that the number of macrophages that aggregate 2-6 hours at the site of inoculation are not significantly high, but increase gradually

with time, reaching a peak between 48-96 hours after inoculation, about the time when the immunosuppressive effect of sand fly saliva wanes (Theodos and Titus, 1991).

In order to determine how an increase in the number of macrophages as a result of recruitment by saliva at the bite site affects the evolution of *Leishmania* lesions, this part of the study was designed, whereby *L. major* was inoculated into mice footpads with different numbers of macrophages.

3.1.2. Materials and methods

3.1.2(a). Parasite cultivation

L. major was aspirated from the footpad lesion of a BALB/c mouse and cultured in Schneider's *Drosophila* medium as earlier described. Stationary phase primary culture promastigotes were counted and adjusted to a concentration of $1x10^6ml^{-1}$ of culture medium. These were washed twice to remove media and then resuspended in 40µl of phosphate-buffered saline (PBS).

3.1.2(b). Preparation of mouse peritoneal macrophages

Weanling 6-8 week old adult male BALB/c mice were sacrificed by cervical dislocation. The bellies of the mice were sterilized with 70% ethyl alcohol. The skins were

dissected out to expose the peritoneum. To obtain peritoneal macrophages, a sterile 10ml syringe fitted with a sterile 21 gauge hypodermic needle was used to introduce 10ml of chilled, sterile incomplete RPMI 1640 medium supplement with antibiotics (250 units ml^{-1} Penicillin and 250µg ml^{-1} Streptomycin) into the unstimulated peritoneum. The abdomen was massaged for 2-3 minutes to circulate the RPMI. The medium containing peritoneal macrophages was then withdrawn. A total of 10 mice was used.

The volume of the cell suspension was topped to 50ml in a centrifuge tube. The cell suspension was centrifuged at 1600 revolutions per minute for 15 minutes at 4°C in a refrigerated centrifuge. The pellet was resuspended in sterile RPMI supplemented with antibiotics and washed twice. The cells were then counted using a haemocytometer, stained with Giemsa to determine the proportion of macrophages and resuspended in fresh sterile RPMI at a concentration of $1x10^6$ cells per millilitre. The viability of macrophages was determined using the trypan blue dye exclusion technique. Only peritoneal macrophages showing over 85% viability were used in the study. Prior to inoculation in mice, macrophage concentrations were adjusted to $1x10^6$, $1x10^4$ and $1x10^2$ cells per 20µl of RPMI respectively.

3.1.2(c). Inoculation of mice with promastigotes mixed with mouse peritoneal macrophages

Weanling 6 week old 60 male BALB/c were individually ear-tagged for easy identification and consistence in recording results prior to inoculation. Thicknesses of left and right hind footpads were measured with a direct reading vernier caliper. The 60 mice were divided into four groups, each consisting of 15 mice. The first group of mice was inoculated subcutaneously in the left hind footpad with $1x10^6$ *L. major* promastigotes mixed with $1x10^6$ peritoneal macrophages. The total inoculum was 60µl. The second group was inoculated with $1x10^6$ promatigotes mixed with $1x10^4$ macrophages, the third group $1x10^6$ promastigotes mixed with $1x10^2$ macrophages, and the control group was inoculated with $1x10^6$ parasites without macrophages.

In all the four groups of mice, the right hind footpad was left to serve as a contralateral control, so as to facilitate calculations of lesion sizes. From the time of inoculation up to 12 weeks post-inoculation when the study was terminated, footpad thicknesses were measured on a weekly basis. Lesion size was obtained by subtracting thickness of the uninfected footpad from the thickness of the infected footpad. In week 13, all mice were sacrificed. Cultures of the control footpad were made in Schneider's medium to detect metastatic spread. Similarly, liver and spleen

cultures were made to detect visceralization. Cultures were examined every day for 14 days before they were discarded.

3.1.3. Results

Results shown in Figure 5 indicte that mice inoculated with $1x10^6$ *L. major* with or without $1x10^2$ macrophages initially developed lesions slowly during the first and second weeks. During this period, mice inoculated with *L. major* mixed with either $1x10^6$ or $1x10^4$ macrophages exhibited larger lesions. From week 3, mice inoculated with *L. major* alone or *L. major* mixed with $1x10^2$ macrophages developed lesions that were larger and progressed fast into ulceration after week 9. At this time, loss of necrotic tissue became evident, leading to a slight decline in mean lesion size. Groups of mice that were inoculated with *L. major* either with $1x10^6$ or $1x10^4$ macrophages displayed a slow pattern of lesion development. Throughout week 3 to 12, these two groups maintained a low grade infection. Some mice in these two groups did not develop lesions. When analysed using the multiple t-test, lesion sizes in mice inoculated with *L. major* mixed with $1x10^6$ macrophages and $1x10^4$ macrophages had significantly lower lesions ($P < 0.01$) than those inoculated with *L. major* alone, or *L. major* with $1x10^2$ macrophages. Standard errors shown in the graphs are the deviations of mean lesion sizes for every weeks

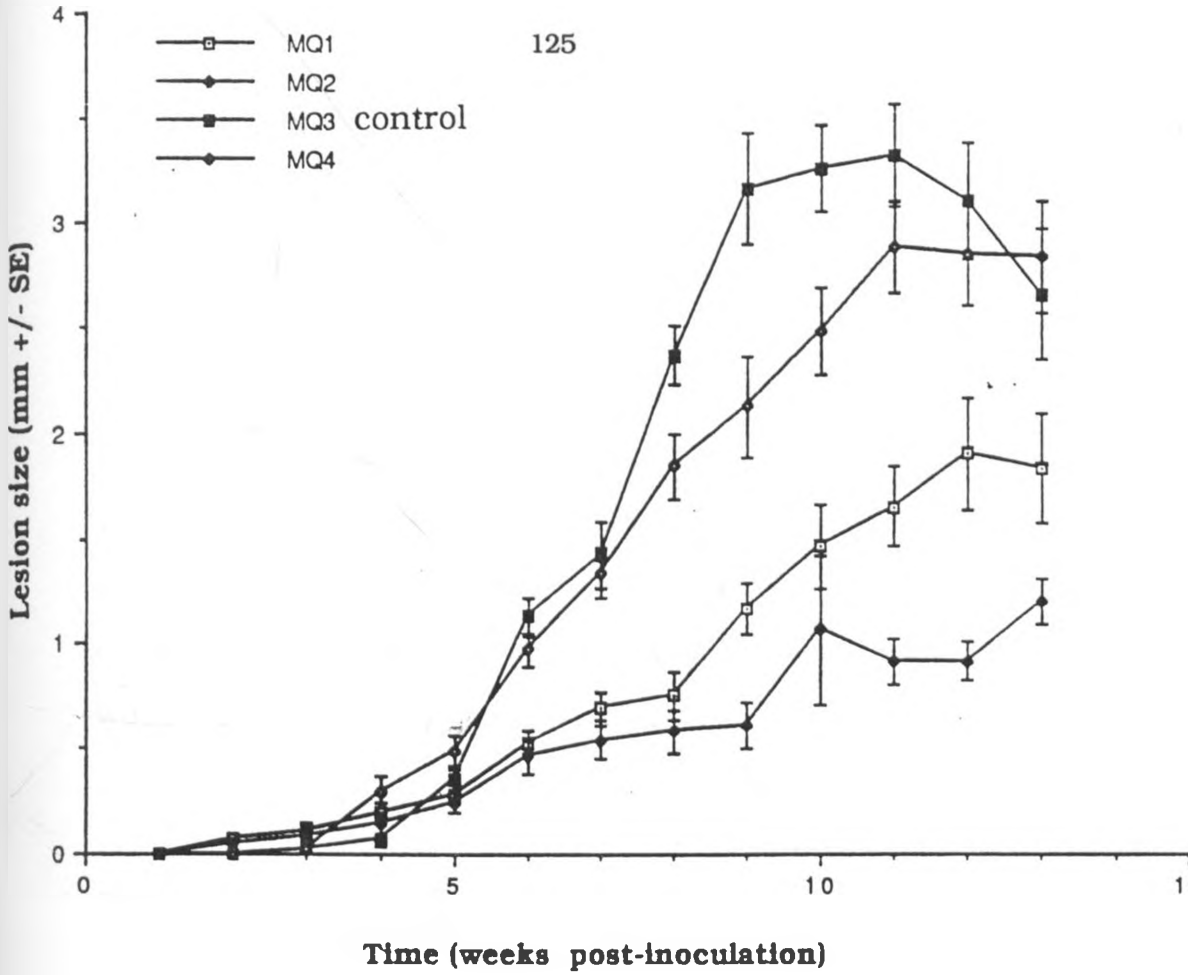

Figure 5. Results of co-inoculation of *L. major* promastigotes with different numbers of mouse peritoneal macrophages. MQ1-mice inoculated with $1x10^6$ *L. major* mixed with $1x10^6$ macrophages, MQ2-mice inoculated with $1x10^6$ *L. major* mixed with $1x10^4$ macrophages, MQ3- mice inoculated with $1x10^6$ *L. major* only, and MQ4- mice inoculated with $1x10^6$ *L. major* mixed with $1x10^2$ macrophages. MQ3 is the control group.

The difference in lesion sizes between mice inoculated with *L. major* only, and *L. major* mixed with 100 macrophages was not significant ($P > 0.01$) as was the difference between lesion sizes of mice inoculated with *L. major* mixed with $1x10^6$ macrophages and *L. major* mixed with $1x10^4$ macrophages. Development of visceral or metastatic disease in viscera and control footpads had not occured when the four groups of mice were sacrificed at week 13.

3.1.4. Discussion

Larger lesions observed in mice inoculated with *L. major* mixed with either $1x10^6$ or $1x10^4$ mouse peritoneal macrophages during week 2 to 3 may be as a result of oedema and the large number of parasites and cells that were inoculated. A notable observation made in this experiment is that inoculation of parasites with few macrophages or without leads to an exagerated pathology that results in development of larger lesions that ulcerate earlier.

Provision of parasites with large numbers of macrophages leads to slow development of lesions that take a long time to ulcerate. The cause of these differences is as yet unclear, but modification of inflammatory reactions at

the site of inoculation may be one of the reasons. Peritoneal macrophages have been shown to exhibit a high triggering response to both amastigotes and promastigotes compared to resident tissue macrophages (Blackwell and Alexander, 1983; Murray, 1982).

Under serum-free conditions, promastigotes are known to elicit a strong respiratory burst response in murine resident peritoneal macrophages (Murray, 1981, 1982). It is possible that the process of phagocytosis started soon after mixing promastigotes with macrophages before inoculation into the mice, thus triggering the generation of toxic oxygen metabolites that killed the parasites. When processing macrophages *in vitro*, they become activated (Modabber, personal communication). This activation of macrophages may mean that development of smaller lesions in mice inoculated with parasites mixed with either $1x10^6$ or $1x10^4$ macrophages was caused by destruction of most of the parasites by the activated cells. The few parasites that survived the respiratory burst are the ones that may have caused lesions. Groups of mice inoculated with *L. major* with $1x10^2$ macrophages or without macrophages developed larger lesions possibly because few of the parasites were destroyed by peritoneal macrophages. More promastigotes were therefore available in intracellular spaces where they activated complement and were able to enter the macrophages without triggering the respiratory burst.

Another product of the activation may be the generation of the chemotactic C5a, which in turn attracted more macrophages to engulf the parasites. C5a has been shown to be generated following complement activation by *L. mexicana* promastigotes (Bray, 1983). Generation of this complement component leads to recruitment of mouse macrophages that are important in the establishment of *Leishmania* lesions. It has been shown *in vitro* that attraction between *L. donovani* promastigotes and hamster monocytes is reciprocal and instantaneous (Pulvertaft and Hoyle, 1960). This kind of reaction may ensure that there is successful parasitization of macrophages by promastigotes.

Of interest in this study is the finding that when many macrophages are provided at the same time together with parasites, there is slow development of lesions. In this case, mice do not develop very destructive infection. They instead develop a low grade infection that is similar to that observed following bites of *L. major*-infected sand flies. In natural infections initiated by the sand fly bite, few parasites are inoculated but they cause lesions, most probably due to immunosuppressive effects of saliva. This may be an adaptation to ensure that there is minimal destruction of host tissue while at the same time ensuring parasite survival. The presence of sand fly saliva in the inocula may probably have changed the patterns of lesion development. The effect of sand fly saliva on host complement and how it

possibly may affect macrophages in relation to *Leishmania* infection has been studied in the next section.

3.2. The effect of *Phlebotomus duboscqi* saliva on vertebrate host complement

3.2.1. Introduction

Saliva of the ixodid tick *Ixodes dammini* has been shown to exhibit anti-complement activity. It is able to prevent haemolysis of rabbit erythrocytes by the human alternative pathway of complement. Deposition of C3b to activating surfaces and concomitant C3a release are inhibited. C3b deposition to activating surfaces is inhibited regardless of the origin (human, rat, mouse, guinea pigs, and hamster) of the serum (Ribeiro, 1987). The mechanism of action of *I. dammini* salivary anti-complement remains to be established, because it prevents C3 hydrolysis even after C3 is fixed to a surface. It is thought that it could disrupt the system controlling C3 conversion through factors B and H or inhibit the associated C3 convertase (Pangburn and Muller-Eberhard, 1984). Following these observations, the author concluded that salivary anti-complement activity of saliva may contribute to succesful feeding of this tick in their natural host.

Saliva of this tick has also been shown to inhibit neutrophil function particularly aggregation, enzyme and superoxide secretion, and phagocytosis of pathogens. At single concentrations of 5µl of saliva per 100µl of reaction media used, inhibitions ranged from about 40-80% (Ribeiro *et al.*, 1990). Neutrophils are among the earliest leukocytes to accumulate in areas of tissue injury, and are abundant in tick feeding sites (Tatchell and Moorhouse, 1970). A similar phenomenon has been demonstrated in hamsters fed on by uninfected and *L. major*-infected *P. duboscqi* in an earlier study (Chapter 2). Neutrophils are thus an important cellular component of the vertebrate's first line of defense and repair mechanisms. Enzymes released from neutrophil granules include lysozyme, glucouronidase, and proteases, which have antimicrobial activity and contribute to inflammation. In addition to aiding in the feeding, neutrophil-inhibiting activity is also thought to play a role in facilitating pathogen transmission (Ribeiro *et al.*, 1990).

One of the mechanisms that has been suggested to explain how promastigotes inoculated by the sand fly into the vertebrate host evade lysis by complement is the inactivation of complement by sand fly saliva (Pearson *et al.*, 1983). There is however no evidence to support or discredit this hypothesis. This part of the study was carried out to examine *in vitro* the possibility that saliva of the sand

fly *P. duboscqi* antagonizes host complement by blocking lysis of sheep red blood cells.

3.2.2. Materials and methods

3.2.2(a). Preparation of *P. duboscqi* salivary gland lysates

P. duboscqi salivary gland lysates were prepared as previously outlined. 100 pairs of glands dissected and transfered to 50µl of sterile phosphate-buffered saline. Throughout the dissection, ice-cold microscope slides were used in order to prevent or minimise denaturing of proteases in the salivary glands. To prevent possible denaturation of proteins, salivary gland lysates were used soon after dissection.

3.2.2(b). Collection and washing of sheep red blood cells

Alsever's solution (an anti-coagulant blood preservative) that permits storage of whole blood at 4°C for up to 10 weeks was prepared as described by Kabat and Mayer (1971), by dissolving 20.5g dextrose, 8g sodium citrate dihydrate, 0.55g monohydrate citric acid, and 4.2g sodium chloride in 1000ml of sterile distilled water. The solution was filter-sterilized and the pH adjusted to 6.1. An adult sheep was restrained and bled from the jugular vein.

Equal amounts of blood and Alsever's solution were mixed and stored at 4°C until ready for use.

Prior to use, sheep red blood cells (SRBC) were washed well in order to remove plasma and haemoglobin from lysed cells. The buffy coat, containing white blood cells (WBC) was removed from the top of the rbc pellet between each wash. Washing of the SRBC was done by removing an aliquot from the suspension stored in Alsever's solution. The volume was made-up to 10ml with PBS (pH 7.2) gently. SRBCs were centrifuged severally at 2500 revolutions per minute (rpm) for 15 minutes in order to wash them, packing cells between each wash until no trace of haemolysis was visible in the supernatant. In order to stabilize and make uniform the susceptibility of the erythrocytes to lysis by antibody and complement, SRBCs were preserved in Alsever's solution and allowed to age for one week at 4°C prior to use (Kabat and Mayer, 1971).

3.2.2(c). Preparation of rabbit anti-sheep red blood cell serum

Sheep red blood cells (SRBC) were washed three times in phosphate-buffered saline at 2000 rpm for 15 minutes in a refrigerated centrifuge set at 4°C. A 10% solution of SRBC was prepared in saline. One millilitre of this solution was injected intravenously in the ear vein of a

rabbit per kilogram body weight. On the third day , the rabbit was boosted with a similar dose. Boosting of the rabbit was then carried out once a week for four weeks. Seven days after the last booster, the rabbit was bled, and the antiserum tested for haemolytic activity.

3.2.2(d). Preparation of veronal buffer and sheep red blood cells for use in the haemolytic assay

Veronal buffer saline (VBS) was prepared by dissolving 8.5mg sodium chloride (NaCl), 0.575gm Barbituric acid, 0.185gm sodium diethylbarbiturate, 0.168gm magnesium chloride ($MgCl_2.6H_2O$), 0.028gm calcium chloride ($CaCl_2$), and 1gm gelatin in 1000ml distilled water. The pH was adjusted to 7.2 using 0.1N hydrochloric acid (HCl) and 1.0 M sodium hydroxide and confirmed using pH 7.0, 7.2 and 7.4 standards (Fisher Scientific, New Jersey, USA). Into 20ml of VBS, 5ml of 50% SRBC were added and centrifuged at 2000 rpm for 10 minutes. The procedure was repeated until the supernatant became clear (3 washes). Packed SRBC were resuspended to a concentration of 4% in VBS and kept on ice until ready for use.

3.2.2(e). Titration of rabbit anti-SRBC antibody (amboceptor)

A healthy male adult guinea pig was anesthetized and bled intracardially. Blood was kept at room temperature for one hour and then it was transfered to 4°C overnight for the serum to separate. The serum was centrifuged at 1500 rpm for 5 minutes and then kept at -80°C until the time it was used. Various dilutions of the rabbit anti-SRBC antibody (amboceptor) were added to the SRBC in the presence of an excess of guinea pig serum as a source of complement, the limiting factor being the amount of anti-SRBC antibody present. A 1:100 dilution of the amboceptor (0.1ml in 10ml VBS) was prepared and kept on ice. Tubes were numbered 1 to 7 and in tubes 1 and 2, 0.2ml of the 1:100 dilution of the amboceptor was added, and into tube 7, only VBS was added. A serial doubling dilution was carried out from tube 2 to 6, with the last 0.2ml being discarded. The last dilution was therefore 1:3200. Into all the tubes, 0.2ml of 4% washed SRBC was added, mixed well and left to stand at 37°C for 30 minutes. These were then transfered to an ice bath. The contents were mixed well and 25µl from each tube transfered to 2 wells of a microtitre plate. Into each well, 100µl of guinea pig complement serum (diluted 1:20 in VBS) was added. The contents were incubated for 1 hour at 37°C. Lysis was then read and scored as negative (no lysis),

partial and complete lysis. The optimal concentration of the amboceptor was found to be 1: 1600.

3.2.2(f). Titration of guinea pig complement serum

Sensitized SRBC were prepared by incubation of 10ml of 4% SRBC and 10ml of 1:1600 dilution of the amboceptor for 1 hour in a water-bath set at 37^{O}C. Dilutions of guinea pig complement serum were carried out using fresh serum diluted 1:40 in VBS (0.5ml complement serum in 20ml VBS). Dilutions were made up to give 1:2, 1:4, 1:8, 1:16, 1:64, 1:128 and 1:256. These were mixed well and from each tube, 0.1ml aliquots of the dilutions were added and transfered to 2 microtitre plate wells. Into each well, 25µl of sensitized SRBC were added. Two wells with the amboceptor and SRBC in VBS were left to serve as negative controls. The contents were gently mixed and incubated for 1 hour at 37°C. The plate was read and scored for lysis as previously described. The last dilution of complement which gave complete haemolysis was 1:64. This was chosen as optimal for use in the complement fixation assay.

3.2.2(g). Complement fixation test using *P. duboscqi* saliva

Before use, salivary gland lysates were mixed with VBS and adjusted such that 1 pair of glands was contained in 1µl of reaction media. The complement fixation assay involved three sets of reactions: (i) the test reaction involving salivary glands (ii) the positive control for complement-mediated lysis, and (iii) the negative control for the salivary gland lysates. The complement fixation assay was perfomed in 96-well flat-bottomed microtitre plates in duplicate. In the test reaction, 100µl guinea pig complement was first mixed with either 5 pairs of salivary glands in 5µl buffer, 10 pairs of salivary glands in 10µl , 25 pairs of glands in 25µl or 40 pairs of glands in 40µl of veronal buffer. After mixing thouroughly, 25µl of 4% antibody sensitized SRBC were added and again mixed.

The positive control reaction involved mixing 25µl complement with 25µl sensitized SRBC. In the negative control, 25µl sensitized SRBC was mixed with an equal amount of salivary gland lysates. In another reaction to control for the volumes, equal amounts (40µl) of salivary gland lysates, SRBC and complement were used. All reactants were incubated for 1 hour at 37°C. Results were then scored as either complete lysis, partial lysis or no lysis. Five replicates of these reactions were perfomed to test the reproduciblity of the assay. An additional complement fixation control assay was carried out using *P. duboscqi* saliva

that had been heat-inactivated by incubation in a water-bath at 56°C for 1 hour. This control was carried out to denature enzymes in saliva.

3.2.3. Results

In all the complement fixation test reactions in which guinea pig serum was incubated with 5, 10, 25, 40µl of *P. duboscqi* salivary gland lysates followed by sheep erythrocytes, all the erythrocytes were lysed by complement. Increasing the amount of saliva did not therefore prevent lysis of sheep red blood cells. In a reaction whereby equal amounts of complement serum, *P. duboscqi* salivary gland lysate and sheep red blood cells were incubated together, complete lysis still occured. In the positive control where 25µl of guinea pig serum was incubated with 25µl SRBC, lysis was detected but not in the negative control where 25µl SRBC were incubated with 25µl of salivary gland lysates. In all replicates, results were reproducible. These complement fixation test results are are summarised in Table 9.

Table 9. Results of complement fixation test using *P. duboscqi* salivary gland lysates, guinea pig complement and sheep erythrocytes.

Amount of reagents incubated			Lysis score
P. duboscqi saliva	SRBC	Guinea pig complement	
5µl	25µl	100µl	complete
10µl	25µl	100µl	complete
25µl	25µl	100µl	complete
40µl	25µl	100µl	complete
40µl	40µl	40µl	complete
[a]25µl	25µl	0µl	No lysis
[b]0µl	25µl	25µl	complete

a - negative control

b - positive control

SRBC - antibody sensitized sheep red blood cells

3.2.4. Discussion

Results from this experiment indicate that saliva of the sand fly *P. duboscqi* does not prevent haemolysis of sheep red blood cells by guinea pig complement. This finding demonstrates that saliva of sand flies is chemically different from that of ticks that is able to inactivate host complement as reported by Ribeiro (1987). The inability of sand fly saliva to prevent the generation of the complement enzyme cascade may be benefitial to *Leishmania* promastigotes that are inoculated by an infected sand fly into the vertebrate host. Promastigotes of various species of *Leishmania* have been shown to activate the alternative pathway of the complement cascade when they are exposed to normal human or guinea pig serum (Mosser and Edelson, 1984).

The ability of fresh sera to cause the lysis of *Leishmania* was recognized as early as 1912 by Patton. Later studies have shown that complement-mediated lysis is dependent on the developmental stage of the parasite with logarithmic phase promastigotes being highly susceptible and metacyclic promastigotes being more resistant to lysis (Franke *et al.*, 1985; Sacks *et al.*, 1985; Puentes *et al.*, 1988).

Leishmania surface glycoconjugate lipophosphoglycan (LPG) on metacyclic promastigotes activates complement

efficiently, resulting in extensive deposition of C3b. This molecule (LPG) is also the major acceptor for the C3b (Sacks, 1989; Turco, 1990). LPG is thought to prevent lysis of metacyclic *Leishmania* promastigotes by sterically hindering access of large molecules to the cell membrane, and therefore prevents channel formation and lysis by the C5-C9 membrane attack complex (Turco, 1990). The release of C5-C9 by *L. major* metacyclics has recently been demonstrated (Sacks, 1989). Promastigotes take advantage of complement activation by rapidly converting C3b to C3bi, and entering the macrophage via respiratory burst non-triggering CR1 and CR3 macrophage receptors for complement components (Blackwell *et al*., 1985; Mosser and Edelson *et al*., 1985,1987).

Considering that *Leishmania* require host complement components for entry into the macrophage and for intracellular survival, any factor that will prevent fixation of C3 will also prevent the successful establishment of *Leishmania* infections. Failure of *P. duboscqi* saliva to inactivate host complement may be a sand fly-parasite co-evolutionary strategy to enable *Leishmania* to establish a successful infection. This part of the study has shown that there is no evidence to support the suggestion by Pearson *et al.* (1983) that sand fly saliva locally inactivates complement to protect lysis of promastigotes.

CHAPTER 4

EFFECTS OF SAND FLY SALIVA ON THE COURSE OF *LEISHMANIA DONOVANI* INFECTION IN THE SYRIAN GOLDEN HAMSTER

4.1. Introduction

Studies carried out by Theodor (1935) and Mellanby (1946) on the phenomenon of sensitization against arthropod derived antigens showed that sensitivity acquired to *P. papatasi* and *Ae. aegypti* bites and saliva respectively, began with a delayed papular reaction. Mellanby (1946), described the 5 stages of immunity inherent in reactions to arthropods. Following the bite, there was initially no reaction in persons never before exposed to the arthropod (or to arthropods of that particular species). Then with repeated bites they began to develop sensitivity, which was initially manifested by a delayed reaction some 24 hours after a bite (Theodor emphasized that this delay could be up to 3 weeks or longer). This reaction subsided after about one week. At a later stage, which might be weeks, months or even years later, a bite was followed by an immediate reaction (a wheal), which subsided in a few hours to be succeeded by the delayed itching papule. After a further period of exposure, the delayed reaction no longer occured but only the immediate wheal and finally no reaction appeared at all. At this stage, the individual was considered sensitized.

In a related study, Gordon (1950) showed that there was no reaction to mosquito bites if the insect's salivary glands were extirpated or the ducts cut, an indication that

the sensitizing component is present in saliva. A similar finding was reported by Hudson *et al.* (1960). Gordon pointed out that sensitization did not develop if mosquito bites were regular and continuous but only if repeated irregularly. He reported that the small itchy, erythematous papules of the delayed response are characterized histologically by minimal dermal oedema, possible focal necrosis and a dense cellular infiltrate of lymphocytes, histiocytes and numerous eosinophils. The sensitization reaction to sand fly saliva has been noted in hamsters that have been bitten by laboratory-bred *P. duboscqi*, but whether or not it has any biological importance in the uptake and transmission of intradermally inoculated *L. donovani*, it is not known.

Highly invasive routes of parasite inoculation such as intracardiac, intrasplenic, intraperitoneal, intravenous, intrahepatic and intratesticular when used to inoculate *L. donovani* promastigotes into unsensitized Syrian hamsters (*Mesocricetus auratus*) usually lead to the development of a severe visceral disease (Schnur and Jacobson, 1987). The progression towards development of visceral infection in the hamster closely parallels that seen in human kala-azar such that this similarity in disease progression makes the hamster a good animal model for studying human infection with *L. donovani*. Despite the high susceptibility shown by hamsters that have been inoculated using the artificial

methods described above, these parasites do not invade the skin (Schnur *et al.*, 1973).

A study carried out by Chulay *et al.* (1985) showed that even in the presence of heavy splenic parasitization, peripheral blood parasitaemia is also usually very low in Kenyan kala-azar patients. Similar observations have been reported in experimentally infected hamsters (Gajree and Goedbloed, 1968: Van Joost and Sluiters, 1972) who found that cultures of heart blood of *L. donovani*-infected hamsters were unreliable in the detection and monitoring of infection.

Lack of parasite metastasis to skins of experimentally infected hamsters, and the very low peripheral blood parasitaemia in both hamsters and human beings has for many years made it not possible to infect sand flies by feeding them on skins of infected patients and hamsters. Using *P. martini*, the local vector for visceralizing *L. donovani*, Beach (1983) was unable to infect this sand fly species by feeding it on skins of *L. donovani*-infected hamsters. Successful infection of these sand flies could only be acheived by feeding them through an artificial membrane on splenic homogenates prepared from infected hamsters. It is therefore not known how *P. martini* acquires *L. donovani* infections by biting infected animal reservoirs. It is also not known whether the site of inoculation may influence parasite presentation to the vector, metastatic spread and the subsequent development of visceral disease.

In a preliminary study, 10 hamsters were sensitized against bites and saliva by exposing them to bites of 100-300 uninfected *P. duboscqi* once a week for 6 weeks. During the feeding time, all hamsters were shaved in the belly to provide a wider feeding area for the sand flies. Sand flies were allowed to feed for 1 hour. After the last biting time, the hamsters were reshaven and then inoculated intradermally in the belly with $1x10^6$ *L. donovani* stationary phase culture promastigotes. Four sensitized hamsters died under anesthesia. Another ten hamsters not sensitized against sand fly saliva were inoculated with *L. donovani* and were used as controls. One hamster from each group was sacrificed opportunistically at 28, 44, 60, 76 and 84 days post-inoculation (DPI). At necropsy, nose skin, belly skin (site of inoculation), liver, spleen, blood were cultured in Schneider's medium supplemented with 20% heat-inactivated foetal bovine serum, 250 units ml^{-1} Penicillin, 250 $\mu g ml^{-1}$ Streptomycin and 500 $\mu g\ ml^{-1}$ 5-fluorocytosine arabinoside.

Two sensitized hamsters were found to harbour parasites at the site of inoculation and in the nose skin at 28, and 44 and in the spleen at 28 and 84 DPI. The other 4 sensitized hamsters had parasites at the site of inoculation when sacrificed on days 44, 60, 76 and 84. Only 1 hamster developed visceral disease by 84 DPI. Control hamsters harboured parasites only at the site of inoculation throughout

the sampling period, and neither metastasis nor visceralization occured. In both groups of animals, at no time was heart blood found to contain parasites. From these results, it was hypothesized that sensitization against sand fly bite and hence saliva may modify the course of *L. donovani* infection. This part of the study was therefore conducted to test whether inoculation of hamsters with *L. donovani* mixed with *P. duboscqi* salivary gland lysates has any effect on metastatic spread and the rate of visceralization, and whether sensitization to sand fly saliva may influence metastatic spread of *L. donovani* to different cutaneous sites. Other objectives of this study were (i) to determine how long *L. donovani* is able to survive in skin sites where amastigotes can be picked up by sand flies, (ii) to determine whether the site of inoculation influences parasite retention in the skin, the course of the disease, and (iii) to determine whether sand flies are able to pick up parasites in *L. donovani*-infected immunosuppressed hamsters.

4.2. Materials and methods

4.2.1(a). The effect of inoculation of *L. donovani* mixed with *P. duboscqi* saliva on the course of *L. donovani*

Twenty weanling male hamsters were intradermally inoculated with $1x10^6$ *L. donovani* (Strain MHOM/KE/82/LRC-L445=NLB-065) stationary phase culture promastigotes in 40µl PBS mixed with 5 pairs of *P. duboscqi* salivary glands in 20µl PBS. Salivary glands were prepared as earlier described in Chapter 2. The site of inoculation was the nose. Every 14 days for 70 days, subcutaneous saline aspirates were taken from the site of inoculation and cultured in NNN/Schneider's *Drosophila* medium supplemented with 20% heat-inactivated foetal bovine serum, antibiotics and antifungal agents. This was done to determine parasite retention at the site of inoculation.

All hamsters were sacrificed 90 days post-inoculation. At necropsy, spleen, liver, nasal skin, left hind footpad, right fore footpad, left mandibular lymph node and right popliteal lymph node, belly skin and heart blood were cultured to determine the pattern of metastatic spread to other cutaneous sites and visceralization. In order to control for sand fly saliva, another group of 20 weanling male hamsters were intradermally inoculated in the nose skin with $1x10^6$ stationary phase *L. donovani* promastigotes without sand fly

saliva. Skin aspirates and sacrificing was carried out as described above.

4.2.1(b). Sensitization of hamsters against bites and saliva of uninfected *P. duboscqi*

Twenty naive male hamsters weighing 138-140 grams each were anesthetized with sodium pentobarbitone and shaven in the belly. Each hamster was exposed to bites of 200 laboratory-bred 3-day old uninfected *P. duboscqi* twice a week for 5 weeks. Sand flies were allowed to bite the shaven bellies, fore and hind footpads, mouthparts and the nasal areas of the hamsters.

4.2.1(c) Inoculation of sensitized hamsters with L. donovani culture-derived stationary phase promastigotes

Primary culture stationary phase promastigotes were counted and adjusted to a concentration of $1x10^6ml^{-1}$ of Schneider's medium. The parasites were centrifugally washed twice in sterile phosphate-buffered saline (PBS) at 3000 rpm for 15 minutes and then resuspended in 60µl PBS. These were inoculated intradermally in the shaven bellies of the sensitized hamsters. Additional twenty naive hamsters were similarly inoculated with *L. donovani* to serve as controls. To monitor parasite persistence,

subcutaneous saline aspirates were taken every two weeks from the site of inoculation and cultured in Schneider's medium with 250µg ml^{-1} Penicillin, 250 units ml^{-1} streptomycin and 500µg ml^{-1} 5-fluorocytosine arabinoside. At 10 weeks post-inoculation, all hamsters were sacrificed. Site of inoculation, the right fore footpad, left hind footpad, right popliteal lymph nodes, left mandibular lymph nodes and nose skin were all cultured to determine the degree of metastatic spread. Liver, spleen and heart blood were cultured to determine the level of visceralization.

4.2.1(d). Inoculation of control naive hamster with *L. donovani* promastigotes mixed with sand fly saliva at different sites

To serve as controls for the sensitized hamsters, 20 naive weanling male hamsters were each inoculated intradermally with $1x10^6$ *L. donovani* stationary phase culture promastigotes in the shaven belly skin as described above for the sensitized group. In order to determine whether the site of parasite inoculation can markedly influence the outcome of the infection as is the case with *L. major* (Kirkpatrick *et al.*, 1987), 3 additional groups of 20 hamsters each were inoculated with $1x10^6$ stationary phase culture promastigotes in 40µl PBS. The first group of hamsters was intradermally inoculated in the right fore

footpads, the second group in the left hind footpads and the third group in nose skin. All the four groups of hamsters were aspirated at the sites of inoculation fortnightly at 14, 28, 42, 56, 70, 84, 98, 112, 126, 140, 168, 182, 196 and 210 days post-inoculation. Saline aspirates were cultured in NNN/Schneider's medium with antibiotics.

All hamsters were sacrificed at 294 days post-inoculation. Portions of site of inoculation, one hind footpad, a fore footpad, nose skin, liver, spleen, heart blood, popliteal and mandibular lymph nodes were cultured in NNN/Schneider's medium. These were examined every day to detect parasite persistence at the site of inoculation, metastasis and visceralization.

4.2.1(e). Xenodiagnosis using *P. duboscqi* and *L. donovani*-infected hamsters

In order to determine whether the sand fly is able to pick up parasites persisting at the site of inoculation, one group of 10 hamsters was inoculated in nose skin with $1x10^6$ *L. donovani* stationary phase promastigotes and left to develop infection for 8 weeks. These were then anesthetized and each one of them exposed to bites of 10 putatively infected *P. duboscqi*. Sand flies were allowed to feed on nose, footpads and mouthparts for 1 hour. These

sand flies were then dissected and examined for the presence of parasites.

In order to determine whether immunosuppression may aid a sand fly in picking up parasites, two groups of hamsters were immunosuppressed before and after inoculation with *L. donovani* respectively. One group of 20 weanling male hamsters was immunosuppressed prior to infection. Each hamster was inoculated intraperitoneally with 10mg Kg^{-1} body weight Cyclophosphamide (Mead Johnson Laboratories, Indiana, U.S.A.) once a day for 5 weeks. These were then inoculated intradermally in nose skin with $1x10^6$ stationary phase *L. donovani* culture promastigotes in 60µl PBS and then left for 4 weeks for the infection to develop. The other group of 20 hamsters was inoculated with $1x10^6$ *L. donovani* in the nose skin as described above.

During the fifth, sixth and seventh weeks post-inoculation, these hamsters were immunosuppressed with cyclophosphamide as described above. During the 8^{th} week, each hamster in the two groups was anesthetized. The nose and footpads of each hamster were exposed to bites of uninfected adult unfed female *P. duboscqi*. For each infected hamster, 10 sand flies were used. The duration of feeding was 1 hour. Fed sand flies were dissected and fresh bloodmeals cultured in NNN/Schneider's medium. This was done to prevent lysis of parasites by lytic enzymes in the gut

of the non-vector *P. duboscqi* which was used because there were no *P. martini* Parrot for use in the xenodiagnosis. Replicates of the xenodiagnosis were carried out after 8 and 10 weeks of *L. donovani* infection. Blood meal cultures were examined every day for 14 days before they were discarded. Hamsters were sacrificed in week 10. Cultures and impression smears of belly skin, nose skin, right fore footpads, left hind footpads, left mandibular lymph nodes, right popliteal lymph nodes, liver, spleen and heart blood were all cultured to detect metastasis and visceralization.

4.3. Results

4.3.1(a). The effect of inoculation of *L. donovani* mixed with *P. duboscqi* saliva on the course of the disease

Results of aspirated cultures taken to determine the persistence of *L. donovani* in nasal skin of hamsters inoculated with $1x10^6$ *L. donovani* mixed with *P. duboscqi* salivary glands and control hamsters inoculated with *L. donovani* only are summarised in Table 10. There was no significant difference (Kolmogorov-Smirnov 2-sample test, $P > 0.05$) in the duration of parasite retention in the skin site of inoculation between hamsters inoculated with $1x10^6$ *L. donovani* promastigotes mixed with salivary gland lysates, and the group of hamsters inoculated with $1x10^6$

promastigotes in the absence of salivary gland lysate. When sacrificed at 90 DPI, cultures of belly and nose skins, left mandibular and right popliteal lymph nodes, liver, spleen and heart blood showed that the pattern of metastasis and visceralization does not differ (Kolmogorov-Smirnov 2-sample test, $P > 0.05$) results are summarised in Table 11. There was no difference in the pattern of visceralization between the 2 groups of hamsters, where 3 out of 19 (15.7%) developed spleen infections in hamsters inoculated with parasites mixed with salivary gland lysates compared to 3 out of 20 (15%) for the control group.

Table 10. Results of aspirate cultures taken from hamsters inoculated with 1x10^6 *L. donovani* promastigotes mixed with *P. duboscqi* salivary gland lysates (SGL) and control hamsters inoculated with *L. donovani* only and sacrificed after 90 days.

Days post-inoculation	ASPIRATE CULTURES WITH PARASITES	
	SGL group	Control group
14	*12/20(60%)	10/20(50%)
28	10/19(52.6%)	8/20(40%)
42	10/19(52.6%)	9/20(40%)
56	8/19(42.1%)	9/20(45%)
70	9/19(47.4%)	8/20(40%)

* Number of cultures with parasites over total cultured

SGL group - hamsters inoculated with *L. donovani* mixed with salivary gland lysates.

Table 11. Results of cultures of organs and tissue of hamsters inoculated with 1x10^6 *L. donovani* promastigotes mixed with *P. duboscqi* salivary gland lysates and control hamsters inoculated with 1x10^6 *L. donovani* only.

	HAMSTER ORGANS AND TISSUES CULTURED								
Group	Belly skin	Nose skin	RFFD	LHFD	LMLN	RPLN	LIV	SPLN	BL
SGL	0/19	10/19	2/19	0/19	7/19	1/19	2/19	3/19	0/
Control	0/20	8/20	1/20	1/20	4/20	0/20	1/20	3/20	1/

SGL - hamsters inoculated with *L. donovani* mixed with salivary gland lysates; RFFD - right fore footpad; LHFD - left hind footpad; RPLN - right popliteal lymph node; LMLN - left mandibular lymph node; LIV - liver; SPLN - spleen, BLD - blood.

4.3.1(b). The course of *L. donovani* infection in hamsters sensitized against sand fly saliva

During the time of inoculation with *L. donovani* stationary phase culture promastigotes, 3 sensitized and 1 naive control hamsters died under anesthesia. Results of cultures of aspirates taken from the site of inoculation in order to detect parasite persistence at the site of inoculation are shown in Table 12. After 28 days, few hamsters from both the sensitized and the control groups had parasites at the site of inoculation. Most of the cultures of the subcutaneous saline aspirates failed to reveal the presence of parasites. Parasite retention at the site of inoculation did not significantly differ between the two groups of hamsters (Kolmogorov-Smirnov 2-sample test, $P > 0.05$).

When the sensitized and control hamsters were sacrificed, cultures of various organs and tissues revealed that only a small percentage of the animals ever developed visceral infection. Blood, left hind footpads and liver cultures did not reveal the presence of parasites. Culture results are shown in Table 13. Most of the hamsters eliminated the parasites and did not develop visceral disease.

Table 12. Results of aspirates taken from belly skins of sensitized and naive control hamsters inoculated intradermally with 1x10^6 *L. donovani* stationary phase culture promastigotes.

Days post-inoculation	CULTURES OF ASPIRATED BELLY SKINS	
	Sensitized hamsters	Control hamsters
14	*5/17(29.4%)	3/19(15.9%)
28	3/17(17.6%)	2/19(10.5%)
42	3/17(17.6%)	4/19(21.1%)
56	1/17(5.9%)	1/19(5.3%)
70	1/17(5.9%)	1/19(5.3%)

* Number of cultures with parasites over total cultured.

Table 13. Results of cultures of organs and tissue of hamsters inoculated with 1 x 10^6 *L. donovani* stationary phase culture promastigotes intradermally and sacrificed at 90 days post-inoculation.

Group	HAMSTER ORGANS AND TISSUES CULTURED								
	Belly	Nose	RFFD	LHFD	LMLN	RPLN	LIV	SPLN	BLD
Sens	*1/17	2/17	3/17	0/17	2/17	1/17	0/17	2/17	0/17
Cont	0/19	0/19	2/19	0/19	1/19	0/19	0/19	1/19	0/19

* Number with parasites over total cultured; RFFD-right fore footpad; LHFD-left hind footpad; LMLN-left mandibular lymph node; RPLN-right popliteal lymph node; Sens-sensitized; Cont-control; SPLN-spleen; BLD-blood

4.3.1(c). The effect of the site of inoculation on the course of *L. donovani* infection in unsensitized naive hamsters

Results of cultures of subcutaneous saline aspirates taken to monitor parasite persistence at different sites of inoculation in control naive hamsters inoculated with *L. donovani* in nose, hind footpad, fore footpad and belly skin are summarised in Table 14. Throughout the 210 days during which aspirates from these sites were cultured, persistence of *L. donovani* was more pronounced in exposed, non-hairy skin sites such as nose and footpads. At 42 days post-inoculation, parasites were not demonstrable in cultures of belly skin aspirates.

Kruskal-Wallis 1-way analysis of variance (ANOVA) performed on data on *L. donovani* retention in skins of the four groups of hamsters separately inoculated with the parasite either in nose skin, belly skin, fore-footpad or in hind footpad skins revealed that these groups of animals differed significantly ($F_{0.05[3,56]} = 76$, $P < 0.01$). Parasite retention in hamsters inoculated with *L. donovani* in belly skin was significantly different from retention in hamsters inoculated in fore footpad and hind footpad ($P < 0.01$), and those inoculated in nose skin ($P < 0.01$). The two groups of hamsters that were inoculated in fore footpads and hind footpads were not significantly different in their retention of parasites ($P > 0.05$).

When all the hamsters were sacrificed at 294 days post-inoculation with *L. donovani*, cultures of liver, spleen, blood, mandibular and popliteal lymph nodes, nose skin, fore- and hind footpads and belly skin showed that the pattern of metastatic spread and visceralization were not very different regardless of the site of inoculation. In all the four groups of hamsters, parasite metastasis to belly skin was not evident. Results of cultures of organs and tissue are summarised in Table 15. More than half of hamsters inoculated in nose, right fore footpad and left hind footpad had parasites at the site of inoculation but no visceral infection. At the time of sacrificing, all hamsters appeared healthy.

Table 14. Results of cultures of subcutaneous saline aspirates taken from skin sites of control naive hamsters inoculated intradermally with $1x0^6$ *L. donovani* stationary phase culture promastigotes.

Days post inoculation	CULTURES OF ASPIRATED HAMSTER SKIN SITES			
	NOSE	BELLY	RFFD	LHFD
14	*13/19	3/20	14/20	15/20
28	9/18	3/20	15/20	17/20
42	6/18	5/19	15/20	16/20
56	8/18	0/19	11/20	12/20
70	8/18	0/19	11/20	14/20
84	6/18	2/19	14/20	14/20
98	12/18	0/19	12/20	11/20
112	9/18	0/19	9/20	14/20
126	8/18	0/19	15/20	9/20
140	10/18	0/19	10/20	14/20
154	7/18	0/19	12/20	10/20
168	9/18	0/19	12/20	13/20
182	9/18	1/19	9/20	8/20
196	8/13	1/19	8/20	9/20
210	9/13	0/19	12/20	8/20

* Number of cultures with parasites over total cultured; RFFD-right fore footpad; LHFD-left hind footpad.

Table 15. Results of cultures of organs and tissues of hamsters inoculated with $1x10^6$ *L. donovani* stationary phase culture promastigotes in nose, belly skin, right fore footpad and left hind footpad and sacrificed at 294 days post-inoculation.

Inoculation site	HAMSTER TISSUES AND ORGANS CULTURED								
	Belly	Nose	RFFD	LHFD	LMLN	RPLN	LIV	SPLN	BLD
Nose	*0/13	10/13	1/13	1/13	7/13	0/13	2/13	1/13	0/13
Belly	0/13	0/13	0/13	0/13	1/13	1/13	1/13	4/13	0/13
RFFD	0/17	0/17	12/17	2/17	3/17	2/17	3/17	6/17	1/17
LHFD	0/17	0/17	0/17	10/17	3/17	6/17	0/17	3/17	0/17

* Number with parasites over total cultured; RFFD-right fore footpad; LHFD-left hind footpad; LMLN-left mandibular lymph node; 'RPLN-right popliteal lymph node; LIV-liver; SPLN-spleen; BLD-blood.

4.3.1(d). Results of xenodiagnosis using *P. duboscqi* and *L. donovani*-infected hamsters

Subcutaneous saline aspirates taken from noses (site of inoculation) and cultured revealed the presence of parasites in 6/10 of the first group of 10 hamsters, at 8 and 10 weeks post-inoculation. When *P. duboscqi* were allowed to feed on the nose and footpads of these 6 hamsters at weeks 8 and 10, none of the 200 bloodmeals cultured from these sand flies yielded parasites in culture. When sacrificed at week 10, cultures of nose skin and mandibular lymph nodes revealed the presence of parasites in 5/10 and 4/10 hamsters respectively. Liver, spleen, blood, right popliteal lymph nodes, left hind footpads and right fore footpads did not yield any parasites by culture. Impression smears of liver and spleen revealed no parasites.

The 20 hamsters that were immunosuppressed with cyclophosphamide before inoculation with *L. donovani* did not yield any parasites in cultures of subcutaneous saline aspirates taken from the site of inoculation at 8 and 10 weeks post-inoculation. Similarly, when sacrificed at week 10, liver, spleen, blood, popliteal and mandibular lymph nodes, nose skin and footpads did not reveal the presence of parasites both by culture and smear. All the 400 blood meals cultured from the 400 *P. duboscqi* used in the xenodiagnosis yielded no parasites both at week 8 and week 10 post-inoculation.

Results of the xenodiagnosis carried out on the 20 hamsters that were inoculated with *L. donovani* and later immunosuppressed with cyclophosphamide were not different from those described above. None of the 200 blood meals cultured from *P. duboscqi* after the week 8 xenodiagnosis and a similar number cultured in week 10 yielded parasites. Subcutaneous saline aspirates taken from the site of inoculation revealed the persistence of parasites in 12/20 hamsters at week 8 and 10/20 at week 10 post-inoculation. When sacrificed during week 10, cultures showed that 13/20 and 9/20 hamsters had parasites at the site of inoculation (nose) and in the left mandibular lymph nodes respectively. Cultures and smears of blood, spleen, liver, right fore footpad, left hind footpads and popliteal lymph node did not reveal the presence of parasites.

4.4. Discussion

Studies carried out by Nong *et al.* (1989) showed that exposure of mammalian macrophages to calcitonin gene-related peptide (CGRP) of synthetic or rat origin, or exposure to *Lu. longipalpis* saliva renders these cells incapable of antigen presentation for at least 4 days and are refractory to activation by gamma interferon for at least 2-3 days. In the experiment reported here, much as leaving the

sensitized hamsters for 7 days before inoculation with *L. donovani* may have led to the disappearance of the immunosuppression at macrophage level as reported by Nong *et al.* (1989), in a related study utilizing nonhuman primates, intradermal co-inoculation of *P. duboscqi* saliva with 1 x 10^8 culture-derived stationary phase *L. donovani* promastigotes in 4 sites failed to produce consistent visceral leishmaniasis in 4 naive vervet monkeys, *Cercopithecus aethiops* in a related study.

Unlike in *L. major* cutaneous infections where *P. duboscqi* saliva produces clearcut exacerbative infection in both vervet monkeys (Anjili *et al.*, 1992) and in BALB/c mice, the same may not necessarily apply for visceral parasites such as *L. donovani.* Sand fly saliva does not influence the course of *L. donovani* in the Syrian golden hamster. A similar finding was recently reported by Paranhos *et al.* (1993) who found that intradermal inoculation of healthy mongrel dogs with $2x10^5$ stationary phase *L. chagasi* culture promastigotes mixed with *Lu. longipalpis* salivary gland lysates did not lead to development of kala-azar in the dogs. The dogs only developed intense eosinophilia. Sensitization against sand fly saliva may not also influence the course of *L. donovani* infection, as there was no difference in the course of disease between sensitized and control naive hamsters.

Another new finding in the experiments described in this part of the study is the observation that, *L. donovani* when inoculated intradermally in cutaneous sites like nose skin and footpads, amastigotes are able to stay alive and dormant in these sites for over 10 months without visceralizing in the hamster, an animal that has always been thought to be highly susceptible to this parasite These cryptic infections are not easy to detect by culturing subcutaneous aspirates from the site of inoculation, but are readily detected by culturing skin from the site of inoculation, an indication that the parasites persisting in the skin are very few. Some hamsters especially those inoculated in belly skin were able to eliminate the parasites completely while others had amastigotes only in lymph nodes draining the site of inoculation. Unlike nose skin and footpads, belly skin is poor at *L. donovani* retention.

Experimental inoculation of human volunteers with a Kenyan strain of *L. donovani* showed that the incubation period from time of inoculation to the onset of symptoms varies from six to eight months (Manson-Bahr *et al.*, 1963). Marsden (1979) reported that the long period of latency may be due to amastigotes that remain dormant but alive in the body. Serological testing of populations has indicated large numbers of cryptic and oligosymptomatic infections (WHO, 1990). A similar finding has been reported by Jahn *et al.* (1986). Apart from lymph nodes that are known to be

able to retain amastigotes causing *Leishmania* lymphadenitis in the absence of visceral leishmaniasis in man (Azadeh, 1985), other sites where amastigotes are able to stay dormant without causing classical kala-azar in susceptible animal hosts are not known. Results obtained in this study suggest that the skin is one of the sites where *L. donovani* can stay dormant for a long time.

Mechanisms by which *L. donovani* amastigotes are able to persist in the skin without visceralizing are not well known, but it is known that while in the skin, most parasites except cutaneous forms are partially protected from the host's immune system due to low temperature (29-33^{o}C). At temperatures below 37^{o}C, the ability of macrophages to exhibit cytocidal activity is significantly reduced due to poor response towards cytokine activation (Scott, 1985). Howard *et al.* (1987) showed that at leg temperatures of 29^{o}C, complement activation to kill *L. donovani* is much reduced. The limited microbicidal capacity of resident skin macrophages together with the ability of *Leishmania* to deplete lysosomes in infected macrophages (Barbieri *et al.*, 1990) may play a role in survival of *L. donovani* in cutaneous skin tissue as a cryptic infection. These skin infections may be an important source of infection for sand flies. The failure of *P. duboscqi* to pick up *L. donovani* amastigotes may be attributed to the low grade parasitaemia observed in all the animals that were used in the xenodiagnosis.

Immunosuppression of *L. donovani*-infected hamsters with cyclophosphamide does not necessarily lead to an increase in skin infections with *L. donovani*. It should be noted that even for a parasite that sometimes causes both visceral and cutaneous leishmaniasis like *L. infantum*, only a small fraction of its natural vector sand fly ever gets infected after feeding on a resevoir host. A good example is seen in the work of Pozio *et al.* (1985) who were only able to infect 4/67 (5.9%) specimens-of *P. perniciosus* after feeding them on the black rat *Rattus rattus* (the reservoir for this parasite in Italy) infected with *L. infantum* and later immunosuppressed with cortisone acetate. The mechanism by which the sand fly takes up East African *L. donovani* still remains an enigma, and more studies should be carried out to unravel the mechanism.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1. General discussion

The previous four chapters have dealt with studies on the effects of *P. duboscqi* saliva on the establishment of *L. major* and *L. donovani* infections. This chapter examines the overall situation regarding interactions between sand fly saliva, *Leishmania* parasites and the mammalian host. The present studies have demonstrated that like saliva of the New World sand fly *Lu. longipalpis*, saliva of the Old World sand fly, *P. duboscqi* contains bioactive substances that manipulate vertebrate host immune components at the site of inoculation thus enhancing infectivity of the parasite it transmits, *L. major*. Basing on *in vivo* and *in vitro* experimental evidence obtained in this study, it is now possible to postulate how *P. duboscqi* saliva enhances *L. major* infections, when the two are co-inoculated in footpads of inbred BALB/c mice.

The *in vivo* observations to the effect that intradermal inoculation of *P. duboscqi* salivary gland lysates in hamster skin generates an inflammatory reaction ranging from haematoma formation to an accumulation of macrophages and that saliva of this sand fly possesses anticoagulant and chemotactic properties has not been previously demonstrated. The inflammatory reaction was shown not to differ, whether inoculation of saliva was done using the sand fly bite or by needle. Considering that a similar observation

has recently been made following intradermal inoculation of *Lu. longipalpis* saliva in hamsters (Laurenti *et al.*, 1992), it appears like the salivary bioactive components are conserved in both *Lutzomyia* and *Phlebotomus* species. An *in vitro* assay showed that *P. duboscqi* saliva is chemotactic to mouse peritoneal macrophages. This is the first time sand fly saliva has been shown to be chemotactic to macrophages. It is however not clear whether or not saliva is directly chemotactic *in vivo* as it is *in vitro*, or whether it also acts as a cytotaxigen by inducing generation of C5a, a powerful chemotactic agent that attracts macrophages to the site of antigenic activity.

Metacyclic *Leishmania* promastigotes have been shown to utilize vertebrate host complement C3b to enter macrophages (Mosser and Edelson, 1985,1987; Blackwell *et al.*, 1985; Wozencraft *et al.*, 1986). In this study, *P. duboscqi* saliva has been shown not to inactivate host complement. This non-inactivation of the complement cascade suggests that vector saliva does not prevent activation of complement by *Leishmania* surface glycoconjugate lipophosphoglycan and hence opsonization by C3b. Another interesting finding in this study is the observation that intradermal inoculation of *P. duboscqi* saliva with *L. donovani* does not influence the course of metastatic spread and visceralization. A similar observation was made in dogs that were intradermally

inoculated with *L. chagasi* mixed with *Lu. longipalpis* salivary gland lysates (Paranhos *et al.*, 1993).

Unlike in *L. major*, it appears that the presence of saliva may only aid *L. donovani* to establish a successful skin infection, with visceralization occuring only as a result of the parasite's intrinsic viscerotropic tendencies. It became evident in this study that intradermally inoculated visceralizing *L. donovani* is able to remain dormant in the skin of a hamster for 10 months without visceralizing. The skin is therefore suggested as one of the sites where *L. donovani* amastigotes can remain dormant in cases of asymptomatic infections and possibly oligosymptomatic infections. This finding casts doubt on the common belief that the hamster is highly susceptible to *L. donovani* (Schnur and Jacobson, 1987), a conclusion that was coined out of highly invasive artificial routes of parasite administration such as intracardial, intrahepatic, intratesticular and intravenous inoculations. The only reasonable model of susceptibility to a parasite of any animal can only be based on a route of inoculation that closely resembles the natural vector inoculation. Thus, hamsters when inoculated intradermally with *L. donovani*, displayed a course of infection similar to that described in humans by Manson-Bahr *et al.*, (1963). This is most likely the course of parasite development after vector inoculation.

Earlier studies, and results obtained in this study, have shed light on the mechanisms through which *L. major* establishes a successful infection and how *P. duboscqi* saliva aids the parasite to overcome host defense mechanisms. Maxadilan, the most potent vasodilator ever described (Lerner *et al.*, 1991) has been identified in saliva of *Lu. longipalpis* as the component that exacerbates *Leishmania* infection (Lerner *et al.*, 1991; Theodos *et al.*, 1991). This vasodilator peptide has been shown to immunosuppress macrophage activation by gamma interferon and antigen presentation (Nong *et al.*, 1989). It is possible that immunization of a host against maxadilan may limit pathology caused by *Leishmania* promastigotes inoculated by the sand fly vector. Another component in sand fly saliva that may aid *Leishmania* in causing an infection is the anticoagulant enzyme ATP diphosphohydrolase (Ribeiro, 1986, 1987).

Incorporating these earlier findings with the results of this study, it can be inferred that, following inoculation during the probing phase, *P. duboscqi* saliva initially prevents blood from clotting. Continuous bleeding, together with saliva-induced chemotaxis attracts macrophages to the site of the bite and saliva inoculation. Macrophages arriving at the site of inoculation are then immunosuppressed by saliva. In this way, C3b opsonized metacyclic promastigotes are able to enter the immunosuppresed cell without

triggering the generation of toxic reactive oxygen metabolites. Sand fly saliva may therefore exacerbate *Leishmania* infections by manipulating the vertebrate host and not by directly acting on the parasite.

5.2. Conclusion

The immediate impression gained from results of this study is that, saliva of *P. duboscqi* contains pharmacologically and immunologically active molecules that cause vasodilation, are chemotactic to macrophages, and also enhance *L. major* infection. The inflammatory reactions generated in hamster skins by *P. duboscqi* saliva, and also its exacerbation of *L. major* infection in BALB/c mice, are similar to those reported for *Lu. longipalpis* (Titus and Ribeiro, 1988,1990), and *P. papatasi* (Theodos *et al.*, 1991).

The bioactive component in saliva of *Lu. longipalpis* that has been shown to exacerbate *L. major* infection has been identified as maxadilan (Theodos *et al.*, 1991; Lerner and Shoemaker, 1992). Maxadilan is a potent vasodilator peptide that is very similar to calcitonin gene-related peptide (CGRP) and calcitonin, both of which are neuropeptides produced by cells that originate from the neural crest in man and animals (Zaidi *et al.*, 1987). CGRP is also immunosuppressive to macrophages, but sand fly

maxadilan has been shown to be 100-fold more potent (Theodos *et al.*, 1991).

Genomic clones and cDNA encoding maxadilan have been characterized but its structure is not yet known. In this study, maxadilan was not positively identified as the component in *P. duboscqi* saliva that exacerbates *L. major* infection. Previous studies utilizing saliva from *P. papatasi* (Theodos *et al.*, 1991) and *Lu. longipalpis* from Costa Rica (Warburg *et al.*, 1994), showed that enhancement of infection by sand fly saliva does not depend entirely on the vasodilatory activity of maxadilan, which has not been detected in saliva of *P. papatasi* (Theodos *et al.*, 1991). This sand fly is closely related to *P. duboscqi* and its saliva has been shown to exacerbate *L. major* infection (Theodos *et al.*, 1991). Other than maxadilan, it is possible that there may be other bioactive substances in saliva of Old World sand flies that are able to exacerbate *Leishmania* infection.

Maxadilan, is now considered a potential vaccine (Theodos *et al.*, 1991; Lerner and Shoemaker, 1992). It is proposed that protection against *Leishmania* and reduction of pathology caused by the parasite, might be achieved if the enhancement factor could be neutralized by immunizing against the bioactive components in sandfly saliva (Theodos *et al.*, 1991). This study has demonstrated that this kind of immunization can be carried out against *P. duboscqi* saliva and hence protect against disfiguring *L. major* infection.

Considering that *P. duboscqi* saliva is chemotactic to macrophages *in vitro* and *in vivo*, this saliva can be used as cytotaxin in chemotaxis assays and immunosuppression studies. More studies are in progress to try and determine how sandfly saliva modulates host immune system in the establishment of visceral and cutaneous *Leishmania* infections.

REFERENCES

Adler, S. and Theodor, O. (1926). The mouthparts, alimentary tract and salivary apparatus of the female *Phlebotomus papatasi. Annals of Tropical Medicine and Parasitology*, **20**, 10-142.

Adler, S. and Ber, M. (1941). Transmission of *Leishmania tropica* by bite of *Phlebotomus papatasi. Indian Journal Medical Research*, **29**, 803-909.

Al Mofleh, I.A. (1987). A macrophage immunosuppression induced by *Leishmani major* in BALB/c mice. *Tropical Medicine and Parasitology*, **38**, 93-96.

Alexander, J.O. (1984). *Arthropods and human skin.* Springer-Verlag Berlin Heidelberg.

Allen, A.C. (1948). Persistent 'insect bites' (dermal eosinophilic granulomas) simulatinf lymphoblastomas, histocytoses and squamous cell carcinomas. *American Journal of Pathology*, **24**, 367-387.

Anjili, C.O., Olobo, J.O., Githure, J.I, Reid,G.D.F.and Mbati, P.A. (1992). Evaluation of the optimum dose of culture derived *Leishmania major* promastigotes required to assess the immune status of vervet monkeys. *Proceedings of the 13th Annual Medical Scientific Conference of KEMRI/KETRI.*

Azadeh, B. (1985). 'Localized' *Leishmania* lymphadenitis: A light and electron microscopic study. *American*

Journal of Tropical Medicine and Hygiene, **34**(30, 447-455.

Barbieri, C.L., Doine, A.I. and Freymuller, E. (1990). Lysosomal depletion in macrophages fron spleen and foot lesions of *Leishmania*-infected hamsters. *Experimental Parasitology*, **71**, 218-228.

Barral-Netto, M., Barral, A., Brownell, C.E., Skeiky, Y.A.W., Ellingsworth, L.R., Twardzik, D.R. and Reed, S.G. (1992). Transforming growth factor-β in leishmanial infection: A parasite escape mechanism. *Science*, **257**, 545-548.

Beach, R. (1983). Localization of *Leishmania donovani* in experimentally infected sand flies: An indication of vectorial competence. *Proceedings of the 4th Annual Medical Scientific Conference of KEMRI/KETRI*, Nairobi,Kenya. P.M. Tukei and A.R. Njogu (editors).

Beach, R., Kiilu, G., Hendricks, L., Oster, C. and Leeuwenburg, J. (1984). Cutaneous leishmaniasis in Kenya: transmission of *L. major* to man by the bite of a naturally infected *Phlebotomus duboscqi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **78**, 747-751.

Beach, R., Kiilu, G. and Leeuwenburg, J. (1985). Modification of sand fly biting behaviour by *Leishmania* leads to increased parasite transmission. *American*

Journal of Tropical Medicine and Parasitology, **34**(2), 278-282.

Berberian, D.A. (1938). Successful transmission of cutaneous leishmaniasis by bites of *Stomoxys calcitrans*. *Proceedings of the Society for Experimental Biology and Medicine*, **38**, 254-256.

Bissonnette, E.Y., Rossignol, P.A. and Befus, A.D. (1993). Extracts of mosquito salivary gland inhibit tumour necrosis factor alpha release from mast cells. *Parasite Immunology*, **15**, 27-33.

Blackwell, J.M. and Alexander, J. (1983). The macrophage and parasitic protozoa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **77**, 636-645.

Blackwell, J.M. (1985). Receptors and recognition mechanisms of *Leishmania* species. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 606-612.

Blackwell, J.M. (1985). Role of macrophage complement and lectin-like receptors in binding *Leishmania* parasites to host macrophages. *Immunological letters*, **11**, 227-232.

Blackwell, J.M., Ezekowitz, R.A.B., Roberts, M.B., Chanon, J.Y., Sim, R.B. and Gordon, S. (1985). Macrophage complement and lectin-like receptors bind

Leishmania in the absence of serum. *Journal of Experimental Medicine*, **162**, 324-333.

Bordier, C. (1987). The promastigote surface protease of *Leishmania*. *Parasitology Today*, **3**, 151-153.

Bouchek, M.M. and Snyderman, R. (1976). Calcium influx requirements for human neutrophil chemotaxis: Inductions by lanthanum chloride. *Science* **193**, 905-907.

Boyden, S. (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes *Journal of Experimental Medicine*, **115**, 387 - 466

Bray, R.S. (1972). Leishmaniasis in the Old World. *British Medical Bulletin*, **28**, 30-34.

Bray, R.S. (1983). *Leishmania* : Chemotactic responses of promastigotes and macrophages *in vitro*. *Protozoology*, **30**(2), 322-329.

Bretscher, P.A., Wei, G., Menon, J.N. and Bielefeldt-Ohmann, H. (1992). Establishment of stable cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major*. *Science* **257**, 539-542.

Chenoweth, R.E. and Hugli, T.E. (1978). Demonstration of a specific C5a receptor on intact human polymorphonuclear leucocytes *Proceedings on the National Academy of Science*, **75**, 3943-3947.

Chenoweth, R.E., Rowe, J.G. and Hugli, T.E. (1979). A modified method for chemotaxis under agarose. *Journal of Immunological Methods*, **25**, 337-353.

Chulay, J.D., Adoyo, M.A. and Githure, J.I. (1985). *Leishmania donovani* parasitaemia in Kenyan Visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 218-222.

Cohen, S. and Wand, P.A. (1971). *In vitro* and *in vitro* activity of a lymphocyte and immune complex - dependent chemotactic factor for eosinophils. *Journal of Experimental Medicine*, **133**, 133-146.

Cross, M.L., Cupp, M.S., Cupp, E.W., Ramberg, F.B. and Enriquez, M.J. (1993a). Antibody responses of BALB/c mice to salivary antigens of haematophagous black flies (Diptera: Simuliidae). *Journal of Medical Entomology*, **30**, 725-734.

Cross, M.L., Cupp, M.S., Cupp, E.W., Ramberg, F.B. and Enriquez, M.J. (1993b). Modulation of murine immunological reponses by salivary gland extract of *Simulium vittatum* (Diptera: Simuliidae). *Journal of Medical Entomology*, **30**(5), 928-935.

Cutler, J.E. (1974). A simple *in vitro* method for studies on chemotaxis. *Proceedings of the Society for Experimental Biology and Medicine*, **147**, 471-474.

Davies, C.R., Cooper, A.M., Peacock, C., Lane, R.P. and Blackwell, J.M. (1990). Expression of LPG and GP63

by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. *Parasitology*, **101**, 337-343.

Ding, A., Nathan, C.F., Graycar, J., Derynck, R., Stuehr, D.J. and Srimal, S. (1990). Macrophage deactivating factor and transforming factor β1, β2 and β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. *Journal of Immunology*, **145**, 940-944.

Dostrovsky, A. (1953). Urticaria multiforme endemica (Harara). In: Simmons R.D.G. (ed). Handbook of tropical dermatology and medical mycology. Elsevier, Amsterdam, Pp. 889 - 894.

Eilam, Y., El-On, J. and Spira, D.T. (1985). *Leishmania major:* Excreted factor, calcium ions, and the survival of amastigotes. *Experimental Parasitology*, **55**, 161-168.

El-On, J., Bradley, D.J. and Freeman, J.C. (1980). *Leishmania donovani.* Action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. *Experimental Parasitology*, **49**, 167-174.

Franke, E.D., McGreevy, P.E., Katz, S.P. and Sacks, D.L. (1985). Growth cycle-dependent generation of complement-resistant *Leishmania* promastigotes. *Journal of Immunology*, **134**(4), 2713-2718.

Gajree, T.N. and Goedbloed, E. (1968). *Annual Report of the Medical Research Centre*, Nairobi. Ed. Royal Tropical Institute, Amsterdam, The Netherlands Pp. 20.

Gallin, J.I. and Rosenthal, A.S. (1974). The regulatory role of divalent cations in human granulocyte chemotaxis. *Journal of Cell Biology*, **62**, 594-609.

Gallin., E.G. and Gallin, J.I. (1977). Interaction of chemotactic factors with human macrophages. *Journal of Cell Biology*, **75**, 277-289.

Garnham, P.C.C. (1965). The leishmanias with special reference to the role of animal reservoirs. *American Zoologist*, **5**, 141-151.

Giannini, M.S.H. (1986). Suppression of pathogenesis in cutaneous leishmaniasis by UV irradiation. *Infection and Immunity*, **51**(3), 838-843.

Glaser, T.A., Baatz, J.E., Kreishman, G.P. and Mukkada, A.J. (1988). pH homeostasis in *Leishmania donovani* amastigotes and promastigotes. *Proceedings of the National Academy of Sciences, USA*, **85**, 7602-7606.

Goldman, L., Rockwell, E. and Richfield, D.F. (1952). III. Histopathological studies on cutaneous reaction to bites of various arthropods. *Journal of Tropical Medicine and Hygiene*, **1**, 514-525.

Gordon, R.M. (1950). Reactions produced by arthropods directly injurious to the skin of man. *British Medical Journal*, **II**, 316-318.

Gottlieb, M. and Dwyer, D.M. (1981). Protozoan parasites of humans: Surface membrane externally disposed acid phosphatase. *Science*, **212**, 939-941.

Gramiccia, M., Gradoni, L. and Pozio, E. (1987). *Leishmania infantum sensu lato* as an agent of cutaneous leishmaniasis in Abruzzi region (Italy). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **81**, 235-237.

Haidaris, C.G. and Bonventre, P.F. (1983). Efficacy of combined immunostimulation and chemotherapy in experimental visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene*, **32**, 286-295.

Hendricks, L.D. and Wright, N. (1979). Diagnosis of cutaneous leishmaniasis by *in vitro* cultivation of saline aspirates in Schneider's *Drosophila* medium. *American Journal of Tropical Medicine and Hygiene*, **28**, 962-964.

Hill, J.O. (1988). Pathophysiology of experimental leishmaniasis: The role of parasite physiology in the development of metastatic disease. *American Journal of Tropical Medicine and Hygiene*, **39**(3), 256-260.

Hommel, M. (1978). The genus *Leishmania*: biology of the parasites and clinical aspects. *Bulletin de L'Institut Pasteur*, **75**, 5-102.

Howard, M.K., Sayers, G. and Miles, M.A. (1987). *Leishmania donovani* metacyclic promastigotes: Transformation *in vitro*, lectin agglutination, complement resistance, and infectivity. *Experimental Parasitology*, **64**, 147-156.

Hudson, A., Bowman, L. and Orr, C.W.M. (1960). Effects of absence of saliva on blood feeding by mosquitoes. *Science*, **131**, 1730-1731.

Jahn, A., Lelmett, J.M. and Diesfield, H.-J. (1986). Seroepidemiological study on kala-azar in Baringo District, Kenya. *Journal of Tropical Medicine and Hygiene*, **89**, 91-104.

Johnson, P.T., McConnel, E. and Herting, M. (1963). Natural infections of leptomonad flagellates in Panamanian *Phlebotomus* sand flies. *Experimental Parasitology*, **14**, 107-122.

Jones, L.D., Hodgson, E.Williams, T., Higgs, S. and Nuttal. P.A. (1992). Saliva activated transmission (SAT) of Thogoto virus: relationship with vector potential of different haematophagous arthropods. *Medical and Veterinary Entomology*, **6**, 261-265.

Kabat, E.A. and Mayer, M.M. (1971). *Experimental immunochemistry*, Second edition. Ed. C.C. Thomas, Springfield.

Katakura, K. and Kobayashi, A. (1985). Enhancement of infectivity of *Leishmania donovani* promastigotes by serial mouse passages. *Journal of Parasitology*, **71**(3), 393-394.

Kehrl, J.H., Roberts, A.B., Wakefield, L.M., Jakowlew, S., Sporn, M.B. and Fauci, A.S. (1986). Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. *Journal of Immunology*, **137**(12), 3855-3860.

Keller, H.U. and Sorkin, E. (1967). Studies on chemotaxis. IX. Migration of rabbit leucocytes through filter membranes. *Proceedings of the Society of Experimental Biology and Medicine*, **126**, 677-680.

Keller, H.U., Borel, J.F., Wilkinson, P.C., Hess, M.U. and Cottier, H. (1972). Reassessment of Boyden's technique for measuring chemotaxis. *Journal of Immunological Methods*, **1**, 165-172

Killick-Kendrick, R. (1978). Recent advances and outstanding problems in the biology of phlebotomine sandflies. *Acta Tropica*, **35**, 297-131.

Killick-Kendrick, R. and Molyneux, D.H. (1981). Transmission of leishmaniasis by bite of phlebotomine sand flies: Possible mechanisms. *Transactions of the*

Royal Society of Tropical Medicine and Hygiene, **75**, 152-154.

Killick-Kendrick, R. (1985). Some epidemiological consequences of the evolutionary fit between leishmaniae and their phlebotomine vectors. *Bulletin de la Societe de Pathologie exotique*, **78**, 747-755.

Killick-Kendrick, R., Bryceson, A.D.M., Peters, W., Evans, R.A., Leaney, A.J. and Rioux, J. A. (1985). Zoonotic cutaneous leishmaniasis in Saudi Arabia: lesions healing naturally in man followed by a second infection with the same zymodeme of *Leishmania major*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 363-365.

Killick-Kendrick, R., Lainson, R., Rioux, J.-A. and Saf'janova, V.M. (1986). The taxonomy of *Leishmania*-like parasites of reptiles. *Leishmania*. Taxonomie et phylogenese. Applications eco-epidemiologiques. (*Coll, int.CNRS/INSERM, 1984) IMMEE*, Montpellier, 143-148.

Killick-Kendrick, R., Wallbanks, K.R., Molyneux, D.H. and Lavin, D.R. (1988). The ultrstructure of *Leishmania major* in the foregut and proboscis of *Phlebotomus papatasi*. *Parasitology Research*, **74**, 586-590.

Killick-Kendrick, R. (1990). Phlebotomine vectors of the leishmaniasis: a review. *Medical and veterinary Entomology*, **4**, 1-24.

Kimber, C.D., Evans, D.A., Robinson, B.L. and Peters, W. (1981). Control of yeast contamination with 5-fluorocytosine in the *in vitro* cultivation of *Leishmania* spp. *Annals of Tropical Medicine and Parasitology*, **75**, 453-454.

Kirkpatrick, C.E., Nolan, T.J. and Farrell, J.P. (1987). Rate of *Leishmania*-induced skin-lesion development in rodents depends on the site of inoculation. *Parasitology*, **94**, 451-465.

Kweider, M., Lemesre, J., Darcy, F., Kusnierz, J.P., Capron, A. and Santoro, F. (1987). Infectivity of *Leishmania braziliensis* promastigotes is dependent on the increasing expression of a 65,000 dalton surface antigen. *Journal of Immunology*, **138**, 299-305.

Lainson, R. and Southgate, B.A. (1965). Mechanical transmission of *Leishmania mexicana* by *Stomoxys calcitrans*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **59**(6), 716.

Lainson, R. and Shaw, J.J. (1979). The role of animals in the epidemiology of South American Leishmaniasis. In: *Biology of the Kinetoplastida*, Lumsden, W.H.R. and Evans, D.A. (editors). London and New York: Academic Press, Pp 1-116

Lainson, R. (1982). *Leishmania*. In *CRC handbook series in zoonoses Vol. 2*, Steele, J., Jacobs, H. and Arambulo, P. (editors). Boca Raton, Florida: CRC Press Pp. 41-103.

Lawyer, P.G., Young, D.G., Butler, J.F. and Akin, D.E. (1987). Development of *Leishmania mexicana* in *Lutzomyia diabolica* and *Lutzomyia shannoni* (Diptera: Psychodidae). *Journal of Medical Entomology*, **24**, 347-355.

Lawyer, P.G., Githure, J.I., Mebrahtu, Y., Perkins, P.V., Muigai, R. and Leeuwenburg, J. (1989). Leishmaniasis research in Kenya: Parasite-vector-host associations. In: *Leishmaniasis*. Edited by D.T. Hart, Plenum Publishing Corporation.

Lawyer, P.G., Githure, J.I., Anjili, C.O., Olobo, J.O., Koech, D.K. and Reid, G.D.F. (1990). Experimental transmission of *Leishmania major* to vervet monkeys (*Cercopithecus aethiops*) by bites of *Phlebotomus duboscqi* (Diptera: Psychodidae). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 229-232.

Laurenti, M.D., Corbett, C.E.P., Chebabo, R., Takakura, C.F.H., Brazil, B.G. and Sotto, M.N. (1992). Inflammatory response at the subcutaneous inoculation site in *L. (L.) chagasi* infection with *Lutzomyia longipalpis* salivary gland lysates. *Memorias Do Instituto Oswaldo Cruz, Rio de Janeiro*, Vol. **87**, Supplement II, November, 1992,110.

Lerner, E.A., Vibbard, D.E. and Shoemaker, C.B. (1991). Maxadilan, a potent vasodilator from sand fly salivary

glands. *American Journal of Tropical Medicine and Hygiene.* Supplement to Vol. **45**(3), 117-118.

Lerner, E.A. and Shoemaker, C.B. (1992). Maxadilan. Cloning and functional expression of the gene encoding this potent vasodilator peptide. *Journal of Biological Chemistry*, **267**(2), 1062-1066.

Lewis, D.J. (1975). Functional morphology of the mouthparts in New World Phlebotomine sand flies (Diptera: Psychodidae). *Transactions of the Royal Entomological Society of London*, **126**, 497-532.

Lewis, D.J. (1987). Depth of penetration of vertebrate skin by phlebotomine sand flies (Diptera: Psychodidae). *Annals of Tropical Medicine and Parasitology*, **81**(2), 173-179.

Lewis, D.J. and Ward, R.D. (1987). Transmission and vectors. *The Leishmaniasis in Biology and Medicine. Vol. 1.* (ed. by W. Peters and R. Killick-Kendrick), Pp. 235-262. Academic Press, London.

Lightner, L. and Robert, L.W. (1984). Mechanical transmission of *Leishmania major* by *Glossina morsitans morsitans* (Diptera: Glossinidae). *Journal of Medical Entomology*, **21**(2) ,243.

Lumsden, W.H.R. (1974). Biochemical taxonomy of *Leishmania*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **68**, 74-75.

Maccache, P.H., Showell, H.J., Becker, B.L. and Sha'afi, R.I. (1977). Transport of sodium, potassuim, and calcuim across rabbit polymorphonuclear leucocyte membranes. *Journal of Cell Biology*, **73**, 428-444.

Mann, G.J. and Bates, H.R. (1960). The pathology of insect bites; a brief review and report of eleven fatal cases. *South Medical Journal*, **53**, 1399-1406.

Manson-Bahr, P.E.C., Southgate, B.A. and Harvey, A.E.C. (1963). Development of kala-azar in man after inoculation with a *Leishmania* from a Kenyan sand fly. *British Medical Journal*, **1**, 1208-1210.

Manson-Bahr, P.E.C. and Southgate, B.A. (1964). Recent reasearch on kala azar in East Africa. *Journal of Tropical Medicine and Hygiene*, **67**, 79-84.

Marinkelle, C.J. (1980). The control of leishmaniases. *Bulletin of World Health Organization*, **58** (6), 807-818.

Marsden, P.D. (1979). Current concepts in parasitology. Leishmaniasis. *New New England Journal of Medicine*, **300**, 350-352.

Mellanby, K. (1946). Man's reactions to mosquito bites. *Nature*, **158**, 554.

Minter, D.M. (1984). *Phlebotomus* sand flies. In: *Mansons Tropical Diseases*. Eighteenth edition. P.E.C. Manson-Bahr and Apted, F.I.C (editors). England: Bailliere Tindall, Pp. 734-759.

Molyneux, D.H. and Ashford, R.W. (1983). *The biology of Trypanosoma and Leishmania: Parasites of man and domestic animals.* London: Taylor and Francis.

Molyneux, D.H. and Jefferies, D. (1986). Feeding behaviour of pathogen-infected vectors. *Parasitology*, **92**, 721-736.

Mosser, D.M. and Edelson, P.J. (1984). Activation of the alternative complement pathway by *Leishmania* promastigotes: Parasite lysis and attachment to macrophages. *Journal of Immunology*, **132**(3), 1501-1505.

Mosser, D.M. and Edelson, P.J. (1985). The mouse macrophage receptor for C3bi is a major mechanisms in the phagocytosis of *Leishmania* promastigotes. *Journal of Immunology*, **135**(4), 2785-2789.

Mosser, D.M. and Edelson, P.J. (1987). The third component of complement (C3) is responsible for the intracellular survival of *Leishmania major*. *Nature*, **327**, 329-331.

Mukkada, A.J., Meade, J.C., Glaser, T.A. and Bonventre, P.F. (1985). Enhanced metabolism of *Leishmani donovani* amastigotes at acid pH: An adaptation for intracellular growth. *Science*, **229**, 1099-1101.

Murray, H.W. (1981). Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *Journal of Experimental Medicine*, **153**, 1302-1315.

Murray, H.W. (1982). Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. **129**, 351-357.

Nelson, R.R., Quie, P.G. and Simmons, R.L. (1975). Chemotaxis under agarose. A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *Journal of Immunology*, **115** (6), 1650-1656.

Nelson, W.A., Cliford, C.M., Bell, J.F. and Hestokin, B. (1972). *Polyplax serrata:* Histopathology of the skin of louse-infected mice. *Experimental Parasitology*, **31**, 194-202.

Newstead, R. (1911). The papataci flies (*Phlebotomus*) of the Maltese Islands. *Bulletin of Entomological Research*, **2**, 47-48.

Nolan, T.J. and Farrel, J.P. (1987). Experimental infections of the multimmamate rat (*Mastomys natalensis*) with *Leishmania donovani* and *Leishmania major*. *American Journal of Tropical Medicine and Hygiene*, **36**(2), 264-269.

Nong, Y.-H., Titus, R.G., Ribeiro, J.M.C. and Remold, H.G. (1989). Peptides encoded by the calcitonin gene inhibit macrophage function. *Journal of Immunology*, **143**, 45-49.

Oliver, J.M. (1978), Cell biology leukocyte abnormalities-membrane and cytoskeletal function in normal and defective cells. *American Journal of Pathology*, **93**, 221-259.

Orlandi, P.A. and Turco, S.J. (1987). Structure of the *Leishmania donovani* lipophosphoglycan. *Journal of Biological Chemistry*, **262**, 10384-10391.

Pangburn, M.K. and Muller-Eberhard, H.J. (1984). The alternative pathway of complement. *Springer Seminars in Immunopathology*, **7**, 163-192.

Paranhos, M., Dos Santos, W.C., Sherlock, I., Oliviera, G.G.S. and de Carvalho, L.C.P. (1993). Development of eosinophilia in dogs intradermally inoculated with sand fly saliva and *Leishmania (Leishmania) chagasi* stationary-phase promastigotes. *Memorias Do Instituto Oswaldo Cruz*, Rio de Janeiro, **88**(2), 249-251.

Patton, W.S. (1912). The development of the parasite of Indian kala-azar. Scientific Memoirs Government of India. Vol. 27,31, and 53. In Hindle, E.P., P.C. Hou and W.S. Patton. 1926. Serological studies in Chinese Kala Azar. *Proceedings of the Royal Society of London (Biology)*, **100**, 368-373.

Pearson, R.D. and Steigbigel, R.T. (1980). Mechanisms of lethal effect of human serum upon *Leishmania donovani*. *Journal of Immunology*, **125**, 2195-2201.

Pearson, R.D., Wheeler, D.A., Harrison, L.H. and Kay, D.H. (1983). The immunobiology of leishmaniasis. *Review of Infectious Diseases*, **5**(5), 907-911.

Peters, W., Elbihari, S. and Evans, D.A. (1986). *Leishmania* infecting man and wild animals in Saudi Arabia. 2. *Leishmania arabica* n.sp. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 497-502.

Pozio, E., Maroli, M., Gradoni, L. and Gramiccia, M. (1985). Laboratory transmission of *Leishmania infantum* to *Rattus rattus* by the bite of experimentally infected *Phlebotomus perniciosus*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 524-526.

Puentes, S.M., Sacks, D.L., da Silva, R.P. and Joiner, K.A. (1988). Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *Journal of Experimental Medicne*, **167**, 887-902.

Pulvertaft, R.J.V. and Hoyle, G.F. (1960). Stages in the life-cycle of *Leishmania donovani*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **54**(2), 191-196.

Ramachandra, R.N. and Wikel, S.K. (1992). Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages

and lymphocyte cytokine production. *Journal of Medical Entomology*, **29**, 818-826.

Ribeiro, J.M.C., Rossignol, P.A. and Spielman, A. (1984). Role of mosquito saliva in blood vessel location. *Journal of Experimental* Biology, **108**, 1-7.

Ribeiro, J.M.C., Rossignol, P.A. and Spielman, A. (1985). Salivary gland apyrase determines probing time in anopheline mosquitoes. *Journal of Insect Phsiology*, **31**(9), 689-692.

Ribeiro, J.M.C., Rossignol, P.A. and Spielman, A. (1986). Blood-finding strategy of a capillary-feeding sandfly, *Lutzomyia longipalpis*. *Comparative Biochemical Phsiology*, **83**(4), 683-686.

Ribeiro, J.M.C. (1987). *Ixodes dammini*: Salivary anti-complement activity. *Experimental Parasitology*, **64**, 347-353.

Ribeiro, J.M.C. (1987). Role of saliva in blood-feeding by arthropods. *Annual Review of Entomology*, **32**, 463-478.

Ribeiro, J.M.C., Vachereau, A., Modi, G.B. and Tesh, R.B. (1989). A novel vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. *Science*, **243**, 212 - 214.

Ribeiro, J.M.C. (1989). A trip with sand fly saliva. In: *Host Regulated Developmental Mechanisms in Vector Arthropods. Proceedings of the second symposium.*

Vero Beach, Florida. February 13-16. Ed. D. Borovsky and A. Spielman. Pp. 221-225.

Ribeiro, J.M.C., Weis, J.J. and Telford III, S.R. (1990). Saliva of tick *Ixodes dammini* inhibit neutrophil function. *Experimental Parasitology*, **70**, 382-388.

Ryan, L., Lainson, R., Shaw, J.J. and Wallbanks, K.R. (1987). The transmission of suprapylarian *Leishmania* by the bite of experimentally infected sand flies (Diptera: Psychodidae). *Memorias do Instituto Oswaldo Cruz, Rio de Janeiro*, **82**, 1417-1419.

Sacks, D.L. and Perkins, P.V. (1984). Identification of infective stage *Leishmania* promastigotes. *Science*, **233**, 1417-1419.

Sacks, D.L. and Perkins, P.V. (1985). Development of infective stage *Leishmania* promastigotes within phlebotomine sandflies. *American Journal of Tropical Medicine and Hygiene*, **34**(3), 456-459.

Sacks, D.L., Hieny, S. and Sher, A. (1985). Identification of cell surface carbohydrate and antigenic changes between noninffective and infective developmental stages of *Leishmania major* promastigotes. *Journal of Immunology*, **135**, 564-569.

Sacks, D.L. and da Silva, R.M. (1987). The generation of infective stage *Leishmania major* promastigotes iis associated with the cell-surface expression and release

of a developmentally regulated glycolipid. *Journal of Immunology*, **139**, 3099-3106.

Sacks, D.L. (1989). Metacyclogenesis in *Leishmania* promastigotes. *Experimental Parasitology*, **69**, 100-103.

Sacks, D.L., Brodin, T.N. and Turco, S.J. (1990). Developmental modification of the lipophosphoglycan from *Leishmania major* promastigotes during metacyclogenesis. *Molecular and Biochemical Parasitology*, **42**, 225-234.

Schneider, P., Rosat, J.-P., Bouvier, J., Louis, J. and Bordier, C. (1992). *Leishmania major*: Differential regulation of the surface metalloprotease in amastigote and promastigote stage. *Experimental Parasitology*, **75**, 196-206.

Schnur, L., Zuckerman, A. and Montilio, B. (1973). Dissemination of leishmanias to the organs of Syrian hamsters following intrasplenic inoculation of promastigotes. *Experimental Parasitology*, **34**, 432-447.

Schnur, L.F., Chance, M.L., Ebert, F., Thomas, S.C. and Peters, W. (1981). The biochemical and serological taxonomy of visceralizing *Leishmania*. *Annals of Tropical Medicine and Parasitology*, **75**, 131-144.

Schnur, L.F. and Jacobson, R.L. (1987). Appendix III. Parasitological techniques. In. *The Leishmaniases in*

Biology and Medicine. **Vol.1.** Biology and Epidemiology. W. Peters and R. Killick-Kendrick, R. (Editors). Academic Press. Pp. 499-541.

Scott, P. (1985). Impaired macrophage leishmanicidal activity at cutaneous temperature. *Parasite Immunology*, **7**, 277-288.

Short, H.E. and Swaminath, C.S. (1928). The method of feeding of *Phlebotomus argentipes* with relation to its bearing on the transmission of Kala-azar. *Indian Journal of Medical Research*, **15**: 827-836.

Shortt, H.E., Smith, R.O.A., Swaminath, C.S., and Krishnan, K.V. (1931). Transmission of kala-azar by the bite of *Phlebotomus argentipes. Indian Journal of Medical Research*, **18**, 1373-1375.

Silverstein, S.C. (1977). Endocytic uptake of particles by mononuclear phagocytes and the penetration of obligate intracellular parasites. *American Journal of Tropical Medicine and Hygiene*, **26**(6) supplement, 161-168

Snyderman, R., Shin, H.S. and Hausman, M.H. (1971). A Chemotactic factor for mononuclear leucocytes. *Proceedings of the Society of Experimental Biololgy and Medicine*, **138**, 387-390.

Spelberg, I. and Mehta, J. (1979). Demonstration of a specific neutrophil receptor for a cell derived

chemotactic factor. *Journal of Clinical Investigation*, **63**, 85-88.

Stebbings, J.H., Jr. (1974). Immediate hypersensitivity: A defense against arthropods?. *Perspectives in Biology and Medicine*, **17**, 233-239.

Tatchell, R.J. and Moorhouse, D.E. (1970). Neutrophils: Their role in the formation of a tick feeding lesion. *Science*, **167**, 1002-1003.

Territo, M.C. (1981). Chemotaxis. In *Leukocyte function. Methods in Hematology.* Martin J. Cline (Editor). Churchill Livingstone. Pp. 39-52.

Theodor, O. (1935). A study of the reactions to *Phlebotomus* bites with some remarks on "Harara". *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **29**(3), 273-284.

Theodos, C.M., Ribeiro, J.M.C. and Titus, R.G. (1991). Analysis of enhancing effect of sand fly saliva on *Leishmania* infection in mice. *Infection and Immunity*, **59**(5), 1592-1598

Theodos, C.M. and Titus, R.G. (1991). The effect of *Lutzomyia longipalpis* salivary gland material on the ability of macrophages to present antigen to *Leishmania major* specific T cells. *American Journal of Tropical Medicine and Hygiene.* Supplement to Volume **45**(3), 180.

Thomson, J.G. and Lamborn, W.A. (1934). Mechanical transmission of trypanosomiasis, leishmaniasis, and yaws through the agency of non-biting haematophagus flies. *British Medical Journal*, **2**, 506-509.

Titus, R.G. and Ribeiro, J.M.C. (1988). Salivary gland lysates from the sandfly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. *Science*, **239**, 1306-1308.

Titus, R.G. and Ribeiro, J.M.C. (1990). The role of vector saliva in transmission of arthropod borne disease. *Parasitology Today*, **6**(5), 157-160.

Turco, S.J. (1988). The lipophosphoglycan of *Leishmania*. *Parasitology Today*, **4**(9), 255-257.

Turco, S.J. (1990). The leishmanial lipophosphoglycan: A multifunctional molecule. *Experimental Parasitology*, **70**, 241-245.

Turco, S.J. and Sacks, D.L. (1991). Expression of a stage-specific lipophosphoglycan in *Leishmania major* amastigotes. *Molecular and Biochemical Parasitology*, **45**, 91-100.

Van Joost, K.S. and Sluiters, J.F. (1972). Appearance of *Lesihamania donovani* (Kenya strain) in the blood of experimentally infected golden hamsters (*Mesocricetus auratus*), *Tropical and Geographical Medicine*, **24**, 292-297.

Vargaftig, B.B., Chignard, M., and Benveniste, J. (1981). Present concepts on the mechanism of platelet aggregation. *Biochemical Pharmacology*, **30**, 263-271.

Wallbanks, K.R., Maazoun, R., Canning, E.U. and Rioux, J.A. (1985). The identity of *Leishmania tarentolae* Wenyon, 1921. *Parasitology*, **90**, 67-78.

Walters, L.L., Modi, G.B., Tesh, R.B. and Burrage, T. (1987). Host-parasite relationship of *Leishmania mexicana mexicana* and *Lutzomyia abonnenci* (Diptera: Psychodidae). *American Journal of Tropical Medicine and Hygiene*, **36**, 294-314.

Walton, B.C. (1987). Leishmaniasis: A global problem. In: *International Workshop on Research on Control Strategies for the leishmaniasis*. Ottawa, Canada. Section 1, Paper No. A1.

Warburg, A., Saraiva, E., Lanzaro, G.C., Titus, R.G. and Neva, F. (1994). Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. *Philosophical Transactions of the Royal Society of London. Series B*, **345**, 223-230.

Warburg, A., Hamada, G.S., Schlein, Y. and Shire, D. (1986). Scanning electron microscopy of *Leishmania major* in *Phlebotomus papatasi*, *Zeitschrift fur Parasitenkunde*, **72**; 423-431.

Ward, P.A. (1968). Chemotaxis of mononuclear cells. *Journal of Experimental Medicine*, **128**, 1201-1220.

Ward, R.D., Ribeiro, A.L., Ready, P.D. and Murtagh, A. (1983). Reproductive isolation between different forms of *Lutzomyia longipalpis* (Lutz and Neiva) (Diptera: Psychodidae), the vector of *Leishmania donovani chagasi* Cunha and Chagas and its significance to kala-azar distribution in South American. *Memorias Do Instituto Oswaldo Cruz, Rio de Janeiro*, **78**,269-280.

Wilkinson, P.C. (1974). *Chemotaxis in Inflammation* London: Churchill Livingstone, Edinburgh.

Wikel, S.K. (1982). Influence of *Dermecentor andersoni* infestation on lymphocyte responsiveness to mitogens, *Annals of Tropical Medicine and Parasitology*, **76**, 627-632.

Wikel, S.K. (1984). Immunomodulation of host responses to ectoparasite infestation - an overview. *Veterinary Parasitology*, **14**, 321-339.

Wirtz, H.-P. (1988). Quantitating histamine in the saliva and salivary gland sof two Palaearctic black fly species (Diptera: Simuliidae). *Tropical Medicine and Parasitology*, **39**, 309-312.

World Health Organization. (1984). The leishmaniasis. Report of a WHO Expert Committee, *Technical Report Series*, **701**, 1-140.

World Health Organization. (1987). The leishmaniasis. *Tropical Disease Research. Eighth Programme*

Report. J. Maurice and Pearce, A.N. (editors). World Health Organization, Geneva: Pp. 101-112.

World Health Organization. (1990). Control of the leishmaniasis. Report a WHO Expert Committee. *Technical Report Series No.* **793**.

Wozencraft, A.O., Sayers, G. and Blackwell, J.M. (1986). Macrophage type 3 complement receptors mediate serum-independent binding of *Leishmania donovani.* Detection of macrophage-derived complement on the parasite surface by immunoelectron microscopy. *Journal of Experimental Medicine*, **164**, 1332-1337.

Wright, S.D. and Silverstein, S.C. (1983). Receptors for C3b and C3bi promote phagocytosis but not release of toxic oxygen from human phagocytes. *Journal of Experimental Medicine*, **158**, 2016-2023.

Yorke, W. and Macfie, J.W.S. (1924). The action of the salivary secretion of mosquitoes and of *Glossina tachinoides* on human blood. *Annals of Tropical Medicine and Parasitology*, **18**, 103-108.

Young, D.G. and Lawyer, P.G. (1987). New World vectors of the leishmaniases. *Current Topics in Vector Research*, **4**, 29-71.

Zaidi, M., Breimer, L.H. and MacIntyre, I. (1987). Biology of peptides from the calcitonin genes. *Quaterly Journal of Experimental Physiology*, **72**, 371-376.

Zigmond, S.H. and Hirsch, J.G. (1973). Leucocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor. *Journal of Experimental Medicine*, **137**, 387-410.