# THE EFFECT OF IMMUNE SERA UPON UPTAKE AND INTRACELLULAR SURVIVAL OF *LEISHMANIA DONOVANI* IN MONONUCLEAR PHAGOCYTES

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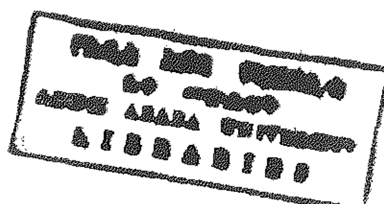

| | PAGE |
|---|---|
| **TABLE OF CONTENTS** | i |
| **ACKNOWLEDGMENTS** | iii |
| **LIST OF TABLES** | iv |
| **LIST OF FIGURES** | vii |
| **LIST OF ABBREVIATIONS** | viii |
| **ABSTRACT** | x |
| **1. INTRODUCTION** | 1 |
| 1.1. Leishmania | 1 |
| 1.2. Leishmaniasis | 2 |
| 1.3. Immune response in visceral leishmaniasis | 6 |
| 1.4. Phagocytosis and mononuclear phagocytes | 8 |
| 1.5. THP-1 cells (monocytic leukaemia cells) | 9 |
| 1.6. Complement | 11 |
| 1.7. Antibodies | 13 |
| **2. MATERIALS AND METHODS** | 17 |
| 2.1. Phagocytosis of Parasites by host cells | 17 |
| 2.1.1. Host cells | 17 |
| 2.1.2. Parasites | 17 |
| 2.1.3. Sera | 18 |
| 2.1.4. Infection | 19 |
| 2.1.5. Harvesting cells after infection | 20 |

2.3. Phagocytosis (uptake) determined by the radiometric method 21

2.3.1. Radioisotope labelling 21

2.3.2. Infection and cell harvesting 22

2.4. Data analysis 23

**3. RESULTS** 24

3.1. Parasite uptake determined microscopically 24

3.2. Viability determined by MTT reduction 30

3.3. Phagocytosis of radiolabelled parasites 34

**4. DISCUSSION** 38

**5. CONCLUSION** 46

**6. REFERENCES** 48

**7. LIST OF ABBREVIATIONS** 60

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## 7. LIST OF ABBREVIATIONS

AVL - Active Visceral Leishmaniasis

CE - Control Endemic

Ci - Curies

CoA - Concanavalin A

CPM - Cycles Per Minute

CR1 - Complement Receptor one

CR3 - Complement Receptor three

DAT - Diffusion Agglutination Test

DCL - Diffuse Cutaneous Leishmaniasis

ELISA - Enzyme Linked Immunosorbent Assay

FCS - Foetal Calf Serum

FnR - Fibronectin Receptor

IFN - Interferon

IL - Interleukin

IUdR - Iodo-Deoxy-Uridine

LK - Lymphokine

LPG - Lipophosphoglycan

mAB - Monoclonal Antibody

MAC - Membrane Attack Complex

MFR - Mannose-Fucose Receptor

MCL - Mucocutaneous leishmaniasis

MNP - Mononuclear Phagocytes

MTT - 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide

OD - Optical Density

PBS - Phosphate buffered Saline

PDGF - Platelet Derived Growth Factor

PKDL - Post kala-azar Dermal Leishmaniasis

PT - Post-treatment

RA - Retinoic Acid

SDS - Sodium Dodecyl Sulphate

SP - Seropositive

TNF - Tumour Necrosis Factor

## ABSTRACT

The effect of sera from patients with acute visceral leishmaniasis on phagocytosis of *L. donovani* by human macrophage-like THP-1 cells was examined using microscopic and radiometric methods. Leishmania antibody-positive sera from clinically asymptomatic subjects, sera from treated individuals, and sera from endemic and non-endemic controls were also tested. In the microscopic phagocytosis assay, enhanced uptake of promastigotes was observed at 3, 6, and 24 hrs after addition of sera from patients with active disease, individuals with treated disease, or asymptomatic subjects with positive antibody titres compared to sera from endemic or nonendemic controls and assays performed without human sera. In some experiments, phagocytosis in the presence of sera from patients with active disease was greater than that observed using sera from asymptomatic individuals with positive, but somewhat lower antibody titres. Enhanced phagocytosis was positively correlated with *L. donovani*-specific ELISA antibody titres and the correlation was higher when data from treated patients was excluded from the analysis. Measurement of phagocytosis using the radiometric method did not detect differences between groups. Numbers of viable parasites 48 hrs after phagocytosis were also greater using serum compared to adding no serum to the cultures. Parasite viability was also positively correlated with the level of phagocytosis, suggesting that intracellular killing was not affected by pretreatment of parasites or phagocytes with different groups of serum.

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## LIST OF TABLES

**Table 1.** ELISA based OD for L. donovani specific antibodies 17

**Table 2.** Microscopic determination of mean percentage of infected cells and mean number of amastigotes per infected cell 3 hrs after infection. 26

**Table 3.** Microscopec determination of mean percentage of infected cells and mean number of amastigotes per infected cell 6 hrs after infection. 27

**Table 4.** Microscopic determination of mean percent of infected cells and mean number of amastigotes per infected cell 24 hrs after infection. 28

**Table 5.** Microscopic determination of mean percent of infected cells and mean number of amastigotes per infected cell 24 hrs after infection at 1:5 cell to parasite ratio. 29

**Table 6.** Viability of amastigotes determined by MTT reduction (parasites pretreated with sera). 32

**Table 7.** Viability of amastigotes determined by MTT reduction (cells pretreated with sera). 33

**Table 8.** Spontaneous release of $^{51}Cr$ by parasites after 24 hrs of labeling. 36

**Table 9.** Phagocytosis of $^{51}Cr$ labelled parasites 24 hrs after infection. 37

## LIST OF FIGURES

**Figure 1.** Incorporation of 3H-Thymidine by parasites 24 hrs after treatment (100$\mu$Ci/20ml of 20x$10^6$ parasites) 35

**Figure 2.** Incorporation of $^{51}Cr$ by parasites 24 hrs after treatment with 200$\mu$Ci/ml of 10x$10^6$ parasites. 35

**Figure 3.** Parasite radiolabelling with $^{125}$IUdR 24 hrs after treatment (40$\mu$Ci/20x106ml$^{-}$). 36

## ABSTRACT

The effect of sera from patients with acute visceral leishmaniasis on phagocytosis of *L. donovani* by human macrophage-like THP-1 cells was examined using microscopic and radiometric methods. Leishmania antibody-positive sera from clinically asymptomatic subjects, sera from treated individuals, and sera from endemic and non-endemic controls were also tested. In the microscopic phagocytosis assay, enhanced uptake of promastigotes was observed at 3, 6, and 24 hrs after addition of sera from patients with active disease, individuals with treated disease, or asymptomatic subjects with positive antibody titres compared to sera from endemic or nonendemic controls and assays performed without human sera. In some experiments, phagocytosis in the presence of sera from patients with active disease was greater than that observed using sera from asymptomatic individuals with positive, but somewhat lower antibody titres. Enhanced phagocytosis was positively correlated with *L. donovani*-specific ELISA antibody titres and the correlation was higher when data from treated patients was excluded from the analysis. Measurement of phagocytosis using the radiometric method did not detect differences between groups. Numbers of viable parasites 48 hrs after phagocytosis were greater using serum compared to adding no serum to the cultures. Parasite viability was also positively correlated with the level of phagocytosis, suggesting that intracellular killing was not affected by pretreatment of parasites or phagocytes with different groups of serum.

# 1. INTRODUCTION

## 1.1. *LEISHMANIA*

*Leishmania* are dimorphic, intracellular protozoan parasites. They exist as flagellated extracellular promastigotes in the invertebrate host (Phlebotomine sandflies) and as aflagellar amastigotes within mononuclear phagocytes of their mammalian hosts (Marsdon, 1984). During its life cycle, each species of *Leishmania* enters its hosts by a common mechanism. Promastigotes released into the skin when the sandfly pierces the epidermis and feeds on blood are internalized by macrophages within which the organisms are transformed into amastigotes. Then lysosomes fuse with the phagosomes containing the organism (Chang and Dwyer, 1976; Berman *et al.*, 1981). Infection is amplified by rephagocytosis of amastigotes released by lysed macrophages. If the amastigotes survive in the phagocytes, the cycle is completed by the sandfly feeding on infected tissues and ingesting amastigotes which subsequently transform into promastigotes (Russell and Talamas-Rohana, 1989; Schlein, 1993).

After phago-lysosome fusion, intracellular parasites have to survive a barrage of anti-microbial systems, including products of oxygen metabolism, lysosomal hydrolases, low pH and cationic proteins (Murray, 1981). As Bogdan *et al.*, (1990) pointed out the fate of the intracellular parasites and the course of the developing disease are highly dependent on the parasite and the response of the host. The ultimate fate depends on the physiological state of the macrophage, but *Leishmania* use a number of strategies to avoid killing. It has been suggested that *Leishmania*

amastigotes which live in phagolysosomes may be acidophilic or acid-tolerant (Mukkard *et al.*, 1985). According to Edelson and Mosser, (1987) deposition of C3b released by activation of the alternative complement pathway by promastigotes actually protects the parasite from the toxic effects of $H_2O_2$. Lipophosphoglycan, LPG (Russo *et al.*, 1992) and the major surface glycoprotein gp63 (Shreffler *et al.*, 1993) are believed to play a role in entry and survival of *Leishmania* into macrophages.

## 1.2. LEISHMANIASES

Leishmaniases are diseases (anthropozoonoses) caused by members of the genus *Leishmania* (Berman *et al.*, 1979; Chang, 1980). Generally leishmanial diseases have a worldwide distribution except in Antarctica and Australia, but occurring predominantly in tropical and subtropical regions. The established number of human incidence is 12 million with annual cases of approximately 400,000 (Reed, 1991). Human leishmaniasis is caused by at least 14 different species or subspecies of *Leishmania.* Leishmaniasis presents as a large variety of disease manifestations differing markedly in their severity and health impact (WHO, 1990; Reed, 1991). Outcome of infection and clinical patterns of the disease vary according to parasite strain, size of inoculum, site of inoculum, host genetics (immunological competence of the host), acquired immunity and nutritional state of the host (Meischer and Belehu, 1982; Molyneux and Ashford, 1983). The spectrum of clinical manifestations of the disease is grouped under three main categories;

cutaneous leishmaniasis (oriental sore), mucocutaneous leishmaniasis (espundia) and visceral leishmaniasis (kala-azar) (Warren, 1982).

Cutaneous leishmaniasis is normally caused by *L. tropica, L. aethiopica and L. major* in the Old World (Bryceson, 1969; Kreir and Baker, 1987). The clinical characteristics of cutaneous leishmaniasis tend to vary from region to region depending on parasite, host and zoonotic cycles involved. The classical disease is characterized by one or more nodular lesions at the site of inoculation. The lesion may heal spontaneously leaving a scar. Non-healing forms are also known. Cutaneous leishmaniases in the New World are caused by members of *L. mexicana* complex. The disease can also be seriously disfiguring particularly on the face ( Hommel, 1978; Sacks *et al.*, 1983). Cutaneous leishmaniasis due to *L. aethiopica* may give rise to oronasal and diffuse cutaneous leishmaniasis. This condition seems to result from cellular immunodeficiency in the patient (WHO, 1990).

It is generally agreed that long-term immunity follows spontaneous cure from cutaneous infections by parasites of the *L. tropica* complex. Experimental infections in volunteers showed that a complex pattern of resistance develops after recovery, provided that the initial infection was allowed to run its full course. Moreover studies indicated that re-infection among a resident population in an endemic area (Old World) is uncommon. Cutaneous leishmaniasis is characterized by the development of marked cell mediated reactions. The demonstration of antibody in cutaneous leishmaniasis has been more problematic than that of cellular reactivity. Antibodies could be detected, however, by the use of immunofluorescence and Enzyme linked immunosorbent assay (ELISA) in a majority of patients with rising

infection results in a systemic fatal disease, visceral leishmaniasis, characterized by parasite invasion of macrophages in all lymphoid tissues in the body (Fernandez-Guero, 1987). The spleen, liver and the bone marrow are heavily infected. As a result these organs are enlarged and infiltrated with infected and uninfected mononuclear phagocytes (MNP). The infection is not localized, but is diffuse throughout the organs. Following the spread of the infection in the viscera, a fatal illness characterized by intermittent moderate fever, swollen abdomen due to splenomegaly, hepatomegaly and general wasting occur. Visceral leishmaniasis in Africa is somewhat similar but there is a variable tendency to cutaneous manifestations as well. In the Mediterranean Basin and South America it is predominantly infants below two years of age who become sick and the disease is known as infantile or Mediterranean visceral leishmaniasis (Molyneux and Ashford, 1983).

A late event that may occur in the course of kala-azar is post kala-azar dermal leishmaniasis (PKDL). Parasites are quite numerous in the body, but are apparently restricted to the skin. The disease responds poorly to chemotherapy. The infection often returns following apparent cure. It does not occur in infantile visceral leishmaniasis. PKDL has been described especially in India, occurring one to ten or more years after apparently successful treatment, but sometimes in the absence of any previous history of kala-azar (Molyneux and Ashford, 1983; Molyneux and Killick-Kendrick, 1987).

Visceral leishmaniasis caused by *L. donovani* is known to occur In the lowland areas of western and southern Ethiopia mainly in the region of Gamo Gofa.

It occurs principally in arid and semi-arid lowlands below 1300 meters altitude. The Aba Roba focus in Konso (Gamo Gofa) is an endemic area (Ayele, 1982). Visceral leishmaniasis is also reported in Metema-Humera areas, south-west Ethiopia, Segen and Woito valleys and near the Red Sea coast. Ashford *et al.*, (1973) presented a few cases of leishmaniasis in a village in the Lake Tana basin at 1800m altitude. Case fatality rate of 56% in hospitalized patients in northern Ethiopia, and 92% in Aba Roba region of among untreated cases has been reported. There were a very few cases of relapse (Mengesha and Abuhay, 1978; Ayele *et al.*, 1988).

## 1.3. IMMUNE RESPONSE IN VISCERAL LEISHMANIASIS

Immunological dysfunction is the hallmark of visceral leishmaniasis. The absence of antigen-induced lymphocyte proliferative responses, negative delayed hypersensitivity reactions and poor generation of lymphokines (LK) are well documented (Beutler *et al.*, 1982; Barral *et al.*, 1986). The pathogenesis of visceral leishmaniasis does not appear to be well understood (Haldar *et al.*, 1983; Murray *et al.*, 1984). In an area endemic for visceral leishmaniasis, it has also been observed that early in the infection some patients have the ability to respond to *Leishmania* antigens by lymphocyte blastogenesis and intradermal skin test, and some do not.

It is possible that susceptibility to the development of clinical visceral leishmaniasis is related to an inability to generate Interleukin 2 (IL-2) and gamma

interferon (IFN-$\gamma$) to *Leishmania* antigen during the infection (Carvalho *et al.*, 1985). As Kemp *et al.*, (1993) reported, in murine models of *L. major* infection, it was possible to direct the disease into a visceral leishmaniasis-like syndrome by IL-4 producing Th2 cells, or cure of the disease with Th1 cells secreting IFN-$\gamma$. Th1 and Th2 cells are subsets of CD4$^+$ T helper/inducer lymphocytes first described in murine models by Mosmann (1986) and lately by Romagnani, (1991) in humans. Th1 cells produce IL-2 and IF-$\gamma$ but not IL-4, IL-5 and IL-10 while Th2 cells produce IL-4, IL-5 and IL-10 but not IL-2 or IFN-$\gamma$. In their study Kemp *et al.*, (1993) examined the potential of human T cells to generate Th1 and Th2 responses to *L. donovani*. Results suggest that in analogy with murine models, there is a dichotomy in the human T cell response to *L. donovani* infections although other studies have not shown a dichotomy (Karp *et al.*, 1993). Preferential activation of IL-4 producing Th1 cells may be involved in the exacerbation of human visceral leishmaniasis, activation of IFN-$\gamma$ producing Th1 cells may protect the host from severe disease. Although Th1-like responses occur during control of leishmanial infections, the data acquired so far do not permit extrapolation to the role of Th2 cells in mediating progressive disease in humans at present (Locksley and Louis, 1992).

## 1.4. PHAGOCYTOSIS AND MONONUCLEAR PHAGOCYTES

A foreign element that has managed to break through the body's bulwarks and has entered the tissues underlying the surface layer encounters the second line of defence, the phagocytes. Their main method of combat is to internalize and digest the intruder. It is this engulfment of the foreign elements by phagocytes which is known as phagocytosis (Klein, 1990). Phagocytosis is enhanced by antibodies and complement. The coating of parasites with any substance that enhances phagocytosis is known as opsonization (Golub, 1987). According to Roitt (1984) and Male *et al.* (1987), phagocytic cells include the polymorphonuclear cells, blood monocytes and the various cells of the reticuloendothelial system. Among the latter are the tissue macrophages, Kupffer cells of the liver, microglial cells of the brain and mesangial cells of the kidney. They play a vital role in innate and acquired immunity. Mononuclear phagocytes are essential for integrating the immune system and for host defence. They are involved in antigen presentation. They also serve as cytotoxic cells against extracellular and intracellular microorganisms (Gordon, 1982; Durum, 1986; Hogg, 1989).

The factors causing susceptibility of mononuclear phagocytes to intracellular infection in visceral leishmaniasis are not well understood (Karp *et al.*, 1993). TNF-$\alpha$ and INF-$\gamma$ produced by mononuclear cells are important regulatory and effector molecules that mediate defense against *Leishmania*. Monocytes fail to produce TNF-$\alpha$ and IL-1 in response to challenge with *Leishmania* whereas preinfected cells produce diminished amounts of IL-1 and normal amount of TNF-$\alpha$.

Cells pretreated with IFN-γ released IL-1 and TNF-α when exposed to *Leishmania*. These results show that *L. donovani* has evolved the capacity to infect MNP without stimulating the production of TNF-α and IL-1 (Reiner *et al.*, 1990). IL-10 may contribute to the pathogenesis of kala-azar by inhibiting the cytokine-mediated activation of host phagocytes that is necessary for control of leishmanial infection (Karp *et al.*, 1993). Ghalib, *et al.*, (1993) presented data which indicate the antagonistic effect of IL-10 upon IFN-γ. It was pointed out that IL-10 was produced by tissues from *L. donovani* infected patients, whereas individuals produced no detectable IL-10 after successful therapy.

The state of differentiation or tissue source of macrophages may also determine whether chronic infection is established. Parasites like *L. mexicana* and *L. donovani* are destroyed more rapidly in bone marrow derived macrophages than in resident peritoneal cells. It is believed that recently derived blood monocytes contain peroxidase positive organelles. Resident macrophages on the other hand have peroxidase activity restricted to the nuclear envelope and granular endoplasmic reticulum and from there it would appear not to enter phagosomes (Bray and Alexander, 1987).

## 1.5. THP-1 CELLS (MONOCYTIC LEUKAEMIA CELLS)

Monocytes and macrophages cannot be grown in a continuous culture systems. In addition, harvesting them is time consuming, the number of cells obtained is limited and the cell population is not uniform. Several lines of human

leukaemia cells, which are blocked at certain steps of the differentiation process and which can be induced to differentiate into macrophage by several stimuli, are available for *in vitro* studies (Auwerx, 1991). These lines allow the investigation of relatively homogeneous groups of cells during different stages of maturation and differentiation. Tsuchiya *et al.*, (1980) have established and characterized a monocytic leukaemia cell line THP-1. This cell line was isolated from a one year old boy suffering from acute monocytic leukaemia. The criteria for classifying these cells as monocytic include: (a) the presence of alpha naphthyl butyrate-esterase; (b) lysozyme production; (c) phagocytic activity of the sensitized sensitized red blood cells and (d) the capacity to restore T lymphocyte response to concanavalin A (Con A). Generally THP-1 cells resemble the human monocytes with respect to numerous criteria such as morphology, expression of membrane antigens and expression of genes involved in lipid metabolism (Auwerx, 1991). These cells express Fc receptors (Tsuchiya *et al.*, 1982), the third component of complement (C3) receptors and can be induced to produce IL-1, TNF and platelet derived growth factor (PDGF). The differentiation of THP-1 cells is associated with a dramatic alteration in cell morphology. After the initiation of this differentiation process, cells acquire a variety of shapes, their nucleus becomes more irregular, and many phagocytic vacuoles can be recognized in their cytoplasm and they show increased adherence to tissue culture plates (Auwerx, 1991). To analyze the interactions between human host cells and parasites, Ogunkolade *et al.*, (1990) used THP-1 cells. They used the cells because they display a significant basal level of phagocytosis and when exposed to a differentiating agent, retinoic acid

(RA) undergo a maturation process resulting in functional macrophage-like cells which remain in suspension.

## 1.6. COMPLEMENT

It is assumed that promastigotes delivered into the mammalian host are immediately exposed to complement present in the body fluids. Complement cytotoxicity might be a factor in limiting the spread of leishmanial parasites in the mammalian hosts. Promastigotes of all species of *Leishmania* so far tested are susceptible to lysis by fresh, non-immune serum. Parasite killing is generally due to the activation of the alternative pathway (Hoover *et al.*, 1984) except in the case of *L. donovani* whose destruction involves natural antibodies and complement activation by the classical route (Pearson and Steigbigel, 1980; Barral-Netto *et al.*, 1987). *L. mex. mexicana* and *L. donovani* are known to be the most resistant and *L. major* the most susceptible to lysis by complement. Complement activation results in the deposition of C3 on the parasite surface resulting in the formation of the membrane attack complex (MAC). MAC is a complex with cell lysing capacity formed when the terminal complement components (C6-C9) assemble around the fifth complement component (C5). Amastigotes of *L. major* were killed even when an incomplete MAC was formed in one study (Hoover et al., 1985). As it was demonstrated by Wozencroft and Blackwell (1987), the greater infectivity of stationary phase promastigotes of *L. donovani* is related to increased complement fixation on the parasite surface, resulting in increased binding to host MNP via

complement type 3 receptors (CR3). It was predicted that the increased infectivity of stationary-phase promastigotes does not relate to enhanced intracellular survival but simply to an increased ability to target to host macrophages. Sacks and Perkins (1984), on the other hand, related infectivity of stationary- phase *L. major* promastigotes to enhanced intracellular survival in resident peritoneal macrophages. They also indicated that there was no measurable difference in parasite:macrophage binding ratios between log- and stationary phase populations. Evidence indicated that complement components fixed on the infective promastigotes failed to mediate cytokine release but instead helped their entry into macrophages (Blackwell *et al.*, 1985). It was speculated that *Leishmania* species may play an active role by changing their surface to fix complement effectively via the complement receptor 1 (CR1) which is known to provoke less respiratory burst, thereby ensuring better intracellular survival. Non-infective promastigotes may enter macrophages through CR3 (receptor for C3) which may activate a high level of respiratory burst (Da Silva *et al.*, 1989; Puentes *et al.*, 1990). Data available from different laboratories do not clarify whether the entry of infective *Leishmania* into macrphages is always mediated by CR1 and not by CR3 and/or other receptors. Binding and internalization of *L. donovani* promastigotes or amastigotes by mouse resident macrophage *in vitro* was considerably inhibited by monoclonal antibody (mAb) to CR3 and by Fab fragments of mAb to C3 (Bray, 1983). Attachment in the absence of opsonins could be mediated by gp63 and LPG which interact with CR1, and CR3 and $p^{150,95}$ respectively. Producion of C3 by macrophages may opsonize *Leishmania* parasites locally under serum free conditions (Blackwell *et al.*,1985). In certain

cases of visceral leishmaniasis significant reduction in C3 was reported (Ghose *et al.*, 1980; Galvao-Castro *et al.*, 1984).

## 1.7. ANTIBODIES

If an infectious agent that has broken the bodies defence bulwarks withstands the onslaught by complement and phagocytes, it is likely to face the class of protective molecules called antibodies (immunoglobulins). Antibodies prepare the foreign elements for phagocytosis (opsonization) by providing surfaces for attachment to Fc receptors expressed on the surface of phagocytes. Since FcR-mediated phagocytosis often elicits a strong respiratory burst, the entry of *Leishmania* parasites into macrophages via this pathway is thought to be unfavourable to the survival of the parasite (Chang *et al.*, 1990).

A marked humoral response is the main immunological characteristic of visceral leishmaniasis. A considerable increase in serum Ig level was observed mostly of the IgG and IgM classes resulting in a reversal of the albumin/globulin ratio (Ghose *et al.*,1980). Much of the increase in Ig appeared to be non-specific and the result of polyclonal activation. Higher titres of specific antibody, however, could be detected by a variety of serological tests (Galvao-Castro *et al.*, 1984). Hailu (1990) showed that levels of *L. donovani*-specific antibodies from visceral leishmaniasis patients in Ethiopia remained high using the direct agglutination test (DAT) in most of patients, whereas ELISA antibodies declined with time after treatment in most patients considered to be clinically cured. Singla *et al.*, (1993)

on the other hand, reported a significant decline in parasite specific antibody titre after chemotherapy using DAT. Elassad *et al.*, (1994), employed an ELISA test to detect *Leishmania*-specific antibody in visceral leishmaniasis patients and others from the Sudan. The visceral leishmaniasis patients showed significantly higher IgG and relatively high IgM responses. In all groups low IgA level was recorded. Following treatment the IgG levels were reduced significantly.

High titres of *L. donovani* specific antibodies are also detected in some clinically asymptomatic individuals. Badaro et al. (1986) studied 86 children with antibody to *L. donovani* but no history of visceral leishmaniasis. Of these 23% never showed evidence of disease, 17% subsequently developed visceral disease and 60% had subclinical disease which resolved without treatment in 85% of the cases. Similar evidence of asymptomatic infection has been found in other areas (Holaday et al., 1993).

In leishmaniasis of humans and experimental animals specific cell mediated reactions greatly influence the course, degrees and probably the final outcome of the diseases (Herman, 1980). The role of specific humoral factors (Karp, 1991) in the initiation of the disease is not clear. Previous studies have indicated that immune sera from humans, rabbit and guinea pig enhanced parasite *(L. mexicana)* promastigote phagocytosis by murine macrophages (Bray, 1983). Similarly, heated immune sera from BALB/c mice (susceptible) and C57BL/6 (resistant) mice were found to increase attachment and subsequent internalization of promastigotes of *L. mex. amazonensis* (Reis *et al.*, 1987). Herman (1980) demonstrated that macrophages treated with mouse immune sera in an *in vitro* macrophage culture

system bound and subsequently phagocytized more *L. donovani* parasites than macrophages treated with control sera. It was concluded that enhancement was due to the opsonizing and cytophilic capacity of the sera. Such *in vitro* enhancement of phagocytosis or intracellular parasitization may be a prelude to the destruction of parasites by antibody dependent cell-mediated cytotoxicity or by activation of macrophages invoked through contact with sensitized lymphocytes (Parrillo and Fauci, 1977). On the other hand, inhibition of phagocytosis of *L. donovani* amastigotes by human macrophages in the presence of immune sera from rabbits was observed (Chang, 1981). Nothing is known about the effect of immune sera from humans upon uptake and killing of *L. donovani* parasites by human macrophages.

The precise nature of the immune effector mechanisms mediating protection against *Leishmania*, and the causes for the dramatic failure of such mechanisms in certain hosts remain obscure despite many years of careful studies. Generally self-healing cutaneous leishmaniasis of man is characterized, from an immunological stand point, by high skin reactivity and low antibody titres. Such parameters are reversed in DCL or kala-azar. There is overwhelming evidence that cell-mediated mechanisms are essential to healing of established leishmanial infections whether cutaneous or visceral. The positive skin test reactions to the parasite antigens in spontaneously cured individuals or in those cured after treatment testifies to this argument. The presence of large amounts of antibodies in man or animals with active visceral infection appears to be of no benefit to the host. Although the available information points to cell-mediated reactions being the crucial immune

defence components in leishmanial diseases, a role for humoral factors as modulators of the host parasite interaction has not been excluded (Mauel and Behin, 1987). This research was aimed at investigating the effects of *L. donovani* specific and decomplemeted immune sera upon in vitro phagocytosis and killing of the parasite by mononuclear phagocytes.

# 2. MATERIALS AND METHODS

## 2.1. Phagocytosis of parasites by host cells.

### 2.1.1. Host Cells

THP-1 cells, the human leukaemic monocytic cell line (American Type Culture Collection, USA) were grown in RPMI-1640 (Sigma, St. Louis, USA) containing 100U/ml penicillin, 100$\mu$g/ml streptomycin, 2mM L-glutamine (Gibco, Grand Island, USA) and supplemented with 10% heat inactivated (56°C, 30 min) foetal calf serum (Serva, USA) at 37°C in humidified atmosphere containing 5% $CO_2$. Cell cultures were passaged twice a week at a maximum volume of 30ml in 75cm$^2$ tissue culture flask (Gibco, England) at a density of 2-5x10$^5$ cells/ml (Ogunkolade *et al.*, 1990; Gebrehiwot *et al.*, 1992). Viable cell counting was done with 0.2% trypan Blue (Fluka, Switzerland). Retinoic acid (RA) (Sigma, USA) which is an active metabolite of vitamin A (Matikainen *et al.*, 1991) was used for differentiating the cells. THP-1 cells were treated with 1$\mu$l of 10$^{-6}$ M RA per ml of culture for three days. The RA was removed by washing three times (750xg, 10min) in excess culture medium.

### 2.1.2. Parasites

Promastigotes of *L. donovani* (MHOM/Am/89/399, an Ethiopian isolate

obtained from Arba Minch and provided by The Institute of Pathobiology) were grown in RPMI-1640 supplemented with 10% FCS, 100U/ml penicillin, 100$\mu$g/ml streptomycin and 50$\mu$g/ml kanamycin in 25cm$^2$ tissue culture flasks (Gibco, England) at room temperature. Promastigotes were passaged at a density of 1-2x10$^6$ parasites/ml. Stationary growth phase promastigotes (a subculture grown for 5-7 days) were concentrated by centrifugation and washed twice (1200xg, 10min) before they were resuspended in 0.1% formalin and counted in a haematocytometer (Neubauer, Germany)

**2.1.3. Sera**

Sera were collected from the visceral leishmaniasis-endemic area of the Aba Roba focus, in southwest Ethiopia. The sera were collected and provided by Dr. Nega Berhe (Institute of Pathobiology, IPB, Addis Ababa University). The sera included:

(a) Visceral leishmaniasis patients'sera from 10 subjects

(b) 10 sera from subjects 6 months to two years after treatment

(c) 10 sera from individuals who were seropositive (IgG based ELISA) for antibody to the parasite but without clinical symptoms

(d) 10 sera from healthy controls in visceral leishmaniasis endemic area

(e) Sera of healthy controls from non-endemic area (Armauer Hansen Research Institute, AHRI, foreign as well as local employees) in Addis Ababa.

All sera used were heat-treated at 56$^0$C for 30 min prior to use in

experiments. ELISA antibody titres for serum used in experiments (Table 1) were kindly measured by Asrat Hailu according to published protocols (Hailu, 1990).

| Sera | AVL | SP | PT | CE |
|---|---|---|---|---|
| Mean OD | 0.74 | 0.58 | 0.253 | 0.07 |
| Standard Error | 0.06 | 0.058 | 0.07 | 0.032 |

**Table 1.** ELISA based mean OD for *L. donovani* specific antibodies (IgG) in the various groups of sera (provided by Asrat Hailu). All sera were decomplemented (56$^0$C, 30 min.) before use. OD values were normalized to the positive control (positive control = 1.0). Cut-off for positive value = 0.32.

### 2.1.4. Infection

Parasite concentration was adjusted to $1.5 \times 10^6$/ml or $3 \times 10^6$/ml. Similarly RA treated THP-1 cells were resuspended in the culture medium after three washes (750xg, 10min) and their count adjusted to $3 \times 10^5$/ml. 100$\mu$l of promastigote suspension was seeded into 96 well flat bottomed microtitre plates (Flow Laboratories). $3 \times 10^5$ or $1.5 \times 10^5$ parasites/well were plated for the phagocytosis test and $1.5 \times 10^5$ promastigotes/well were used for the viability to yield cell to parasite ratio of 1:5. assay. Sera were added to the duplicate wells (1$\mu$l/well) to give a concentration of 1%. Promastigotes were pretreated with sera for 30 minutes at room temperature for both phagocytosis and killing assays. After 30 min,

promastigotes were mixed with differentiated THP-1 cells at parasite to cell ratio of 10:1 or 5:1 for phagocytosis and at 5:1 ratio for killing (viability) tests. The lower parasite:cell ratio (5:1) was used in experiments with longer infection periods to avoid lysis of THP-1 cells seen at the higher (10:1) ratio. Then incubation was done in a 5% $CO_2$ humid air at 37°C for 3 hrs, 6 hrs and 24 hrs for the phagocytosis tests and 48 hrs for the killing test.

### 2.1.5. Harvesting cells after infection

After variable times, non-phagocytized parasites were removed by low speed centrifugation (50xg, 10min, three times) in an excess medium. Cells were then resuspended, harvested onto glass slides by cytocentrifrgation (Shandon, Cat. No. 0026, England) at 25xg for 5 minutes, fixed with absolute methanol and stained with Giemsa (Hopkins and Williams, England). Parasite phagocytosis was observed microscopically (x1000, under oil). At least 100 cells were counted and the number of infected cells, uninfected cells and the number of amastigotes per infected cell were determined.

## 2.2. Viability of parasites following phagocytosis

THP-1 cells were infected as described above using a 1:5 ratio of THP-1 cells to parasites. After 48 hour of infection, parasites not internalized by THP-1 cells were removed by three washes as in the phagocytosis test. Instead of

resuspending the pellets (cells), they were lysed with 70μl/well of a lysis medium consisting of warm RPMI + 0.01% sodium dodecyl sulphate (SDS, Sigma, USA). Lysis of the cells was monitored with an inverted microscope within 7-20 minutes. Once cell disintegration was complete, 105 μl/well of Hepes buffered RPMI supplemented with 17% FCS was added to neutralize the SDS. Lysates were incubated for 48hrs at room temperature so as to promote transformation of released parasites to promastigotes.

The relative number of viable parasites/well was determined by measuring parasite dehydrogenase activity. 7μl MTT, [(3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide, (Sigma, USA)] was added per well. MTT was prepared by dissolving MTT 5mg/ml in phosphate buffered saline (PBS). Parasites were then incubated for 8-16 hrs at room temperature, cells were lysed and crystals were solubilized by adding 70μl/well of 10% SDS and incubating an additional 6-16 hrs at 37°C. The relative optical density (OD)/well was determined at a test wavelength of 570nm by blanking against medium + MTT + 10% SDS alone using an automatic microplate reader (Labsystems and Flow Laboratories, Mod. 311AO, Finland) (Kiderlen and Kaye, 1990).

## 2.3. Phagocytosis (uptake) determined by the radiometric method.

### 2.3.1. Radioisotope labelling

The conventional microscopic method used for the study of phagocytosis and

killing of Leishmania by macrophages is a dependable one, but it is time consuming and tedious. Cillari *et al.*, (1990) employed a double radiolabelling method for observing the phagocytosis of *L. major* promastigotes by macrophages. With minor modifications, this radiometric method was used to observe phagocytosis of *L. donovani* promastigotes by THP-1 cells. Cells and parasites were grown and counted as before. Parasites were labelled with three different isotopes for determination of optimal labelling. 40 $\mu$Ci [$^{125}$I]5-Iodo-2-deoxyuridine (IUdR, Amersham, U.K.), 200$\mu$Ci sodium [$^{51}$Cr] chromate or 100$\mu$Ci tritiated thymidine (Amersham, U.K.) was mixed with 10-20x$10^6$ promastigotes/ml in a 50ml polypropylene tube (Falcon, New Jersey). Then growth proceeded for 24 hrs at room temperature. Labelled promastigotes were washed 4 times and resuspended at the required concentration to determine the total number of phagocytized promastigotes. A standard curve was prepared using aliquots of $^{51}$Cr labelled parasites. This was done by serially diluting 1.1x$10^6$ parasites /200$\mu$l. 20 $\mu$l of this was transferred to 180$\mu$l of medium serially which would give approximately 1x$10^6$, 1x$10^5$, 1x$10^4$, 1x$10^3$, 1x$10^2$ parasites/200$\mu$l medium. After $^{51}$Cr labelling of parasites spontaneous release of the radioisotope was also tested at 2 hrs, 6 hrs, 24 hrs and 48 hrs. 20x$10^6$ parasites were suspended in RPMI and counts appearing in the supernatant were measured.

### 2.3.2. Infection and cell harvesting

The infection procedure was identical to that used for the microscopic

method. 24 hrs after infection, non-phagocytized parasites were removed by six washes (50xg, 5min). Cell pellets were resuspended and their contents transferred to 12x75mm propylene tubes (Falcon, New Jersey). The radioactivity in each tube was measured with a Rackgamma 11 (LKB Wallac, Model 1270, Finland) counter.

### 2.4. Data Analysis

All experiments were conducted 2-3 times and usually included replicate wells. Results presented are representative experiments i.e. repeat experiments gave equivalent results. Each table reports the result of one of the experiments. The means, standard deviations and standard errors were calculated for the percentage of infection, average number of amastigotes per infected cell and OD values of the killing assay for each group. Statistical significance was analyzed using one way analysis of variance (ANOVA) and to determine whether any differences among groups was present. Differences between specific pairs of serum groups were determined using Duncan's Procedure.

# 3. RESULTS

## 3.1. Parasite uptake determined microscopically

A total of 46 sera divided into five groups were used for this test. 10 sera from visceral leishmanial patients (AVL), 10 sera from clinically asymptomatic seropositive subjects (SP), 10 sera from treated patients (PT), 10 control sera (seronegative) from endemic area (CE) and 6 non-endemic control sera (CNE) from AHRI were tested.

After the 3 hrs infections, the mean percentages of infected cells for immune sera treated cultures were higher than those of control sera treated cases (Table 2). These differences were also evident for the 6 hr and 24 hr infections (Tables 3 and 4). Similarly, the mean number of amastigotes per infected cell for control sera treated infections was lower than those of post treatment sera, control non endemic sera and asymptomatic (preclinical) positive sera (see Tables 3 and 4).

For the three time points, the mean number of amastigotes per infected cell was greater in cultures treated with visceral leishmaniasis patient sera than cultures treated with control endemic sera. Similar differences were observed between cultures incubated with post-treatment sera and sera from antibody positive but clinically asymptomatic individuals on one side and control endemic sera on the other. Comparisons between asymptomatic seropositive cases and patient sera from antibody positive revealed appreciable differences only for the 6 and 24 hr time points at cell to parasite ratio of 1:10. Cultures which were not treated with sera

gave the least number of amastigotes and percentage infections for the three incubation periods. Tables 2, 3, and 4 give results of infections carried out at 1:10 cell to parasite ratio.

| SERA | AVL | SP | PT | CE | CNE | NS |
|---|---|---|---|---|---|---|
| Mean % infection | 69.4[a] | 62.3[b] | 62.1[c] | 39.1[d] | 45.4[e] | 21.0[f] |
| standard error | 2.0 | 2.8 | 3.2 | 3.5 | 4.1 | 3.3 |
| Mean amast. no./ infected cell | 4.06[1] | 3.9[2] | 3.5[3] | 3.3[4] | 3.8[5] | 2.7[6] |
| S.E. | 0.33 | 0.31 | 0.28 | 0.28 | 0.32 | 0.16 |

**Table 2.** Microscopic determination of mean percentage of infected cells and the mean number of amastigotes per infected cell three hours after infection. $3 \times 10^5$ parasites and $3 \times 10^4$ RA treated cells (1:10 ratio) were incubated in 200$\mu$l of RPMI containing 10% FCS, 1% sera for 3 hrs. Extracellular parasites were removed by centrifugation and cells were stained with Giemsa and examined microscopically. Statistically significant differences ($p<0.05$): a,b,c,d,e vs f; a,b,c, vs d and a,b,c vs e; 1 and 2 vs 6. AVL = active visceral leishmaniasis; SP = seropositive; PT = post-treatment; CE= control endemic; CNE= control non-endemic; NS = no serum; inf. =infection; amast.= amastigote. Correlation between ELISA Ab titres and mean % infection determined by linear regression r = 0.85.

| Sera | AVL | SP | PT | CF | CNE | NS |
|---|---|---|---|---|---|---|
| Mean % infection | 80.9[a] | 66.4[b] | 71.5[c] | 59.8[d] | 52.7[e] | 36.7[f] |
| S.E. | 1.6 | 3.4 | 4.9 | 3.2 | 4.8 | 0.8 |
| Mean amast. no./ infected cell | 6.4[1] | 5.7[2] | 5.2[3] | 4.7[4] | 3.9[5] | 3.0[6] |
| standard error | 0.45 | 0.37 | 0.38 | 0.50 | 0.47 | 0.20 |

**Table 3.** Microscopic determination of mean percentage of infected cells and the mean number of amastigotes per infected cell six hours after infection. $3 \times 10^5$ parasites and $3 \times 10^4$ RA treated cells (1:10 ratio) were incubated in 200$\mu$l of RPMI containing 10% FCS, 1% sera for 6 hrs. Extracellular parasites were removed by centrifugation and cells were stained with Giemsa and examined microscopically. Statistically significant differences ($p<0.05$): a,b,c,e,vs f; a,b,c vs d; a vs e and a vs b; 1,2,3 vs 6; 1,3 vs 5 and 1 vs 4. Correlation between ELISA Ab titres and mean % infection $r = 0.77$.

| Sera | AVL | SP | PT | CE | CNE | NS |
|---|---|---|---|---|---|---|
| Mean % Inf. | 85.0[a] | 69.9[b] | 81.2[c] | 51.9[d] | 69.9[e] | 55.2[f] |
| S.E. | 1.4 | 2.4 | 3.16 | 2.34 | 2.9 | 0.28 |
| Mean amast. no./inf. cell | 8.3[1] | 5.5[2] | 7..1[3] | 5.1[4] | 6.3[5] | 3.53[6] |
| S.E. | 2.7 | 1.3 | 1.8 | 1.7 | 1.9 | 0.04 |

**Table 4.** Microscopic determination of mean percentage of infected cells and the mean number of amastigotes per infected cell twenty four hours after infection. $3x10^5$ parasites and $3x10^4$ RA treated cells (1:10 ratio) were incubated in 200$\mu$l of RPMI containing 10% FCS, 1% sera for 24 hrs. Extracellular parasites were removed by centrifugation and cells were stained with Giemsa and examined microscopically. Statistically significant differences ($p<0.05$): a,b,c,e, vs d and f; a,c,e vs b; 1,2,3,5 vs 6; 1,3 vs 4,2 and 1 vs 5 and 3. Inf.= infection; amast.= amastigotes. Correlation between ELISA Ab titres and mean % infection $r = 0.69$.

| Sera | AVL | SP | PT | CE | NS |
|---|---|---|---|---|---|
| Mean % Infection | 32.2[a] | 28.9[b] | 30.1[c] | 18.0[d] | 17.6[e] |
| standard error | 1.8 | 1.7 | 1.3 | 1.9 | 0.9 |
| Mean amast. no. per infected cell | 3.15[1] | 2.9[2] | 2.68[3] | 2.22[4] | 1.81[5] |
| standard error | 0.142 | 0.114 | 0.164 | 0.177 | 0.108 |

**Table 5.** Microscopic determination of mean percent infection and mean amastigote number per infected cell twenty four hours after infection at 1:5 cell to parasite ratio. 1:5. $1.5 \times 10^5$ parasites and $3 \times 10^4$ RA treated cells (1:5 ratio) were incubated in 200$\mu$l of RPMI containing 10% FCS, 1% sera for 24 hrs. Extracellular parasites were removed by centrifugation and cells were stained with Giemsa and examined microscopically. Statistically significant differences ($p<0.05$): a,b,c vs d and e 1,2,3 vs 5 and 1,2, vs 4 CNE used here was from Ethiopians. Correlation between ELISA Ab titres and mean % infection $r = 0.80$.

Table 5 shows phagocytosis in cultures incubated for 24 hrs at a parasite to cell ration of 5:1. The numbers of amastigotes per infected cell and the related percentage infection between groups obtained for the 24 hrs of infection at this ratio corresponded to the relative values measured after the 3 hr, 6 hr and 24 hr infections conducted at parasite to cell ratio of 10:1. Intergroup comparisons indicated that uptake by cells treated with control sera were lower than the other groups. The highest calculated result was obtained for active visceral leishmaniasis patient sera followed by post treatment sera and clinically asymptomatic positive sera.

Examination of the phagocytosis test results within each group showed an increase that corresponded with incubation periods. The increase with time in the percentage infections calculated for clinically healthy positive sera treated cultures seemed to be less when compared with the other groups. Linear regression analysis revealed that phagocytosis at all time points was correlated with serum ELISA antibody titres. Correlation coefficients determined by linear regression analysis ranged from 0.69-0.85. Indivicual coefficients appear in the legends to tables 2-5.

### 3.2. Viability determined by MTT reduction

To allow a degree of phagocytosis which insured high numbers of intracellular amastigotes (Cillari *et al.*, 1990) a longer incubation time of 48 hrs was used in the viability test. The OD measured for control non-endemic sera and

untreated cells were lower than those obtained for visceral leishmaniasis patient sera. There were slight differences between cultures with pretreated cells and cultures with pretreated parasites. The OD values were slightly greater in sera pretreated cells. Viability was strongly correlated with the mean percent of cells undergoing phagocytosis. Correlation coefficients for comparisons of viability with each of the phagocytosis tests (Tables 2-5 and $^{51}Cr$ assay Table 9) ranged from 0.76 to 0.99. Individual correlation coefficients appear in legend to tables 6 and 7. These results suggested that cytotoxicity did not vary among the groups.

| Sera | AVL | SP | PT | CE | CNE | NS |
|---|---|---|---|---|---|---|
| Mean OD | 0.3334[a] | 0.3062[b] | 0.3114[c] | 0.2916[d] | 0.3084[e] | 0.3084[f] |
| S. E. | 0.02 | 0.012 | 0.012 | 0.012 | 0.001 | 0.0078 |

**Table 6.** Viability of amastigotes determined by MTT reduction (Parasites pretreated with sera) S. E. standard error; OD = optical density. Viability of amastigotes determinedby MTT reduction. Viability was analyzed 48 hrs after infection at cell to parasite ratio of 1:5. (parasites were pretreated with sera before infection). There were no statictical differences between groups.
Correlation coefficients for comparing viability to mean % infection:

Table 2, 3 hr assay: r = 0.88
Table 3, 6 hr assay: r = 0.99
Table 4, 24 hr (1:10 ratio) assay: r = 0.90
Table 5, 24 hr (1:5 ratio) assay: r = 0.86
Table 9, 24 hr $^{51}Cr$ assay: r = 0.92.

| Sera | AVL | SP | PT | CE | CNE | NS |
|---|---|---|---|---|---|---|
| Mean OD | 0.3202[a] | 0.3026[b] | 0.2502[c] | 0.1960[d] | 0.2682[e] | 0.208[f] |
| S. E. | 0.0152 | 0.0050 | 0.0114 | 0.0200 | 0.0152 | 0.0140 |

**Table 7.** Viability of amastigotes determined by MTT reduction (cells were pretreated with sera). OD = optical density; S.E. = standard error; Statistically significant differences: ($p<0.05$): a,b,c,e vs d; a,b,e vs f; a,b vs c and a vs e. Cells were pretreated with sera before infection. Viability was tested 48 hrs after infection at cell to parasite ratio of 1:5. Correlation coefficient for comparison of viability and mean % infection:

Table 2, 3 hr assay: $r = 0.92$
Table 3, 6 hr assay: $r = 0.77$
Table 4, 24 hr (1:10 ratio) assay: $r = 0.76$
Table 5, 24 hr (1:5 ratio) assay: $r = 0.87$
Table 9, 24 hr $^{51}Cr$ assay: $r = 0.96$.

### 3.3. Phagocytosis of radiolabeled parasites.

Attempts were made to label parasites and/or cells with different radioisotopes before infection so as to detect and evaluate phagocytosis. $^{125}$IUdR labelling of parasites based on the report of Mosser and Edelson (1985), incorporation of tritiated thymidine by parasites (Cillari *et al.*,1990) and $^{51}$Cr labelling of parasites were tried (see Figures 1, 2, and 3). The labelling methods all gave linear standard curves. However, $^{125}$IUdR labellings of parasites was too low and $^{3}$H Thymidine incorporation was too variable to be used for uptake test. Spontaneous release of $^{51}$Cr was minimal (Table 8). Of all the radioisotope labellings, $^{51}$Cr was selected for labelling parasites. Therefore, results for assessing phagocytosis under the radiometric method were based on $^{51}$Cr labelling of parasites and their subsequent incorporation by the cells.

As determined by radioactivity measurements (CPM) of cells infected for 24 hrs, no significant differences were seen between control sera treated uptake results and immune sera treated ones. Contrary to the results seen in the microscopic assay, cultures without serum had high uptake values. As with the microscopic assay, phagocytosis determined using $^{51}$Cr was correlated with serum ELISA titres ($r = 0.93$).

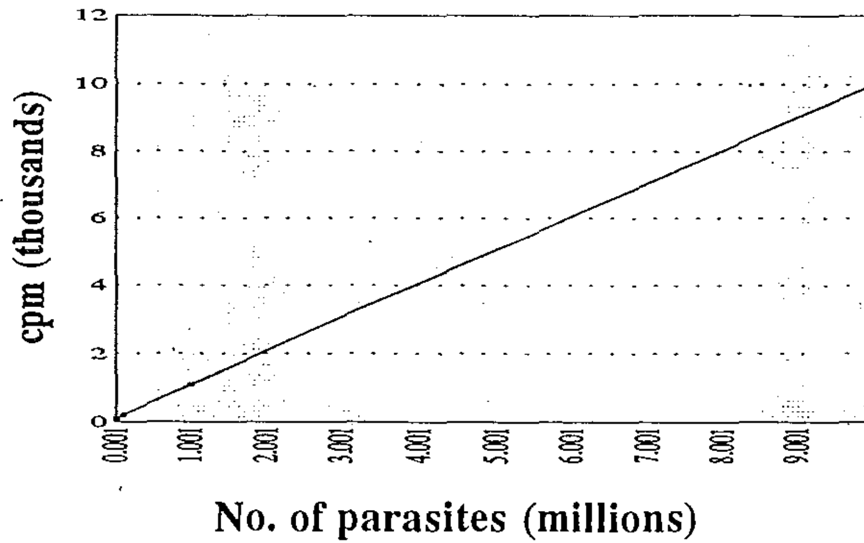


**Figure 1.** Incorporation of $^{3}$H-Thymidine by parasites 24 hrs after treatment (100$\mu$/20 ml of 20x$10^6$ parasites).

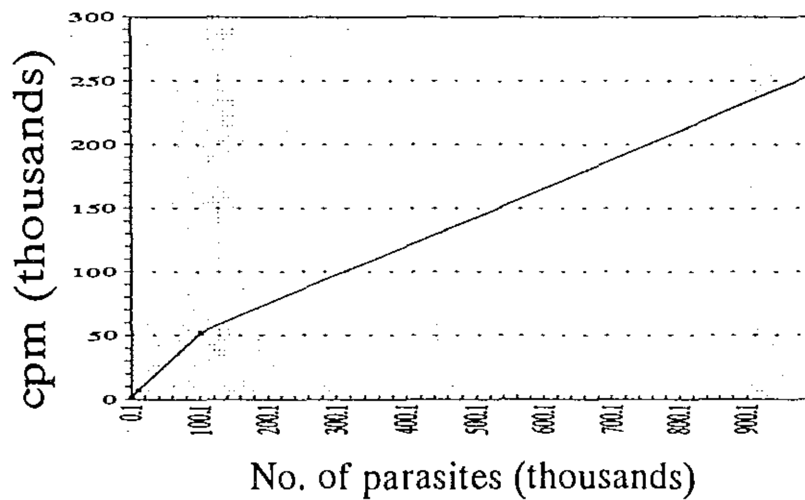


**Figure 2.** Incorporation of $^{51}$Cr by parasites 24 hrs after treatment (200$\mu$Ci/ml of 10x$10^6$ parasites).

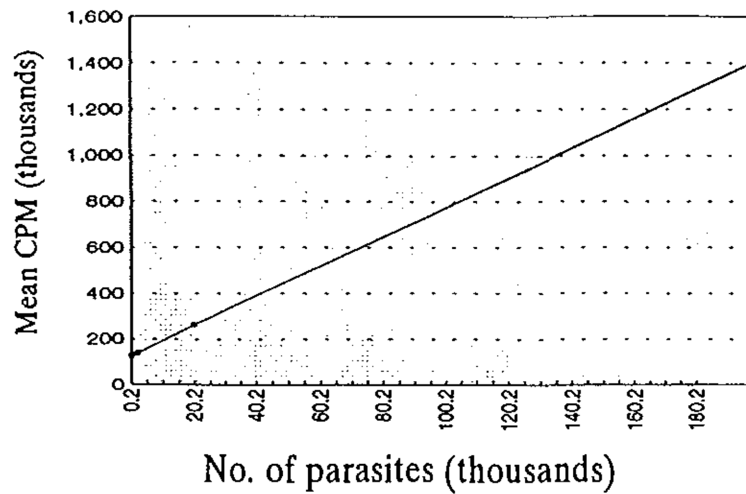


**Figure 3.** Parasite radiolabelling with $^{125}$IUdR 24 hrs after treatment ($40\mu Ci/20x10^6$ parasites).

| Time (hrs) | 2 | 6 | 24 | 48 |
|---|---|---|---|---|
| Spontaneous release (CPM) | 7263 | 5876 | 7227 | 12650 |
| percent release | 2.5 | 2.1 | 2.7 | 4.4 |

**Table 8.** Spontaneous release of $^{51}$Cr by parasites after 24 hrs of labelling. Initial CPM of parasite culture was 285,026. ($20x10^6$ parasites in 1 ml).

| Sera | AVL | SP | PT | CE | NS |
|---|---|---|---|---|---|
| Mean CPM | 2630 | 2570 | 2545 | 2425 | 2620 |
| S. E. | 144 | 137 | 123 | 60 | 258 |

**Table 9.** Phagocytosis of $^{51}Cr$ labelled parasites 24 hrs after infection by THP-1 cells at cell to parasite ratio of 1:5. S. E. = standard error; $^{51}Cr$ labelled parasites twenty four hours after infection by THP-1 cells with cell to parasite ratio of 1:5. No statistically significant differences. Correlation between serum ELISA Ab titres and mean CPM $r = 0.93$.

# 4. DISCUSSION

In all forms of leishmaniasis cure is thought to require an effective cellular immune response capable of activating host macrophages to eliminate the intracellular amastigotes. Alteration of human macrophage internalization and intracellular survival of *L. donovani* in the presence of immune human sera was examined in this study.

Analysis of the results using the microscopic method (Tables 2-5) indicated that *L. donovani* specific immune sera enhanced uptake of the parasites by the phagocytes. This finding is consistent with the findings of earlier works (Herman, 1980; Bray, 1983; Reis *et al.*, 1987). Reis and co-workers used *L. mex. amazonensis* promastigotes, mouse macrophages and 0.5% sera from the same mice for the studies. Herman used amastigotes and promastigotes of *L. donovani* and macrophages and sera from mice. We are unaware of any previous studies of *L. donovani*, human macrophage and human serum.

It has been well established that human visceral leishmaniasis is characterized by a poor lymphoproliferative response to parasite antigens and a failure to generate IFN-$\gamma$ which is an essential macrophage activating lymphokine (Carvalho *et al.*, 1985; 1989). If these basic host immune protective mechanisms are lacking in visceral leishmaniasis patients other factors might have operated to account for the increase in phagocytosis observed in the present studies. The investigation by Bray (1983) revealed that heat inactivated immune sera from humans and rabbits allowed enhanced attachment and engulfment of *L. mex. mexicana* promastigotes by murine

macrophages, compared with heated and unheated normal sera. It was postulated that specific antibodies in the heat inactivated immune sera were the likely factors responsible for raising internalization of promastigotes by the phagocytes. It was also demonstrated that depletion of immune serum components (presumably antibodies) by absorption with the promastigotes markedly decreased parasite entry into the phagocytes (Reis *et al.*, 1987). Analysis of our test results also showed strong correlation (r = 0.63-0.85) between levels of *L. donovani* specific antibodies and phagocytosis.

On the other hand, Chang (1981) reported the results of a study that focused mainly on uptake of amastigotes rather than amastigotes. He used immune sera from rabbits immunized with *L. donovani* promastigotes or amastigotes, human monocytes and neutrophils and the same parasites used to immunize the rabbits. In all cases the number of amastigotes in both types of phagocytes was lower in cultures containing anti-amastigote immune sera than in cultures containing anti-promastigote immune sera and normal immune sera. There was no significant difference between the latter two in contrast to our study showing high number of organisms using immune sera. As in the present work, there was an increase in phagocytosis with time. It was suggested that phagocytosis rather than enhanced leishmanicidal activity accounted for the decrease in the number of intracellular amastigotes observed.

The present studies also showed enhancement of phagocytosis of the parasites in the presence of post-treatment sera compared with control endemic sera (Tables 2-5). Unlike sera from active cases of kala-azar (OD = 0.74 ± 0.06) sera from

treated patients were found to have lower levels of antibodies (OD = 0.253 ± 0.07) of antibody titres determined by ELISA and kindly provided by Asrat Hailu (Institute of Pathobiology, Addis Ababa University). This is consistent with published reports of antibody titres determined by ELISA in Ethiopia (Hailu, 1990) and in the Sudan, (Elassad *et al.,* 1994). However the ELISA test may preferentially measure antibodies to non-surface components or amastigote antigens of *L. donovani* and may not actually reflect levels of opsonic antibody. Since ELISA test results differ from direct agglutination measures of antibody, it is clear that antibodies with different specificities are measured by the two tests. Alternatively, so called cytophilic antibodies which were implicated as mediators of enhanced phagocytosis for immune sera, might not account for all of the increased uptake in post-treatment sera, suggesting that some non-antibody components of sera may be involved. Heat stable cytokines in serum might contribute to enhanced phagocytosis. In a study intended to evaluate the *in vitro* correlates of immunity of patients across the spectrum of clinical manifestations of *L. chagasi* infection, peripheral blood mononuclear cells of the patients were examined (Holaday *et al.,* 1993). The parameters examined were cytokine generation, lymphoproliferation and intracellular killing of parasites by activated macrophages. It was discovered that patients with acute disease were generally unreactive in each of the assays. Treated patients were found to show higher lymphoproliferative responses and released more activating cytokines (IFN-$\gamma$). Peripheral blood mononuclear cells (PBMC) of subjects with positive DTH-responses and no history of kala-azar showed the broadest reactivity in the assays. This finding seems to support the idea that

the process of intracellular parasitism. Complement receptors (CR1 and CR3) (Blackwell *et al.*, 1985); mannose-fucose receptors (MFR) (Wilson and Pearson, 1986) and fibronectin receptors (FnR) (Rizvi *et al.*, 1988) are a few of the essential receptors to which different *Leishmania* species bind. To a certain degree, FnR mediated phagocytosis of *Leishmania* opsonized by fibronectin produced and released locally by phagocytes. Quassi (1988) also showed that macrophages produced C3 which could opsonize *Leishmania* under serum-free conditions. As it has been pointed out previously, in this study emphasis was placed on the observation of phagoocytosis in the absence of serum complement heat-labile components. Although at this stage it is difficult to ascribe particular ligand-receptor interactions to the group test sera, one cannot rule out differences in receptor-ligand pairs between groups of sera. As has been suggested previously, increased fibronectin levels in post-treatment sera might explain the high levels of phagocytosis observed for this group. In addition Karp *et al.*, (1991) reported that in spite of the strong antibody response in kala-azar, metacyclic promastigotes of *Leishmania* were invisible to or unavailable for opsonization by the antibodies due to increased expression of LPG molecules on their surfaces.

Another interesting observation in our study was the increased uptake observed for non-endemic control sera obtained from foreigners (Caucasians) compared with the non-endemic sera from Ethiopians for the 6 and 24 hr infections (Tables 3 and 4). It is difficult to draw conclusions because no information is available about the Ig or cytokine status of the foreigners' sera.

The radiometric uptake assessment was intended to supplement the

microscopic method and to evaluate its potential use. [$^3$H]thymidine and $^{125}$IUdR incorporation by *L. donovani* plotted during preliminary radiolabelling trials were quite linear in spite of low counts. There was also variability in labelling efficiency, some of which seemed to be attributable to the growth phase (early, mid or late log and stationary phase) of the parasite. Labelling was therefore routinely done using 24 hr subcultures which should have been in early log phase. Generally radioactive labelling of parasites with $^{51}$Cr was found to be most useful because specific activity was high enough to give high CPM values and standard curves which were useful to compare phagocytosis effectively. The method was clearly faster and easier to use than the microscopic assay.

No significant differences were seen between any of the serum groups and cultures without serum in 24 hr infections using $^{51}$Cr labeled parasites. This contrasts with the results of all the microscopic analyses of phagocytosis. In addition, the level of uptake in cultures without added human sera was high in radiolabeled experiments but low in experiments using the microscope. The reason for these discrepancies is unclear. Radiolabelling may have altered surface antigen involved in binding of opsonic antibody or there may have been loss of radioactivity under the culture conditions. Chromium diffuses through cell membranes and may have been transferred to THP-1 cells upon contact with labeled parasites that were not ingested. We are unaware of published reports comparing phagocytosis of $^{51}$Cr labeled parasites measured radiometrically with phagocytosis measured microscopically, although Cillari *et al* (1990) found good correlations between phagocytosis of $^{125}$I doexyuridine-labeled parasites measured radiometrically and

microscopically. Before using the method in future experiments, one would need to determine whether labeling with $^{51}Cr$ alters the uptake of parasites by comparing phagocytosis (% of cells infected and number of parasites/cell) of labeled and unlabeled parasites and correlating that to radioactivity taken up by cells.

Immune sera didn't seem to potentiate intracellular killing more than that observed with the other sera. There was a correlation ($r = 0.76 - 0.99$) between the degree of phagocytosis (Tables 2 - 5) and numbers of viable parasites (Tables 6 and 7). As far as visceral leishmaniasis patient sera were concerned, it was unlikely that the high titres of specific antibodies would mediate both uptake and intracellular killing. Maximal killing activity requires the presence of accessory cytokine producing cells and/or their products. Since the present tests were conducted after heating serum, only heat stable products would have been present. The known macrophage activating lymphokines, IFN-$\gamma$ and TNF-$\alpha$ are heat labile. However, other cytokines might have contributed as opsonins or to macrophage activation. The very small differences in OD between infections with sera-pretreated parasites and sera-pretreated cells (Tables 6 and 7) probably don't reflect basic functional differences in the phagocytes. The difference might be caused by variation in timing of measurement taking, i. e. two OD measurements of one plate taken at an interval of a few minutes show slight changes.

# 5. CONCLUSION

*Leishmania* parasites have adapted remarkably in respect of their ability to infect, propagate and persist within MNP (Pearson *et al.*, 1984). MNP and cytokines play key roles in leishmanial diseases. The extraordinary preference of the parasites for MNP explains the chronic nature of leishmanial disease. Verifying the interplay between humoral factors and MNP in leishmaniasis is fundamental to the immunobiology of the disease and for vaccine development (Nacy *et al.*, 1989). It is in this perspective that this research has been carried out. The present work showed that *L. donovani* immune sera enhanced phagocytosis more than non-immune sera. The most likely component responsible for uptake enhancement is the high titres of specific antibodies, although other heat-stable serum components may have been involved. The correlation between antibody titre and level of phagocytosis ($r = 0.69$-$0.99$) supports the hypothesis that specific antibodies in sera enhance phagocytosis. Radioactive isotopes were found to be promising for studies of this kind, although the method did not show differences in phagocytosis among groups of sera. Reasons for the discrepancy between the microscopic and radiometric method need to be elucidated through experimentation. In conclusion I would like to suggest that future investigations of this kind should focus on determining whether the parasite specific antibodies in immune sera are directly involved in enhancing phagocytosis. This could be done either by blocking the Fc receptors of phagocytes and by showing that purified serum antibodies can mediate enhanced phagocytosis. In addition similar experiments should be performed using

amastigotes rather than promastigotes since *in vivo* infection is maintained and amplified by release of amastigotes from infected phagocytes and reinfection of additional cells.

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