# *LEISHMANIA AETHIOPICA* INFECTIONS IN *CERCOPITHECUS AETHIOPS*: INITIATION OF INFECTION AND IMMUNE PROFILES.



By

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# ABBREVIATIONS

| | |
|---|---|
| Ab | antibody |
| Ag | antigen |
| AHRI | Armauer Hansen research institute |
| ALP | alkaline phosphatase |
| ALERT | All African Leprosy Rehabilitation and Treatment Center |
| CD | cluster of differentiation |
| CL | cutaneous leishmaniasis |
| CMI | cell-mediated immunity |
| CPM | counts per minute |
| DCL | diffuse cutaneous leishmaniasis |
| DMSO | Dimethyl sulphoxide |
| DTH | delayed-type hypersensitivity |
| Fig | Figure |
| FPA | formaldeyde fixed promastigote antigen |
| HIV | human immunodeficiency virus |
| IPB | Institute of Pathobiology |
| IG | Immunoglobulin |
| LCL | localized cutaneous leishmaniasis |
| LTT | lymphocyte transformation test |
| NNN | Novy-Nicolle-MacNeal |
| NK | natural killer (cell) |
| OD | Optical density |

| | |
|---|---|
| OPD | Orthophenlene Diamine |
| PBMC | peripheral blood mononuclear cells |
| PHA | phytohaemagglutinin |
| pNPP | p-Nitrophenyl phosphate |
| PROM | promastigote |
| SI | stimulation index |
| SIV | simian immunodeficiency virus |
| Th1 | T helper |
| TNF | tumor necrosis factor |
| VL | visceral leishmaniasis |
| WHO | World Health Organization |

# ABSTRACT

In the search of vaccines and drugs for the control of diseases the availability of animal models is vital. Cutaneous leishmaniasis, caused by *L. aethiopica* is an endemic disease in Ethiopia. Availability of an animal model for *L. aethiopica* could be useful for studying the different aspects of the disease. In this study, monkeys were infected with an isolate of *L. aethiopica* to develop the primate experimental model of the disease. Twelve grivet monkeys (*Cercopithecus aethiops*) were trapped from cutaneous leishmaniasis (CL) non-endemic areas. Experimental infections were initiated in these animals after they were screened for natural infection. Inoculation of grivet monkeys with *L. aethiopica* parasite resulted either in lesion or symptomless infection. One grivet monkey produced clinical lesions following inoculation with promastigotes of diffuse cutaneous leishmaniasis (DCL) strain of *L. aethiopica*. Another grivet monkey produced nodules following inoculation with promastigotes of localized cutaneous leishmaniasis (LCL) strain of *L. aethiopica*. In addition to this, loss of hair at the infection site was seen in two of these animals after inoculation with LCL strain of *L. aethiopica* promastigotes. In order to assess whether grivet monkeys have similar immune responses as humans following infection with leishmaniasis, we investigated the *in vivo* and *in vitro* immune responses of these animals to leishmanial antigens. Delayed type hypersensitivity (DTH) response as measured by skin testing indicated no significant response in the experimental animals. Monkeys were bled and the proliferative response of their peripheral blood mononuclear cells (PBMC) as well as IFN-γ and interleukin IL-10 production were tested *in vitro* in response to leishmanial antigens. The finding indicated that *in vitro* lymphocyte proliferative response of infected animals to live localized cutaneous leishmaniasis strain *L. aethiopica* promastigotes was

significantly higher than controls. However, *in vitro* lymphocyte proliferative response of infected animals to live DCL strain of *L. aethiopica* promastigotes and killed parasites was not significantly higher compared to controls. Low level of IFN-γ was produced after stimulation with leishmanial antigen. There was no detectable level of IL-10 production after *in vitro* stimulation.

# 1. INTRODUCTION

Leishmaniasis is an infectious disease caused by different species and subspecies protozoan parasites belonging to the genus *Leishmania.* They are obligate intracellular parasites of the vertebrate host. The genus *Leishmania* is comprised of different species and subspecies. Previously the taxonomy of the parasite was based on the geographical distribution of the parasite, clinical features of the disease, specificity of the parasite to vectors and hosts and behavior of the parasite in culture or experimental animals (Gardner, 1974). Recently, species specific DNA probes, monoclonal antibodies and isoenzyme pattern are being used for the classification of *Leishmania* parasites.

Leishmaniasis is present in all continents except Australia and Antarctica. It is reported from about 97 countries in the world (Ashford *et al.*, 1992). The estimated numbers of leishmniasis cases surpassed 12 million and those at risk about 350 million people worldwide (WHO, 1998). The global annual incidence is estimated to be 1.5-2 million (Desjeux, 1996).

Infection with *Leishmania* parasite causes a spectrum of diseases depending on the host and the species of the parasite involved. The cutaneous disease pattern ranges from self-healing lesions to disseminated cutaneous disease or highly destructive mucosal lesions. The visceral ranges from asymptomatic infection to fatal visceral dissemination. On the whole, the disease appears in three clinical forms: Visceral leishmaniasis (VL) or Kala-azar, Mucocutaneous leishmaniasis (MCL) and Cutaneous leishmaniasis (CL).

Visceral leismaniasis is caused by parasites of the *Leishmania donovani* complex in the Old

World whereas it is caused by *L. chagasi* in the New World (WHO, 1981). It affects the visceral organs mainly the spleen, the liver and the bone marrow.

Mucocutaneous leishmaniasis or espundia is caused by *L. braziliensis* and *L. panamensis*. It is mainly confined to south and Central America (WHO, 1990). However, a few cases of mucocutaneous leishmaniasis due to *L. aethiopica* were also reported (Belehu, 1982). The primary lesions are similar to other types of CL. Metastatic spread to the mucosa of the nose, mouth and pharynx may occur during the first infection or several years later. Then, ulceration and erosion of the soft tissues occurs.

CL of the Old World is caused by three species of *Leishmania*: *L. tropica, L. major* and *L. aethiopica* (WHO, 1981). In addition to this, cutaneous leishmaniasis due *to L. infantum* have been reported from Africa and the Mediterranean basin (WHO, 1984). *L. braziliensis* and *L. mexicana* also cause New World CL (WHO, 1981).

## 1.1 Life cycle

Leishmania parasites are transmitted by the bite of female sandflies belonging mainly to the *Phlebotomus* and *Lutzomyia* species in the Old and New World, respectively. Human beings are usually accidental hosts of leishmaniasis. They get exposed to infected sand flies in endemic zones. The reservoir hosts of leishmaniasis include rodents, foxes and dogs. The *Leishmania* parasite is a dimorphic protozoan. The life cycle involves intracellular amastigote stage in the macrophage of the vertebrates and an extracellular promastigote stage in the gut of sandflies. The amastigote stage is oval shaped and without flagellum. It is also known as Leishman-Donovan (LD) bodies. The promastigote is slender shaped and with a flagellum. When an infected sandfly bites a susceptible vertebrate host, it injects the

species are zoophilic, feeding mainly on wild or domestic animals and man only as a second choice (Humber *et al.*, 1986).

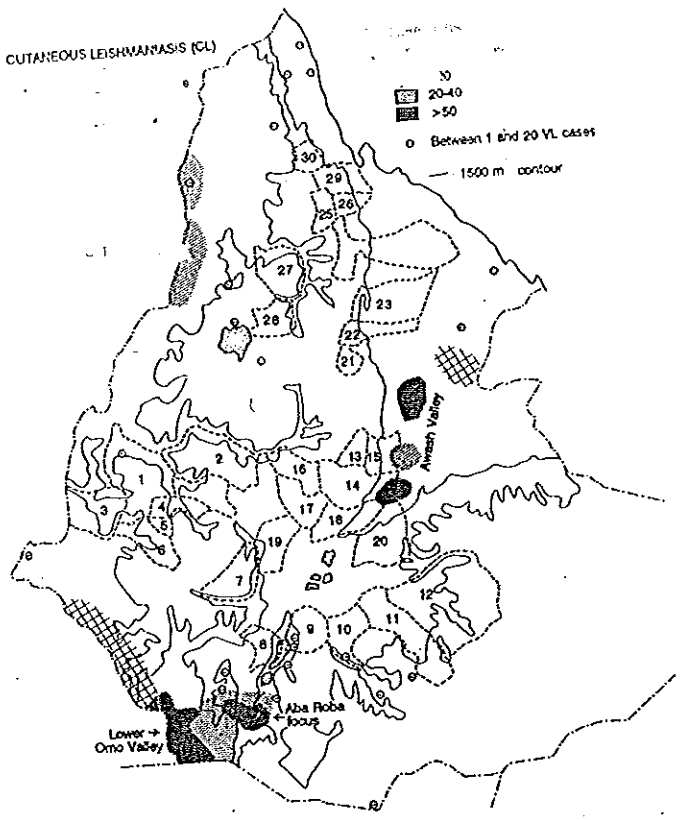
CL due to *L. aethiopica* is zoonotic. The disease is maintained by the natural reservoir hosts of two species of rock hyraxes: *Procavia habessinica* and *Hetrohyrax brucei*. *L. aethiopica* had been also isolated from other animals such as the Giant Rat (*Cricetomys* sp) (Mutinga, 1975) and Ground Squirrel (*Xerus ratilus*) (Abebe, 1990). Mutinga (1989) also reported isolation of *Leishmania* parasite from domestic goat in Kenya, which are known to harbor *L. aethiopica*.

### 1.2.1.2 Geographical distribution

CL is endemic in the highland areas of Ethiopia (Ashford, 1973 and TekleMariam, 1982)). Active infections were reported from villages in Shewa (Ashford *et al.* 1973), Wello (Wilkins,. 1972) and Semen Omo zone (Laskay *et al.*, 1991) regions. TekleMariam (1982) identified new foci of CL at Basona Warena in Tegulet and Bulga localities, Tulu Kuche in Chilalo localities and Goba in Mendayo localities.

Price and Fitzherbert (1965) presented the geographical distribution of 22 cases of DCL. These patients came from the highlands (2000m above sea level) of the former Gonder, Gojam, Shoa, Wellega, Keffa and Arssi administrative regions. Most of them were from the eastern edges of the central plateau of Ethiopia.

In general, clinical cases of leishmaniasis have been reported from 28 localities before 1993 (Hailu and Frommel, 1993) (figure 1).




Localities from where cutaneous leishmaniasis cases have been reported:

| Localities | Region | Localities | Region |
|---|---|---|---|
| 1. Gimbi | Region 4 | 16. Selale | Region 4 |
| 2. Horo Gudru | Region 4 | 17. Menegesha | Region 4 |
| 3. Kelem | Region 4 | 18. Yerer and Kereyu | Region 4 |
| 4. Arjo | Region 4 | 19. Chebo and Gurage | Southern Region |
| 5. Sor and Geba | Region 4 | 20. Arba Gugu | Region 4 |
| 6. Gore | Region 4 | 21. Dese Zuria | Region 3 |
| 7. Jima | Region 4 | 22. Yeju | Region 3 |
| 8. Gamo | Southern Region | 23. Raya and Kobo | Region 3 |
| 9. Sidama | Southern Region | 24. Inderta | Region 1 |
| 10. Genale | Region 4 | 25. Adwa | Region 1 |
| 11. Mendoyu | Region 4 | 26. Agame | Region 1 |
| 12. Wabe | Region 4 | 27. Simen | Region 3 |
| 13. Menz and Gish | Region 3 | 28. Libo | Region 3 |
| 14. Tegilet and Bulga | Region 3 | | |
| 15. Yifat and Timuga | Region 3 | | |

Figure 1 Distribution of visceral leishmaniasis and cutaneous leishmaniasis

(Adopted from Hailu and Frommel, 1993)

### 1.2.1.3 Prevalence

CL is a widespread tropical disease, with major foci in Ethiopia. The disease was estimated to affects almost 0. 5% of the total population (Belehu, 1980). In Ethiopia CL has been documented by authors from results of field surveys and from hospitals as self-reported cases. Bryceson and Nichol (1966) conducted a small field-study in Dembidolo district, Region 4. They found a total of 14 active CL cases and other 12 with old scars. Lemma *et al.* (1969) conducted an epidemiological study of CL in 3 highland areas and in the lake region of the Ethiopian Rift Valley. According to these studies skin-test positivity ranged from 25.5% to 44.2% in Dessie and Karakore towns. Among schoolchildren in Aleku and Shashemene towns, the positivity was 6.7% and 5.5% respectively. Prevalence rates of active infection between 5.5 and 40 per 1000 population were reported from villages in Region 3 and Region 4 (Ashford *et al.* 1973) Wello (Wilkins *et al.* 1972) Semen Omo zone (Laskay *et al.,* 1991) with highest rates in Semen Omo zone.

Wilkins (1972) made an epidemiological study of CL in Meta Abo, about 25 km south-west of Addis Ababa. He found 9 active cases of oriental sore and a prevalence of active infection to be 9/1000.

Ashford *et al.* (1973) conducted a survey of CL in highland areas over the central plateau of Ethiopia. The areas studied included Kutaber in Wello, Aleku in Region 4 and Ocholo in Semen Omo zone. In Kutaber the prevalence of active infection was 9/1000 and in Ocholo, the prevalence was 107/1000.

Eleven active cases of CL were seen in Adigrat Hospital (Northern Ethiopia) alone (Desta, 1982). Four histologically proven cases of CL, from the Sirba area of Blue Nile

Blue Nile Valley, West Ethiopia, were reported (Gundersen, 1982). Nine DCL patients were recorded in ALERT hospital (Sarojini *et al.,* 1984).

### 1.2.2 The disease

#### 1.2.2.1 Clinical manifestations

*L. aethiopica* is taxonomically distinct from other *L*eishmania species. It differs from all other *Leishmania* species by isoenzyme profile (Le Blancq, 1986) by kinetoplastid DNA buoyancy and excreted factor serotype (Chance *et al.,* 1978). It is also clinically unique among the Old World *Leishmania* (Le Blancq, 1986). *L. aethiopica* infection can be similar to leprosy where there is a spectrum of clinical presentations (Barnetson, 1978a; Convit, 1974; Bryceson, 1969). It induces three clinical forms of disease: Localized Cutaneous Leishmaniasis (LCL), Diffuse Cutaneous Leishmaniasis (DCL) and Mucocutaneous Leishmaniasis (MCL). The first form is mostly seen as single lesions which are self-healing over time and localized in areas uncovered by clothing like the face, arms or legs (Belihu, 1982). The parasite load in the lesion is minimal (Nilson, 1987). DCL shows multiple lesions on the face, trunk and extremities and is usually not self-healing. The parasite load in the lesion is very high. There is often considerable similarity between DCL and multibacillary leprosy. MCL affects the mucous membrane of the nose and the mouth. This form may occur or spread to mucous membranes. However, it does not correspond to the metastatic form of MCL found in South America (Barnetson, 1978b).

#### 1.2.2.2 Pathology

The histological changes in simple CL are dominated by evidence of delayed type hypersensitivity to parasite antigens. The histological response must be considered under two headings: Firstly, cellular immune response, which reflects the host immunity. In early

stages of the diseases in patients with persistently low levels of antibodies, there are large numbers of parasite-laden macrophages, some of which are vacuolated and carry numerous parasites. The infiltrate of lymphocytes and plasma cells increases progressively as the lesion evolves. Parasite elimination usually follows destruction of host macrophages, either at the center of circumscribed clusters in the dermis, with the release of amastigotes, or in the subepidermal zone, causing liquefaction of the basal layer and ulceration. Polymorphs are dominant in the necrotic centers followed by Langhans' giant cells and few epithelioid cells. Cytochemical studies suggested that immune complexes might play an essential role in necrosis. Lesions that fail to undergo necrosis become chronic and develop a tuberculoid histology with only a few parasites (WHO, 1990).

LCL lesion show a distinct pattern of cell organization consisting of macrophage granulomas which may also contain epithelioid cells and surrounded by lymphocytes with a mixture of plasma cells (Acherman, 1978).

Secondly, tissue response, which may reflect the effect of released antigen during the period of active destruction of parasites, one or more of the following acute changes are usually seen. There is oedema in the superficial dermis and damage to collagen and elastin with an increase in reticulin, followed by fibrosis. In some cases, there is necrosis of collagen or epidermis, and pseudoepitheliomatous hyperplasia is often severe. At this stage the small capillaries may show endothelial swelling or proliferation, or there may be vasculitis. In the later tuberculoid phase, some vessels may be broken (Ridely, 1979).

The histopathology of DCL reflects the absence of cell-mediated immunity and is remarkable for the enormous numbers of vacuolated, parasite-laden macrophages ("foam

cells"), scarcity of lymphocytes, and absence of necrosis and ulceration (Ridely, 1987).

### 1.2.3 Diagnostic procedures

The diagnosis of leishmaniasis depends upon clinical, parasitological and serological grounds. There is no simple, sensitive and specific diagnostic method for leishmaniasis.

#### a). Clinical aspects

In endemic areas, health workers recognize cases of CL and the diagnosis may be based on the observation of the disease presentation like lesions on exposed areas, presence for several months, resistance to all types of attempted treatments and usually no pain or itching. However, it is not reliable because the disease resembles many other skin diseases like lepromatous leprosy (Belihu, 1980; Sarojini, 1984).

#### B). Demonstration of parasites

Confirmation of the diagnosis of CL is based on demonstration of the parasite. The Parasite may be detected by direct smear or culture. Smears are simple and inexpensive but they have low sensitivity (Kirsten *et al.,* 1987). Culture was found to be the most sensitive diagnostic method for diagnosis of CL (Mengistu *et al.,*1992). Samples of tissue can be obtained by biopsy. However, these methods have limitations. Isolation in culture depends on the presence of sufficient numbers of viable amastigotes and need longer time. There is also a possibility of contamination and the cost is relatively high. The parasitological diagnosis of the disease is also based on the presence of viable amastigotes. However, in chronic LCL lesions due to *L. aethiopica* intact parasites are scarce. Therefore, it is difficult to detect by *in vitro* culture and or direct microscopy (Kristen *et al.,* 1987). In addition, the use of culture as a routine diagnostic procedure may be limited.

**c). Skin test**

The type of test applied for the diagnosis of previous *Leishmania* infection is the leishmanin or Montenegro test. Leishmanin is a suspension of washed promastigotes in phenol saline, which is injected intradermally. The reaction is read after 48-72 hours. This test also has limitations. It is only useful in non-endemic areas as a diagnostic tool and in endemic areas as a test for survey because it becomes positive early in the infection and remains positive for life (Reed *et al.,* 1986).

**d). Serological diagnosis**

Serological assays have also been found to be of limited use for the diagnosis of LCL because differences in antibody titers and fine specificity occur between patients at similar stages of clinical presentation (Jaffe, 1990; Mengistu, 1990). In previous studies, enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT) were evaluated for the diagnosis of leishmaniasis. DAT is the most simple method (Harith *et al.,*1986). However, it shows low sensitivity (El Safi, 1989). ELISA was used to establish the final diagnosis (Mengistu *et al.,*1992). Overall, the serological assays were not sensitive enough to diagnose all parasitologically confirmed cases of localized cutaneous leishmaniasis. Therefore, they serve only as supportive methods.

**e). DNA hybridization**

*Leishmania* species have also been identified by DNA hybridization probes (Laskay, 1991). *Leishmania* can also be identified in tissue or culture by polymerase chain reaction (PCR) using the genus or species specific probes (Laskay *et al.*, 1995). Although this approach is currently available in only few research laboratories, it may become the method of choice for diagnosis in the future. However its application for widespread routine diagnosis would

not be practical.

### 1.2.4. Treatment

Many of the commonly used drugs are not effective against *L. aethiopica* infection. LCL is unresponsive to antimonials at conventional doses (WHO, 1990). As localized cutaneous leishmaniasis is self-healing with time, most ulcers should be left to heal spontaneously. However, it might result in tissue destruction and ulceration that may cause disfigurement. Pentamidine is recommended for treatment of this infection but its high toxicity makes it unacceptable for systemic use in LCL (Bryceson, 1970).

DCL is also poorly sensitive to the pentavalent antimonials (Bryceson, 1969). The second line drugs are pentamidine and amphotericin B but they are difficult to administer and had serious side effects (Bryceson, 1970). Relapses occur unless the patient has acquired *Leishmnia*-specific cell mediated immunity after treatment. Thus there is a need for effective and less toxic drugs.

### 1.2.5. Immune response to CL due to *L. aethiopica*

Leishmania infection sometimes runs a sub-clinical course without symptoms. It is detectable only by the development of specific immunological memory to leishmanial antigens. The immunological features of various human *Leishmania* infections have been evaluated by measuring the levels of antibodies, lymphocyte responses to leishmanial antigens and levels of cytokines.

Resistance to infection with *Leishmania* is largely T cell mediated because T-cells produce cytokines that stimulate the intracellular killing of parasites by macrophages (Murray,

1982; Nacy *et al.,* 1985). Although there is a role for many types of lymphocytes including CD8+ cells, the major effector cell in cell-mediated immunity in leishmaniasis is the CD4+ T lymphocyte. CD4+ T-helper cells can be divided at least into two phenotypes namely T helper 1(Th-1) and T helper 2 (Th-2) cells. Protection against *Leishmania* species requires interferon gamma (IFN-γ) and IL-2 producing Th1 cells while susceptibility is associated with IL-4, IL-5 and IL-10 secreting Th2 CD4 cells (Heinzel *et al.,* 1989). This fact clearly shown in animal model.

The main cytokine that has been implicated in all model systems acting against *Leishmania* is IFN-γ. The mode of action of this cytokine alone or in combination with other cytokines is through the oxidative burst and nitric oxide pathways (Liew *et al.,* 1990). The role of TNF-α in host defense against leishmaniasis is controversial. The serum TNF-α is usually high in the forms of disease that have no cell-mediated immune response to these pathogens. Therefore, a high parasitic load seemed to correlate with an elevated TNF-α serum titer (Pisa, 1990). In contrast to this, TNF-α was suggested to kill *L. major* in synergy with IFN-γ (Liew *et al.,* 1990) while transforming growth factor (TGF-β) and IL-4 were associated with the down-modulation of IFN-γ action (Barral *et al.,* 1992), with resultant enhancement of susceptibility. IL-4 and IFN-γ have antagonistic effects on each other (Heinzel *et al.,* 1989). The cytokine IL-12 is also involved in protection against *Leismania*. It is crucial for the innate and the Th-1 responses to *Leishmania* parasite (Sypek *et al.,* 1993). IL-10, a regulatory cytokine, can block antigen specific proliferative responses of CD4+ T-cell and also suppresses the functions of macrophages.

The diversity in clinical manifestations which develop after infection with *L. aethiopica* has been attributed to a number of factors such as differences in immune response of the

infected patient and differences of the infecting parasites (Bryceson, 1970; Akuffo *et al.*, 1987 and Akuffo *et al.*, 1988).

Previous studies on the immunological aspects of *L. aethiopica* infection have demonstrated marked differences in the specific recognition of *L. aethiopica* antigens by LCL and DCL patients. At the level of T-cell reactivity, unlike LCL patients, DCL patients show anergy to leishmanial antigens on delayed type hypersensitivity skin testing (Bryceson, 1970). The lymphocytes of DCL patients also show reduced proliferative responses and cytokine production *in vitro* to specific leishmanial antigens in contrast to LCL patients (Schurr *et al.*, 1986; Akuffo *et al.*, 1988). There is no impairment of reactivity to other antigens *in vivo* (Bryceson, 1970) or to mitogens *in vitro* (Shurr *et al.*, 1986 and Akuffo *et al.*, 1987). This indicates the presence of an antigen specific anergy in DCL condition. A defect in IL-2 production has also been shown to be involved in the non-healing DCL form of *L. aethiopica* infection (Akuffo, 1992). As DCL patients cured of infection through chemotherapeutic treatment recover their antigen specific reactivities, both *in vitro* and *in vivo* (Bryceson, 1970; Akuffo *et al.*, 1988), the specific anergy may not be due to intrinsic defects in host immune response.

Further studies have shown that the antigen specific anergy observed in DCL patients is in part influenced by the particular promastigote isolate used to stimulate the lymphocytes of a DCL patient. DCL patients show significantly higher levels of proliferation, IL-2 and IFN-γ production in response to antigen preparations of promastigotes cultured from the lesions of LCL patients (PROM-LCL) than to promastigotes cultured from the lesions of DCL (PROM-DCL) patients (Akuffo *et al.*, 1987). Akuffo *et al.* (1987) also identified differences in the capacity of PROM-LCL and PROM-DCL patient biopsies to stimulate

IFN-γ and IL-2 production. Lymphocytes from both LCL and DCL patients showed higher IFN-γ and IL-2 production after stimulation with PROM-LCL than PROM-DCL.

Another factor, which could play a role in disease progression, may be the possible variation in the antigenic properties of the infecting organisms (Akuffo *et al.,* 1988). According to these authors, the immune response which develops in patients may be a reflection of the particular influence exerted by parasite associated determinants. The effects of this *in vivo* modification in immunologic reactivity may point to differences in host immunologic responsiveness. However, the initial event, which separates LCL from DCL type disease, might occur at the level of the infecting parasite.

Experiments showed that cells from normal unexposed individuals can respond to whole promastigotes of *L. aethiopica* by IFN-γ production (Akuffo and Britton, 1992) or proliferation (Akuffo *et al*, 1992; Maasho and Akuffo, 1992). The main cell type that appeared to be activated following such stimulation was $CD3^-$, $CD16^+$ /$56^+$ NK cells (Akuffo *et al*, 1993). Furthermore, subsequent field studies support a role of NK cells and CD8+ cells in protection from healthy and cured Ethiopian LCL patient (Maasho *et al.,* 1998).

The role of antibodies, which are produced in both DCL and LCL, is unknown. The humoral responses in DCL patients are also manifested by the presence of antibodies that are specific to antigens of different molecular-weight, whereas antibodies in sera from LCL patients showed a limited recognition of the low-molecular-weight antigens (Mengistu *et al.*, 1990).

### 1.2.6. Protection of people from infection

As far as man is concerned vaccination is considered to be the simplest, cheapest, most convenient and reliable way of protection from infection. Vaccination trails in animal models, human beings or both, have been performed with virulent promastigotes (Modabber, 1989), attenuated (Howard *et al.,* 1982) or killed promastigotes (Greenblatt, 1986) and specific antigens purified from promastigotes (Jardim *et al.,* 1991; McMahon-Pratt *et al.,* 1992; Olobo et al., 1995). However, no vaccine is conclusively effective against any form of leishmaniasis. Therefore, attempts to develop safe vaccine for leishmaniasis is still underway.

In general, control and prevention of leishmaniasis in the future depends on the development of more efficacious vaccines and convenient, nontoxic therapeutic agents. There is a need for an animal model for vaccine and drug trials.

### 1.2.7 Animal models

The functions of experimental animal model in leishmaniasis include:

1. *in vivo* maintenance of virulent strains of the parasite for the production of the amastigotes;
2. To study the pathogenesis of leishmaniasis and anti-leishmanial immunity;
3. To test anti-leishmanial drugs and vaccines.

Leishmania parasites infect a wide variety of animals. The severity of infection varies, depending on the type of host-parasite combination. For instance, man and dog are considered as accidental hosts in the leishmanial life cycle and exhibit severe clinical signs and symptoms when infected. On the other hand, the natural reservoirs of the various

parasite species, including gerbils, rats and other rodents, various canines (foxes and jackals), hyraxes and lower vertebrates, may become infected naturally by *Leishmania* parasites and exhibit few, minor or no sign of infection (Bray, 1987).

In experimental conditions, parasites have been shown to be capable of inducing an infection in a wide variety of mammals, many of which would not be natural hosts for the parasite.

Animal models are expected to mimic the pathological features and immunological responses that are observed in humans when exposed to a variety of *Leishmania* species with different pathogenic characteristics. Therefore, the usefulness of any animal model of human leishmaniasis is clearly determined by the degree of similarity of such models to the human disease. However, the choice of laboratory models of human leishmaniasis has also been dictated by convenience: mice, guinea pigs and hamsters are the most widely used species. Leishmanial disease caused by *L. donovani, L. major* and *L. mexicana* have been mimicked in animals like hamster (Farrel, 1976), guinea pigs (Mauel *et al.,* 1975), white mice (Trotter *et al.*, 1980) and primates (Githure *et al.,* 1986).

In each particular laboratory animal, the outcome of infection will depend on a combination of factors, including the *Leishmania* species, the virulence of the parasite isolate used, the nature of the inoculum and the route of inoculation. The susceptibility or resistance of each animal species is genetically controlled. However, there are cases where a particular host may be susceptible to one species of *Leishmania* but fully resistant to another.

Of Particular important consideration in experimental models is the influence of inoculum

on the infection and hence a wide variety of inoculation routes have been used. The intra-dermal (i.d), subcutaneous (s.c), intravenous (i.v) and intra-peritoneal (i.p) routes are the most frequently used. The following animals are examples of experimental models for leishmaniasis.

**a) The Guinea pig**

Guinea pigs are usually reported to be a poor host except for their own specific parasite, *L. enriettii*. The guinea pig *L. enriettii* model has been used extensively in the past as a model for immunological studies (Mauel *et al.*, 1975), but has now been outdated because of availability of other models.

**b) The mouse**

A lot of information on the immunology and pathology of leishmaniasis has been obtained from mouse models. Outbred and inbred mouse strains have been shown to be susceptible to most leishmanial parasites of mammals. In these animals like in human beings, dermatropic parasites generally produce cutaneous lesion at the site of inoculation. However, unlike in human beings, it visceralizes or produces metastatic lesion (Trotter *et al.*, 1980). Therefore, *L. major* infection in BALB/C mice cannot be considered as a model for any of human leishmaniasis. The visceralization and associated changes in serum proteins, peripheral blood cells, anergy to skin test antigen and hepato -splenomegaly etc. resemble human Kala-azar (Hommel *et al.*, 1995). On the other hand, outbred mice are less susceptible to *L. donovani* and their lesions tend to self-cure.

**c) The hamster**

The golden hamster is particularly useful for studying VL due to *L. donovani* and different

species of *L. mexicana, L. braziliensis* complexes and *L. chagasi* (Farrell, 1976; Wilson *et al.,* 1979). It is different from other laboratory rodents in its susceptibility to species of the subgenus Viannia. In addition to its use as a model of disease, the hamster is also a favorite laboratory animal for the isolation and laboratory adaptation of field isolates.

**d) The cotton rat**

The cotton rat is one of the most susceptible hosts for *L. donovani* (Fulton *et al.,* 1950). An exquisitely susceptible animal like the cotton rat may have a role in initial studies of protection against VL, by providing the means to screen species-specific vaccines before trials in canine and primate models or in human volunteers.

**e) The dog**

Dogs have been used as experimental models for *Leishmania* infection since the beginning of the last century and experimental infections have been achieved with leishmanial species such as *L. donovani* from India for which the dog is not a natural host (Hommel *et al.,* 1995). *Leishmania* may exhibit different tropisms in the dogs than in humans, and parasites, which normally cause cutaneous disease in humans, may produce visceral infections in dogs (Abranches *et al.,* 1991). Dogs are considered to be important laboratory models for *L. infantum* and *L. chagasi* because they reproduce the natural infection. In addition, vaccination of dogs is considered as a promising strategy for the control of both human and canine disease. The sign of natural and experimental VL in the dog model are very similar (Hommel, 1995). One major problem in using the dog model is the lack of available immunological reagents for monitoring cell subpopulations and cytokine production (Abranches *et al.,* 1991).

**f) Primate model**

Many primate species have been experimentally infected with different *Leishmania* species. Previous report indicated that New World nonhuman primates such as the squirrel monkey and the owl monkey (Chapman and Hanson., 1981) and the marmoset (Marsden *et al.*, 1981) could be suitable animal models for certain types of studies involving leishmaniasis. Primates are readily available in the wild and are considered in most cases as pests. They are adaptable to laboratory colonies. The main drawback is that they are expensive to maintain.

Experimental infection of Old World *L. donovani* have been achieved primarily in grivets (Kirk, 1945) and other East African monkey species (Githure *et al.*, 1986; Lawyer *et al.*, 1990). In the above studies the evolution of cutaneous lesions after infection was followed by visible lesions which eventually self-cured.

The utilization of nonhuman primates susceptible to CL is important because of their phylogenetic closeness to humans and close behavioral biologic and genetic relationships to humans. They should be of benefit for studying the immunological basis of protective response, as well as allowing humane and controlled vaccine and drug development studies. Moreover many of the available anti-human reagents for cell detection cross-react with the monkey system (Olobo,1992).

**G) *L. aethiopica* and animal model**

The availability of an experimental model system for both *L. major* and *L. tropica* has allowed the elucidation of some of the different mechanisms involved in these infections.

Less is known about *L. aethiopica* infection mainly due to the absence of a suitable animal model.

BALB/c mice are highly susceptible to *L. major* (Howard *et al.*, 1980). However, infection of BALB/c mice with *L. aethiopica* in the nose resulted in no clinical signs although the parasites could be recovered from the infected site (Childs *et al.*, 1984). Akuffo *et al.* (1990) suggested that the failure of this species to establish a clinical infection must be due to an effective immune response. Bray *et al.* (1973) reported limited success of infection in hamsters. Humber *et al.* (1989) attempted to infect a variety of laboratory animals with *L. aethiopica*. Clinical lesions were produced only following inoculation of the promastigotes into hamster noses and this parasite was highly selective in both species and site for laboratory animals tested. The lesions produced following inoculation of these parasites into hamster noses were histologically similar to that of DCL in man, but with no evidence of cutaneous dissemination. This lack of spread was probably due to the fact that the nose is the only site that appears to provide appropriate conditions for the growth of the parasite. The reason for this site-specific growth of *L. aethiopica* is not known. However, the commonest sites of persistent or long lasting lesions in man are also the nose and lips. The site and species preference of *L. aethiopica* may be due to differences in temperature between the different sites within the hamster.

Studies on cutaneous leishmaniasis caused by *L.eathiopica* are hampered because of the lack of a suitable animal model in which controlled studies could be conducted. The model could also be useful for testing new drugs against the disease and for vaccine development. Hailu *et al.* (1995) reported successful infection of grivet monkeys with *L.aethiopica* resulting in cutaneous leshmaniasis.

# OBJECTIVES

General objectives:

Examine the possibility of establishing a grivet monkey model of the disease to describe the immunological consequences of infection and assess the possible use of the animal model for drug and vaccine trials.

Specific objectives

(a) To measure *in vivo* and *in vitro* cell mediated immunity of grivet monkeys (*Cercopithecus aethiops)* following infection with *L aethiopica*.

(b) To measure cytokines produced by PBMC during infection with *L.aethiopica*

(c) To measure Plasma cytokine levels before and following infection of the monkeys with *L. aethiopica.*

(d) To measure serum antibody levels following inoculation with *L. aethiopica.*

# 2. MATERIALS AND METHODS

## 2.1 Animal

### 2.1.1 Animal trapping

Monkeys were trapped using locally made collapsible traps and baited vehicles. Traps and cars were set at dawn. They were checked intermittently to see if they had caught monkeys. The traps were baited with bread, banana, orange and maize. The most effective method of capture was the baited car. The collapsible traps were much less successful. Animals caught were then transferred into cages and transported by vehicle to Addis Ababa within one to three days.

### 2.1.2 Maintenance of animals

Upon arrival in Addis Ababa, the caged animas were placed in the animal house at the Institute of pathobiology (IPB). The animals were weighted, sexed and given identification tags. The regular feed was carrot supplemented with maize beans and banana. They were provided with tap water.

### 2.1.3 Inspection of animals for leishmania infections

The animals were closely examined for any sign of prior infection with leishmania and repeatedly checked for development of any external lesion until infection. In addition to this, natural exposure/infection was determined by detection of anti-leishmanial antibodies from sera collected serially over two months.

### 2.1.4 Screening animals for endoparasites.

i) To detect for any endoparasites peripheral blood films were prepared and stained with

Giemsa and examined for the presence of haemo-parasites.

**ii) Stool examination**

Stool samples were collected in vials and examined using direct microscopy and formol ether concentration techniques.

**a) Direct microscopy**

Using a wooden applicator, about 2 mg of stool was transferred into a glass slide and a drop of normal saline was added, and mixed until a thin smooth suspension was made. The preparations were then covered with cover slip and the entire preparation was examined under 25X and 40X magnifying power of a compound microscope.

**b) The Formol-Ether stool parasite concentration method.**

An estimated 2 grams of stool was mixed with 10% formalin solution. The stool formalin mixture was passed through a strainer to remove any large particles, then 3 ml of ether was added and the suspension was mixed and centrifuged. The suspension with debris was discarded and the sediment, which may contain parasite cysts, eggs and larvae, transferred onto glass slide for microscopic examination.

**c) Treatment**

400 mg double dose of mebendazol was administered twice a week to the animals to cure any intestinal parasites.

**d) Stool examination**

Stool examination was done using the above procedures after treatment.

### 2.1.5 SIV test

SIV screening was done using RECOMBIGEN HIV-1/HIV-2 kit because the use of this immunosassay kit was reported to detect both HIV and SIV (Biberfeld *et al.*, 1988).

## 2.2 Parasite and culture conditions

The diphasic medium NNN with Locke's solution as overlay was routinely used for parasite maintenance and cultivation. One hundred units of penicillin and 100 µg of streptomycin (Gibico, Scotland) were added to 1 ml of overlay to control bacterial contaminants. Parasites were also routinely sub-passaged every five days in this medium. The stationary phase promastigotes were harvested from 10 to 12 days old cultures.

The parasites were transferred into RPMI-1640 (Sigma, USA) supplemented with 20% heat inactivated fetal calf serum (Sigma, USA), penicillin 100U/ml and streptomycin 100µg/ml (Gibco, Scotland) and 2 mM l-glutamine (Irvin, Scotland). The cultures were grown until they reached a stationary phase at which time the parasites were harvested.

## 2.3 Experimental infections

One strain originating from LCL patients, strain number 1282/99 (AHRI ref number) and a strain originating from DCL patient, strain number P-16 (IPB ref number) were used. P-16 was isolated from a lesion of a patient from Ocholo, in October 1999. These strains were characterized as *L. aethiopica* by isoenzyme electrophoresis technique. Stationary phase promastigotes grown on NNN medium were inoculated subcutaneously into the tip of the nose at the dose of 5 x $10^6$. All strains used for the experimental infection were recent isolates, passaged not more than five times in cultures. All animals except thwo *C.*

*aethiops*, were infected with LCL strains. Observation of the infected site was made once a week to note emergence and appearance of lesions and subsequent changes.

## 2.4. Immunological tests

### 2.4.1 Antigen preparation

a) Preparation of live promastigote antigen.

Promastigotes were harvested at stationary phase. Then, they were centrifuged at 3000 rpm for 10 min. The pellets resuspended in cold PBS and let to stand for 10 min. The debris was discarded and the supernatants were centrifuged at 3000 rpm for 10 min. The pellet was washed two times as already stated. Parasites were resuspended in RPMI-1640 (Sigma, USA) containing penicillin (100U/ml), streptomycin (100µg/ml) and 10% FCS counted on a haemocytometer before use in the cultures.

b) Preparation of formalin killed promastigote antigens.

Promastigotes were washed three times by centrifuging at 2500 rpm for 15 minutes in phosphate-buffered saline solution (PBS, pH 7.2). The pellet was resuspended to a concentration of $10^6$ /ml in a 1% formalin solution, left overnight at 4 °C, washed three times, and resuspended in complete RPMI-1640.

c) Preparation of parasite lysates

Parasites were washed three times with PBS and resuspended in ice-cold lysis buffer (10mM Tris, 2 mM EDTA) containing protease inhibitors, 1 mM PMSF (phenyl-methyl-sulfonyl fluoride) and 100 units/ml of aprotinin. It was subjected for four cycles of alternate freezing (with liquid nitrogen) and thawing (water bath at 37 °C). The preparation was then sonicated, the suspension was placed on ice and sonicated at 150W for 3 minutes, with a

one minute interval for each minute of sonication (Bransonic company, US). Protein concentration was measured by Lowry method using bovine serum albumin as standard. The suspension was then stored at –70 °C.

### 2.4.2 Lymphocyte stimulation test

#### 2.4.2.1 Isolation of PBMC

Peripheral venous blood was drown from monkeys infected with LCL and DCL strains as well as controls in 15 ml polystyrene tubes containing 2% EDTA. Blood samples were diluted 1: 2 with RPMI-1640 and layered on Ficoll-Hypaque (Pharmacia, Sweden) in the ratio of 1:3 ficoll to blood. It was then centrifuged at 1800 rpm for 30 minutes at room temperature. The layer of PBMC was collected into 50 ml polystyrene tubes and washed two times with cold RPMI-1640 at 4°C. Red blood cells (RBC) were lysed by using lysing buffer (0.15M $NH_4Cl$, 1.0mM $NaHCO_3$ and 0.1mM Titriplex III disodium EDTA). The cell pellet was resuspended in 5 ml of lysing buffer and allowed to stand for 15 minute and centrifuged for 15 minute at 1500 rpm. The supernatant was removed and the pellet resuspended in RPMI-1640 and centrifuged once more at 1500 rpm for 15 minutes. The cell pellet was resuspended in 2 ml of complete RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1% (V/V) of antibiotics (10000U of penicillin, 10000µg/ml streptomycin) and 1% L-glutamine (200 Mm/ml) and counted by diluting 1:10 with 0.2% trypan blue under the 40x magnifying power using an haemocytometer. Then, RPMI-1640 added to the cell suspension and centrifuged once more and cell pellets were resuspended to 1 x $10^6$ cells /ml in 10% DMSO with FCS, transferred into 1 ml Nunc tubes, frozen at -70°C overnight and then stored in liquid nitrogen until use.

### 2.4.2.2 Proliferation assay

Frozen PBMCs were thawed in a water bath at 37°C and washed three times in RPMI-1640. PBMC pellets were resuspended in complete RPMI-1640. Viability of cells and numbers were determined by trypan blue staining using a haemocytometer,

The number of mononuclear cells was adjusted to $1x10^6$ cells /ml. Cells (at 180µl/ wells) and appropriate antigen and mitogen (20µl/ well) were added into wells of a 96 well tissue culture microtiter plate (Flow laboratory, USA) and incubated at 37°C in a 5%$CO_2$ incubator. PHA (5µg/ml), promastigote ($1 \times 10^5$ parasite in 20µl), formaldehyde fixed promastigote ($1 \times 10^5$ parasite in 20µl), and soluble leishmanial antigen (12.5µg/ml) were added independently in triplicate microwell cultures.

Culture supernatant (100µl) were collected into clean tubes for IFN-γ and IL-10 measurement and frozen at -70°C until use. Culture plates were pulsed with 1µCi 3 H-thymidine (Pharmacia, Sweden) on the third day and fifth day for PHA and antigen respectively. Pulsed cultures were harvested on a filter mat (cat.No. 11731) using harvester (Skatron, Norway) and dried in an oven. The incorporation of thymidine in the DNA of proliferating cells was counted in scintillation fluid on β-liquid scintillation counter (1216 Rackbetta II, Finland). Stimulation index was calculated by dividing counts per minute (CPM) of antigen by CPM without antigen.

### 2.4.3 Cytokine analysis

IFN-γ and IL-10 levels were measured using sandwich Enzyme linked immunosorbent assay (ELISA).

### 2.4.3.1 IFN-γ assay

IFN-γ assay was done using Mabtech kit. Wells of Dynatech Immunol plates were coated with primary monoclonal antibody to human IFN-γ (moAb 1-DIK) using 2 μg /ml concentration and incubated overnight at 4°C. After washing 3x with saline-Tween (0.05%) (Tween-20 Sigma). Plates were incubated with phosphate buffered saline (PBS)-Tween at room temperature for 1 hour in order to block unspecific binding. At the end of incubation period, samples and standard titrated in double dilution were added in duplicate to the plates and incubated at room temperature for 1.5 hours. After further washes, with saline-Tween biotinylated secondary monoclonal antibody (7-B6-1.biotin, Mabtech), was added. Streptavidin ALP diluted 1:1000 in PBS-Tween was added to the wells after another washing step and incubated for 1 hours at room temperature. After further washes, a substrate that was prepared by dissolving one p-Nitrophenyl phosphate (pNPP) tablet (Sigma, UK) in 20 ml distilled water was added to the wells. Then the plate was incubated for 30 minutes at room temperature covered with aluminum foil. The reaction was stopped by adding 50μl of NaoH. Absorbance was read at 405nm by ELISA reader (Titertek multiskan plus, Finland). As standard reference, commercially available recombinant IFN-γ (Gibco laboratories) was used. The results were given as the means of duplicate wells.

### 2.4.3.2 IL-10 assay

Three microgram/ml in 100μl/well of monoclonal antibody specific for IL-10 (R & D) was precoated onto a microplate and incubated overnight at room temperature. After washing three times with PBS-Tween , plates were incubated with blocking buffer (PBS containing 1%BSA, 5% sucrose, and 0. 05%$NaN_3$). Two hundred microliter standards and samples were added into wells of microtiter plate and incubated at room temperature for 2 hours.

After washing away any unbound substance using PBS-Tween, 250µg/ml of biotin labeled monoclonal antibody specific for IL-10 (R&D) was added to the well (200µl) and incubated at room temperature for 1 hr. This was followed by additional washing step to remove unbound antibody enzyme reagent and then avidine peroxidase was added. Finally, OPD solution was added to the well. After incubation and another wash, 50µl of stop solution (2N sulfuric acid) was added and optical density was read at 405 nm in an ELISA reader (Titertek multiskan plus, Finland).

### 2.4.4 Antibody assay

All experimental animals were bled every 30 days. Sera were stored at -70 $^{0}$C until time of assays. Antibody assay was based on ELISA. The soluble antigen was diluted in carbonate and bicarbonate buffer. The working dilution of the antigen was determined by using checkerboard titration, with negative and positive control sera. In this manner, a final concentration of 20 µg /ml was determined to be appropriate. It was also determined that the sera should be tested at 1: 500. In each well of the ELISA microtiter plate (Immunol 2, USA), 50µl of the diluted antigen solution was dispensed. The plates were covered with titer plate sealer and left at 4°C overnight. The plates coated with antigen were washed three times with 0.05% Tween-20 in PBS (PBS-Tween ) pH 7.4 and then blocked with the same buffer containing 1% BSA. Fifty µl of test serum diluted 1: 50 in PBS-Tween was added and incubated at 37°C for 2 hours. The plates was washed as above and 50µl of peroxidase enzyme labeled protein A (Sigma, USA), diluted in 1: 500 in PBS-Tween 20 was added to each well and incubated at 37°C for 2 hours. This was followed by washing as above. Finally 50µl of enzyme substrate, OPD was added to each well and incubated for 30 minutes. The reaction was stopped by adding 50µl of 2M $H_2 SO_4$. The result was read

spectrophotometrically at 492nm by using ELISA reader (Titertek multiskan plus, Finland).

### 2.3.5 Skin testing

Leishmanin was a gift from London School of hygiene and Tropical Medicine Laboratory (UK). A single dose contained 5 x $10^6$ *Leishmania major* promastigotes in 0.1 ml of 0.5% phenol saline. As it was indicated before the use of cross-reacting leishmania species (*L. major*) for skin test of *L. aethiopica* (Akuffo *et al.,* 1995), we used standardized commercially prepared antigen. The test was performed by intradermal administration of the test antigen into the surface of the shaved forearm. DTH reactions were read 72 hours after administration.

## 2.5. Culture method

A few drops of cardiac blood and small piece of tissue from lesion and nodules were aseptically transferred to the liquid phase of the NNN slant in screw-capped glass vials. These were incubated at room temperature. A search for promastigotes was made every third day under the X40 phase objective. Culture was examined for 10 days and then discarded.

## 2.6 The impression smear method

Thin blood film from lesions prepared on clean slides. Slides were fixed in absolute methanol and stained with stock Giemsa for 45 seconds. The slides were then examined for amastigotes with the oil immersion objective.

## 2.7. Data analysis

Data were analyzed using Sigma Plot, Sigma Stat, and Excel computer programs and

differences in proliferation responses and cytokine production were compared by StudentS' t-tests. Data was expressed as a means and standard error of the means. Differences were considered significant when the P-value was less than or equal to 0.05.

# 3. Results

## 3.1. Screening

In order to confirm the health status of the monkeys for infection, various screening tests were performed.

### 3.1.1 Screening of monkeys for natural infection with leishmaniasis

To determine whether any of the wild-trapped animal had been exposed to leishmanial infection, a prior screening was done as described in section 2.1.3 (Materials and Methods) Clinical examination revealed no ulcers or wounds suggestive of leishmanial infection in monkeys. No anti-leishmania antibody was detected from pre-infection sera drawn from each monkey.

### 3.1.2 Screening monkeys for endoparasite infections

As described in Materials and Methods (2.1.4) the animals were screened for infection by endoparasites. The most prevalent helminthic infestations were *Trichuris trichura*. Hookworms and strongyloides were also observed. All these animals were treated for all above mentioned helminths.

### 3.1.3 Screening monkeys for SIV

The SIV test using commercially available kit revealed that all animals were seronegative.

All the above tests confirmed that the animal were free of any other infections and could thus be used for infection with *L. aethiopica*.

## 3.2 Course of infection

### 3.2.1. Grivet monkeys infected with LCL strains of *L. aethiopica*

Grivet monkeys infected with LCL strains of *L. aethiopica* are coded as M-56, M-57, M-333, M-335, M- 336 and M-337. Infection of *L. aethiopis* with LCL strain produced a nodule in one monkey and transient indurations in two others (Table 1). Depletion of hair was also seen in two other animals.

### 3.2.2 Grivet monkeys infected with DCL strains of *L. aethiopica*

Grivet monkeys infected with DCL strains of *L. aethiopica* were coded as M-334 and M-338. Infection with DCL strain produced a non-ulcerative localized lesion in one animal and loss of hair and bump in another. The lesion persisted until the end of the study.

### 3.2.3 Controls

Control monkeys used in the experiment are coded as M-320, M-328, M-34O, and M-341. They are sex and weight matched. Controls did not show any lesion development and remained healthy.

TABLE 1 Chronological history of infection

| | Animal code No. | Infection site | Type of strain | No of days after infection to lesional episodes | | | | Lesion size (mm) Length x width |
|---|---|---|---|---|---|---|---|---|
| | | | | Loss of hair | bumps | nodules | lesion | |
| LCL | M-556 | Tip of nose | 1 | 50 | 50 | | | |
| | M-57 | " | " | 50 | | | | |
| | M-333 | " | " | - | | | | |
| | M-335 | " | " | - | 50 | 90 | | |
| | M-336 | " | " | - | 90 | | | |
| | M-337 | " | " | - | | | | |
| DCL | M-338 | " | 2 | | | | 131 | 6 x 8 (160) |
| | M-334 | " | " | 90 | 90 | | | |

1= strain number 1282; 2 = strain p-16; M= monkey; Numbers in parentheses show numbers of days at which lesion size measured.

## 3.3 Lymphocyte proliferation assay

### 3.3.1 Grivet monkey PBMC proliferation following mitogen stimulation.

PBMC from infected and control animals were stimulated with T-cell mitogen PHA as described in (section 2. 3.2.2, Materials and methods). Results obtained showed that PBMC from all animals whether infected or not responded to PHA (Fig 2). There was, however, individual variation from animal to animal. The highest record was in animal number M-334 with a stimulation index of 93 and the lowest stimulation index was 3 in animal number M-335. Both of them were from infected group.

### 3.3.2 Grivet monkey PBMC proliferation following stimulation with live parasite antigen (PROM-LCL, PROM-DCL), formalin killed parasite antigen (FPA) and soluble antigen

As described in materials and methods (section 2.3.2.2) PBMC of experimental and control animals were stimulated with different preparations of leishmanial antigen.

All infected monkeys' PBMC proliferated to live LCL strains of leishmania parasite (Fig 3). However, variation of response was observed with high and low responders. Only three infected animals' PBMC proliferated to live DCL strains. Only two infected animals' PBMC proliferate to FPA. Control animals' PBMC did not proliferate in response to live parasites for all above cases.

Mean proliferative response to PROM-LCL was significantly higher in infected monkeys compared to controls. (P=0.005). This is shown in Fig 4. Although it was not statistically significant, mean proliferative response to PROM-DCL was higher in infected monkeys

compared to controls (Fig 5). Mean proliferative response to FPA was also higher in infected monkeys compared to controls. However, this was also not statistically significant (Fig 6).

To examine the response of PBMC to soluble leishmanial antigen, the cells were harvested and co-cultured with soluble antigen as described in materials and methods. Proliferative responses to the soluble antigen were minimal (Fig 7). Moreover there was no difference between infected and control monkeys. Of the 8 infected monkeys 5 had some proliferative response to soluble *Leishmania aethiopica* antigen that had SI above 2 (maximum SI=8).

## 3.4. Cytokine secretions

### 3.4.1 The levels of IFN-γ in the serum of monkeys before and after infections.

A sandwich ELISA was employed to detect the level of IFN-γ in the monkeys before and after infection with leishmanial parasites. Results showed that almost all experimental animals had elevated but variable serum levels of IFN-γ before infection. However, This decreased after 12 days of infection except animal Number M-335 and M-338 (monkeys that develop clinical sign) then was increased again after 30 days of infection (Fig 8). The levels of IFN-γ in the serum of M-335 and M-338 were still decreasing after 30 days of infection.

### 3.4.2 Production of IFN-γ by PBMC following stimulation with PHA.

PHA was used to stimulate the cells and the amount of IFN-γ in the culture supernatant was measured by ELISA. PBMC from infected animals produced relatively higher levels of IFN-γ following stimulation with PHA. There were high and low responders among them. PBMC from control animals had a relatively lower level of IFN-γ except for monkey

Number M-340 which had a higher level of IFN-γ (Fig 9).

### 3.4.3 Production of IFN-γ following stimulation with soluble antigen

PBMC from the monkeys were stimulated *in vitro* in presence of soluble leishmanial antigen. Infected animals were shown to produce low levels of IFN-γ following stimulation with soluble antigen but the cytokine level in this group of animals was not very different from unstimulated controls. (Fig 10).

### 3.4.4. Levels of IL-10 in serum and culture supernatant of grivet monkey PBMC

As for IFN-γ a sandwich ELISA was conducted using either plasma or supernatants to determine the level of IL-10. There was no detectable level of IL-10 observed neither in serum nor in PBMC culture supernatants of experimental animals after stimulation with mitogen and antigen except animal No M-335 that developed nodules, which produced high level of IL-10 after infection.

## 3.5 Antibody against *Leishmania* parasite

To quantify the level of anti promastigote antibody in serum of animals following infection, a direct ELISA was performed (section 2.3.4). All experimental animals were found to be sero-negative for antibody for *Leishmania* with the exception of animal Number M-335 and M-338.

## 3.6 DTH responses

Following an intradermal injection of leishmanin, positive reactions are characterized by development of induration which is an indication of prior exposure to *Leishmania* antigen. We wished therefore to determine whether infected animals had DTH responses to

leishmanin following injection of the preparation. Results showed that there was no significant response to leishmanin among experimental animals except for erythema on animal numbers M-334, M-333 and M-56, which was observed after infection. All control animals were negative for DTH response.

## 3.7 Diagnosis of leishmania after infection in the experimental animals

A definite diagnosis of leishmaniasis is the demonstration of leishmania parasites on stained smear or in culture. Biopsy material obtained from the site of infection or the edge of the lesion revealed parasite presence in only one of the animals (smear and culture). The others were negative by both tests.

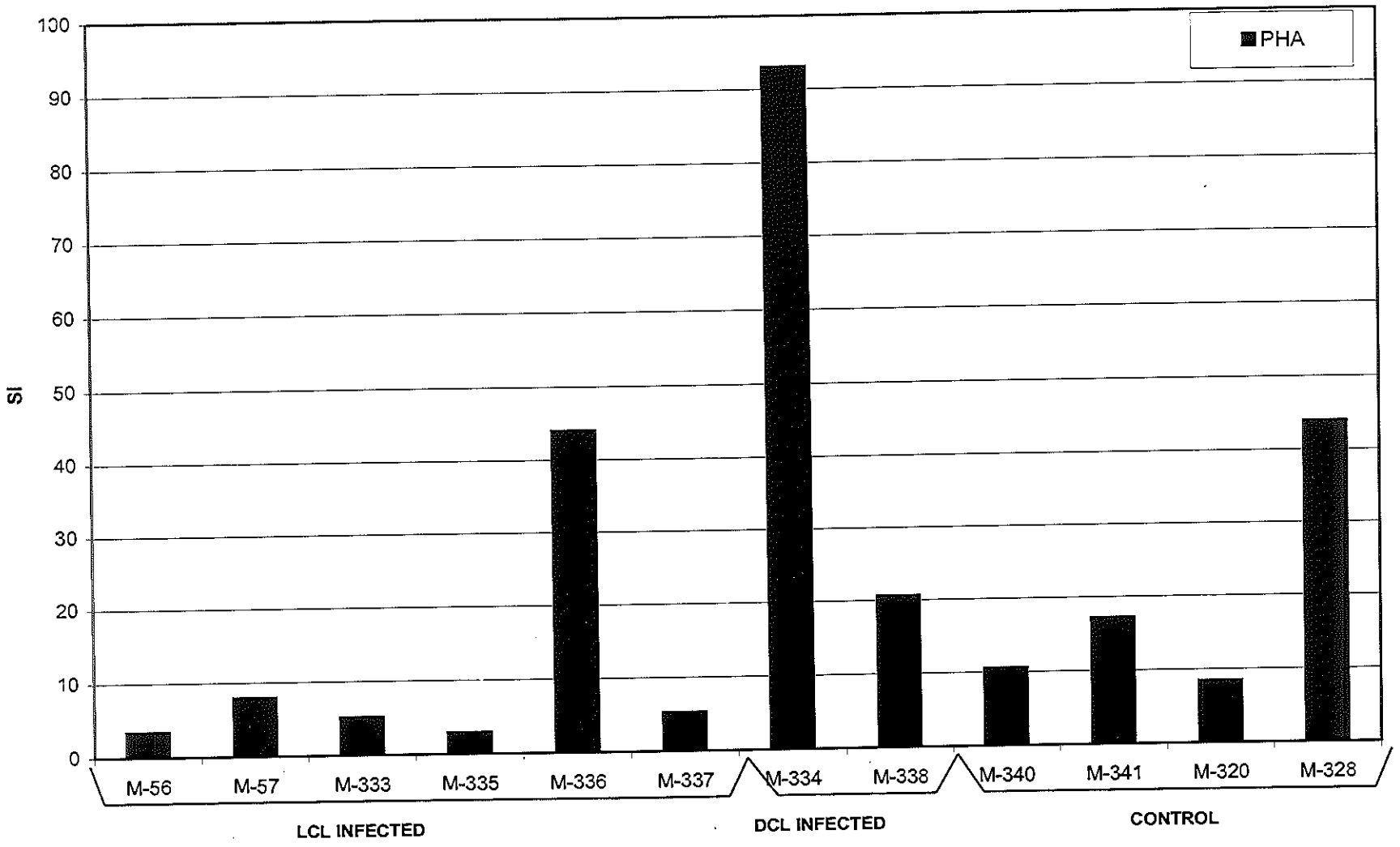
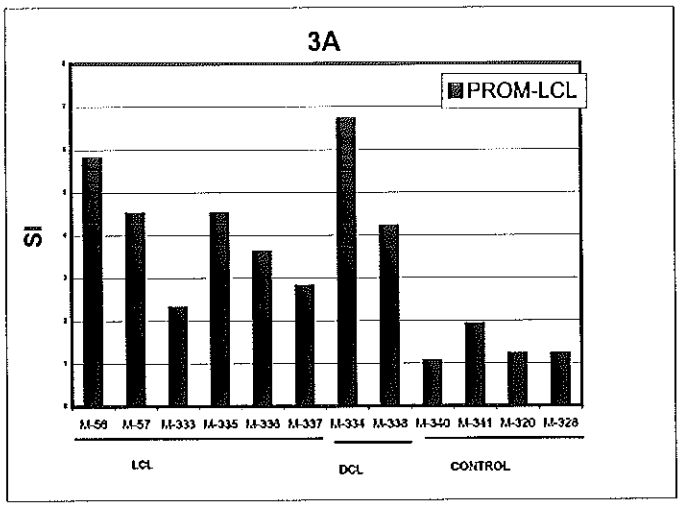
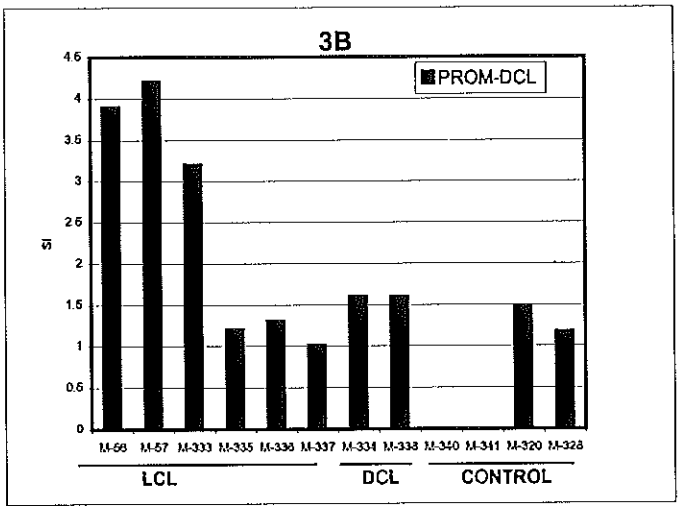



**Fig 2. Proliferative response of PBMC from control and infected animals to PHA**



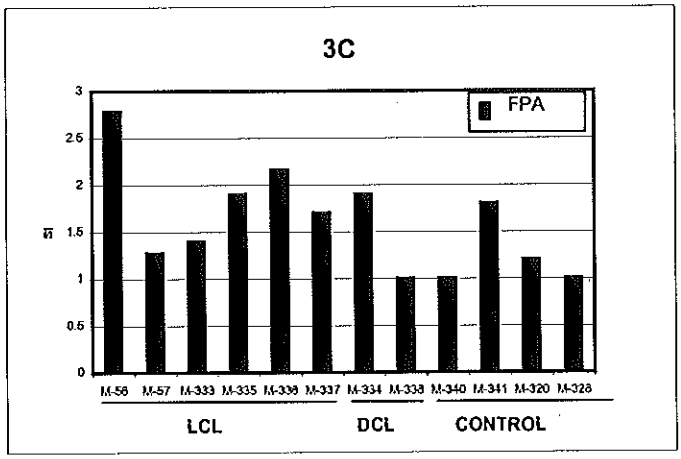




**Fig. 3. Proliferative responses of PBMC from infected and control monkeys to Promatigotes of LCL (3A), DCL (3B) and FPA (3C)**

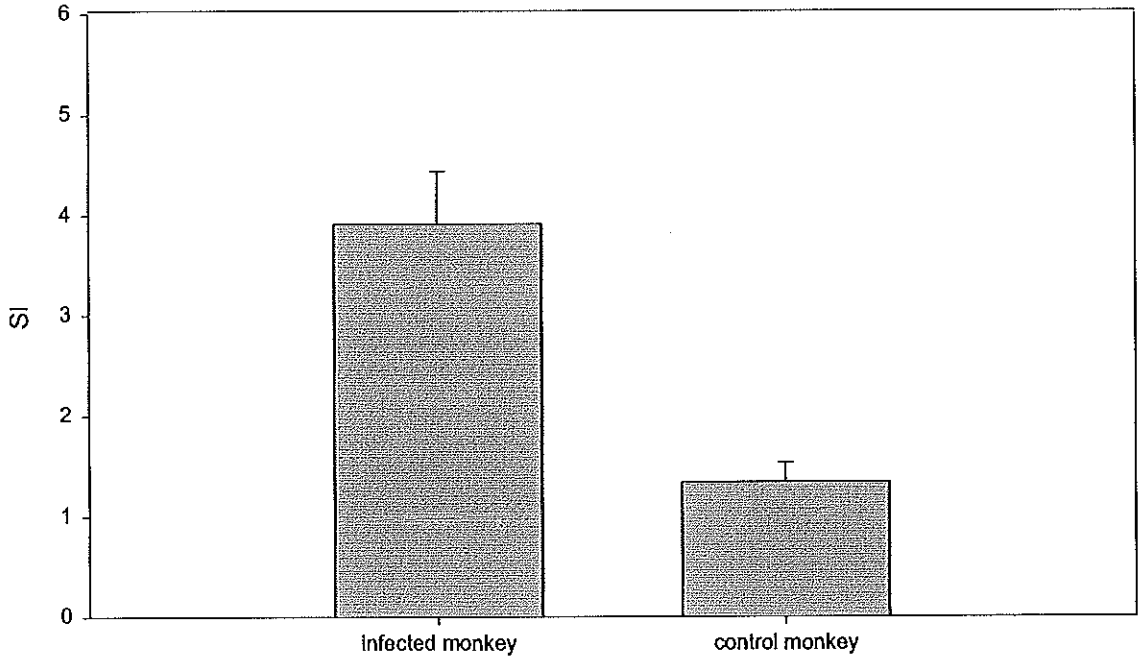



Fig 4. Mean proliferative responses of PBMC from infected and control monkeys to PROM-LCL.

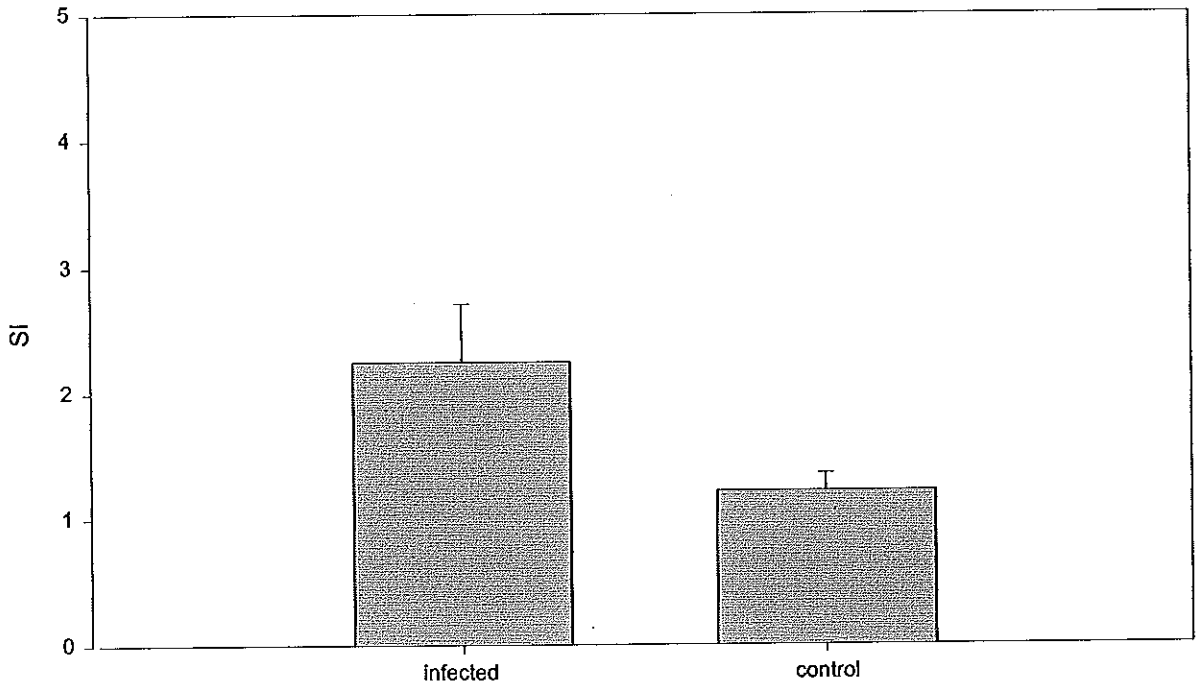



Fig 5. Mean proliferative responses of PBMC isolated from infected and control animals to PROM-DCL

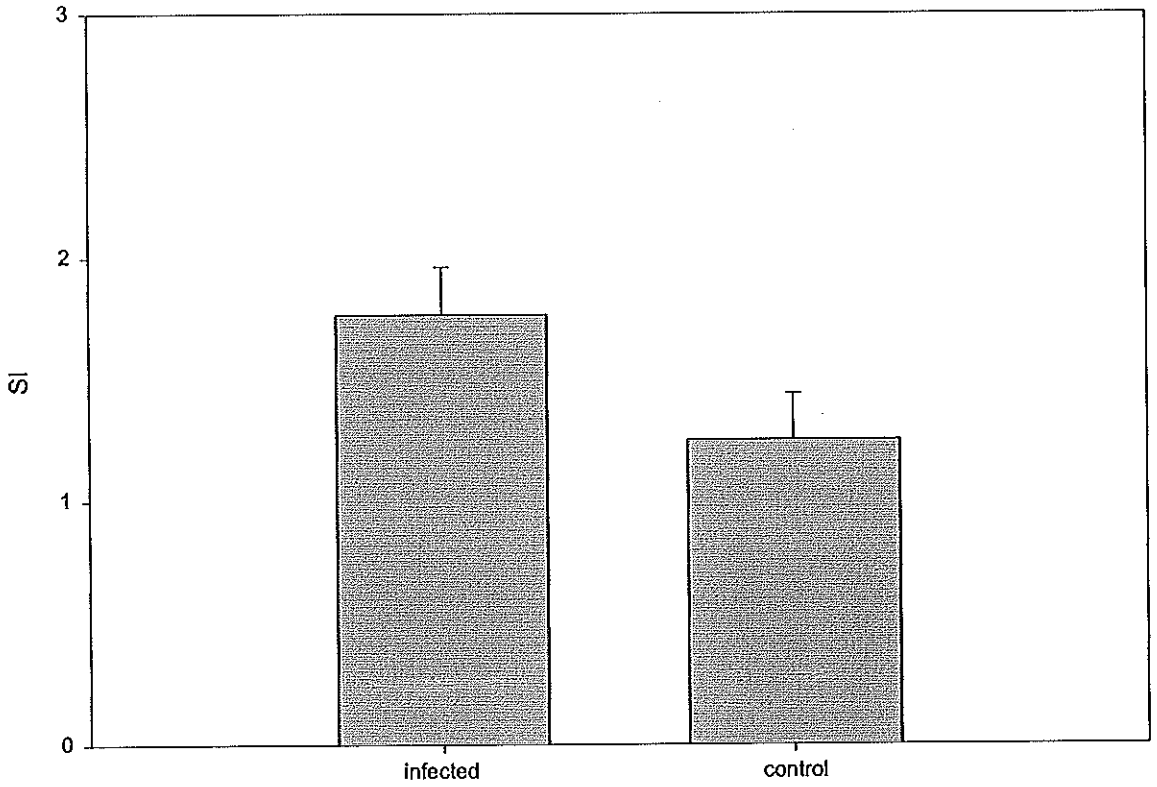



FIG 6. Mean proliferative response of PBMCs isolated from infected and control animals to FPA

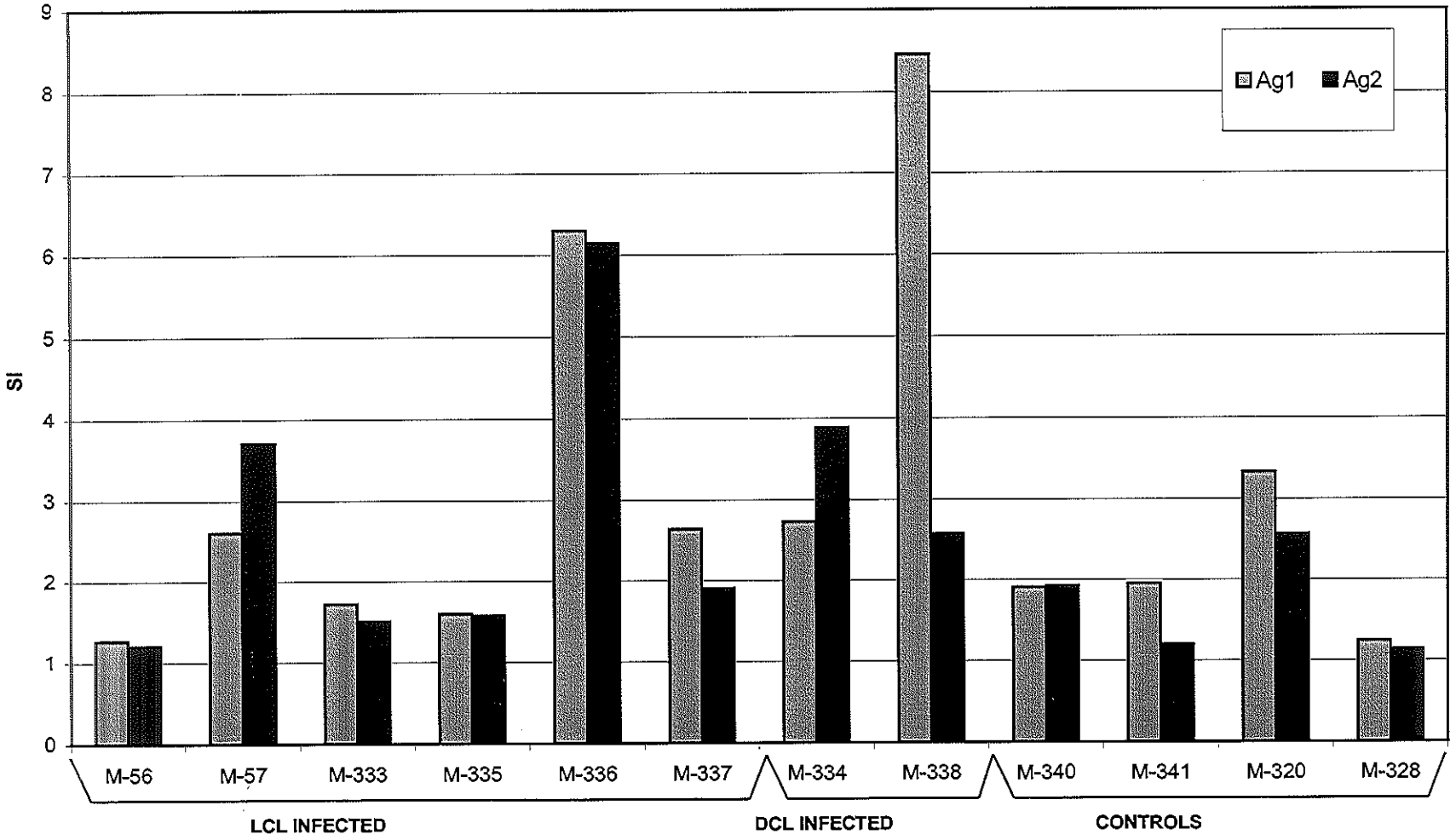



**Fig 7. Proliferative Response of PBMC from Control and Infected Animals to two different concentrations of Leishmanial soluble antigens**

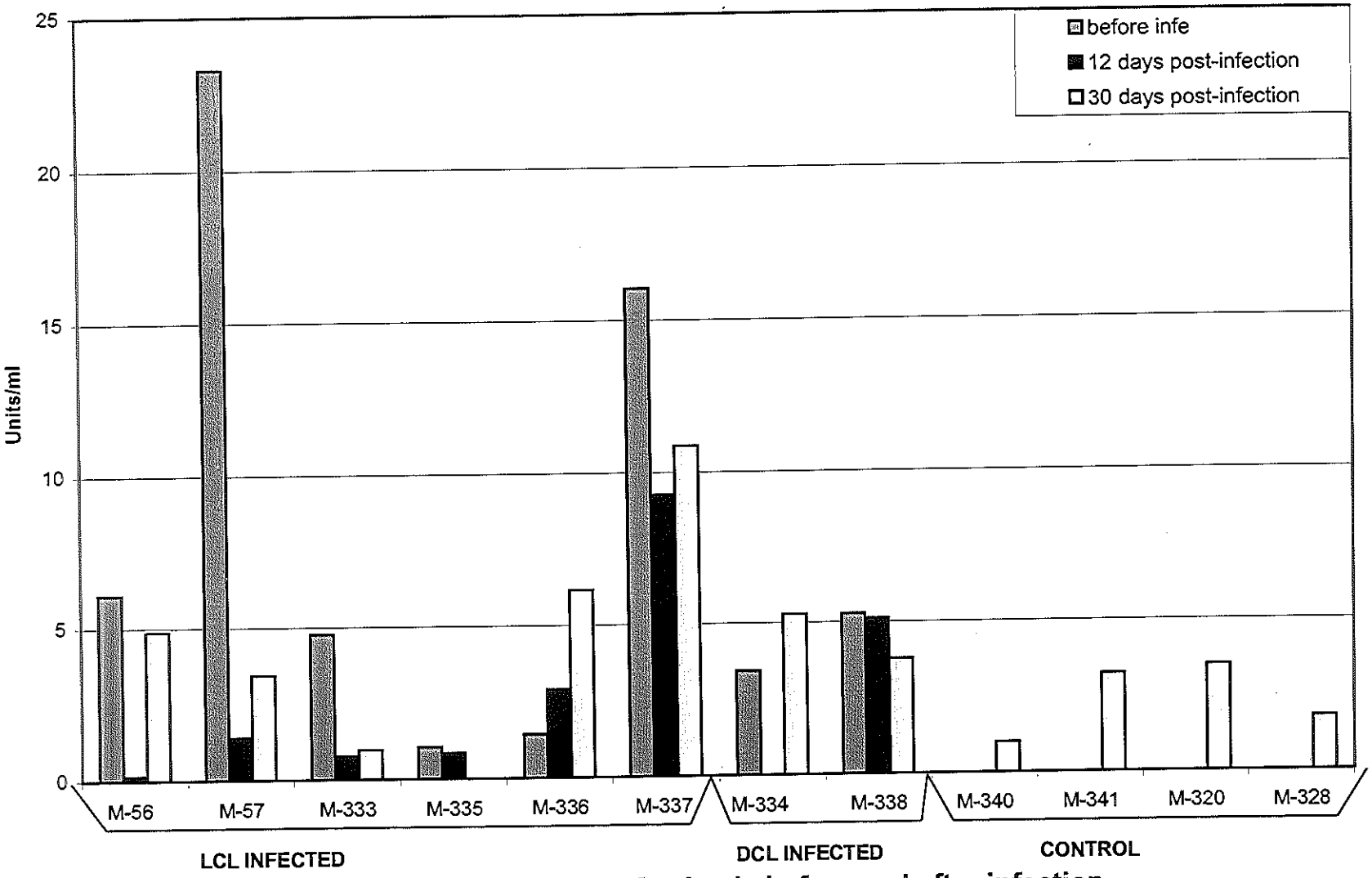



**Fig. 8 Serum level of IFN-g of animals before and after infection**

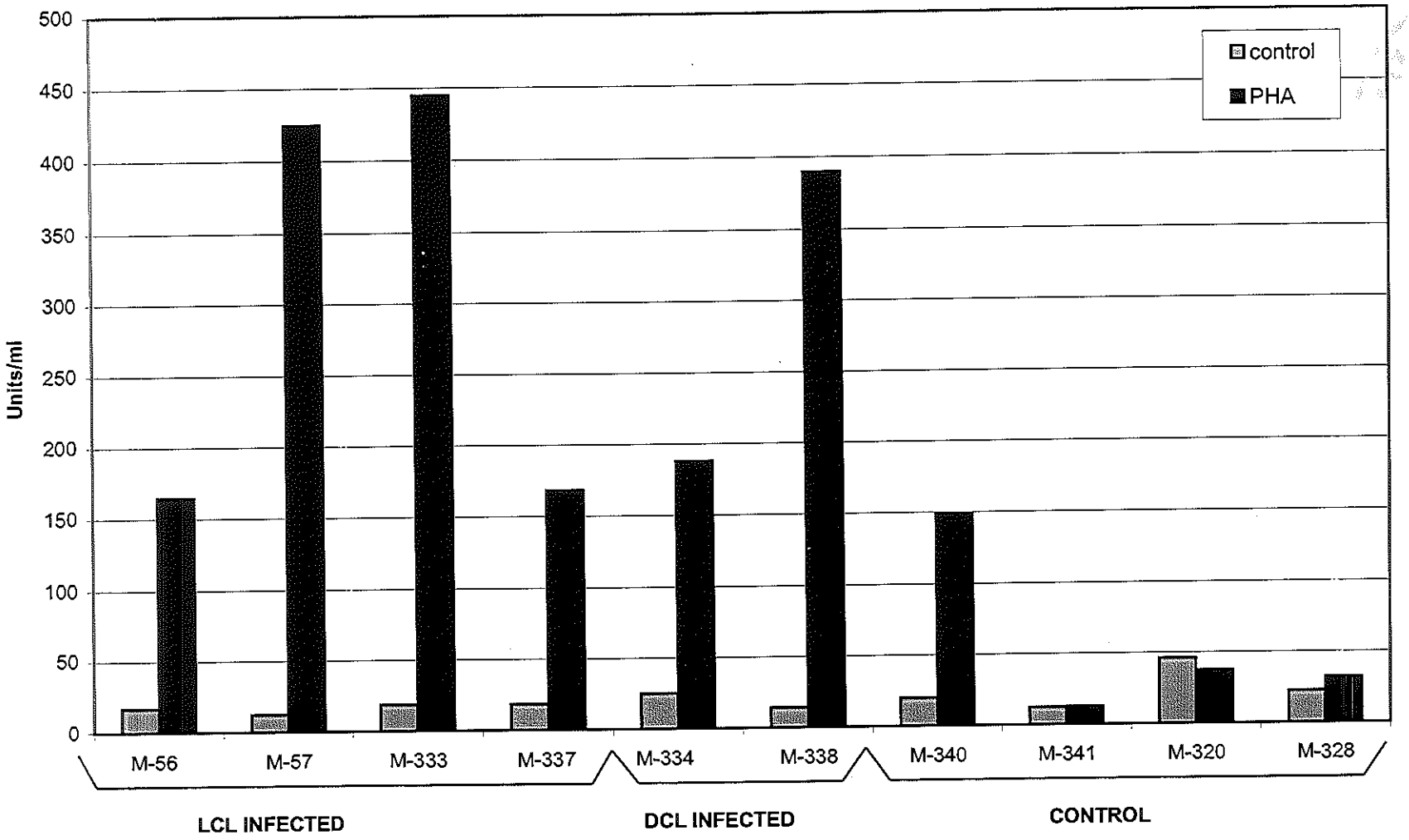



**Fig. 9 In vitro production of IFN-g by PBMC from infected and control animals following PHA stimulation**

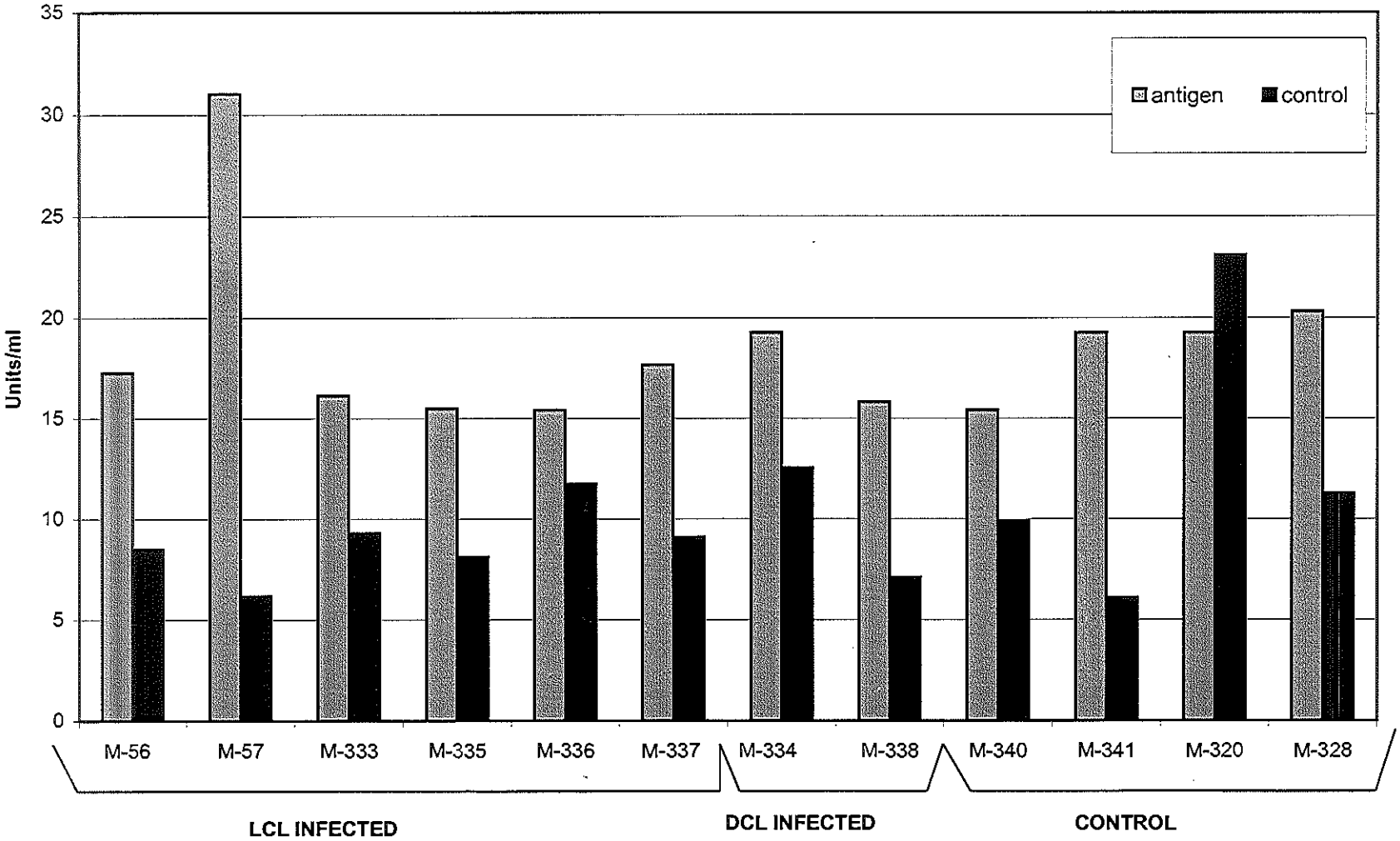



**Fig. 10. In vitro Production of IFN-g from infected and control animals following stimulation with soluble antigens**

# 4. Discussion

The development of a monkey model for *L. aethiopica* infection in which the disease mimic the human situation would be a useful tool for the study of different aspects of the disease, to determine the efficacy of new drugs and in vaccine trials.

In view of this, our study was undertaken to experimentally transmit *L. aethiopica* to grivet monkeys and examine host immune responses. Results showed that not all grivet monkeys could be infected through needle inoculation.

Following injection of *in vitro* derived *L. aethiopica* promastigotes, only one animal from DCL infected group developed a lesion and another one from LCL infected group developed nodules resembling the human disease. In some animals there was hair loss at the site of parasite inoculation but no ulceration or nodule formation was observed.

In a previous study by Hailu *et al.*(1995) it was reported that grivet monkeys developed cutaneous ulcers following inoculation of *L. aethiopica* promastigotes. Our result tend to partially agree with that of Hailu *et al.*(1995) as only few grivets got experimentally infected with *L. aethiopica.*

The pre-patent period is longer similar to that which would be observed in humans (Lemma *et al.* 1969), in whom an initial bump is detected as the first sign of overt disease. After subcutaneous inoculation of virulent parasites, the infection remained sub clinical for more than 24 days whereas Hailu *et al.* (1995) reported that the pre-clinical period varied from 35-161 days. It was difficult to determine the upper limit of pre-patent period in our

experiment because lesions were still under development.

Virulence of infection with *L. aethiopica* varied considerably from previous studies. Such individual variations were reported previously in most primates (Hommel *et al.,* 1995). In addition to this, experimental infections of monkeys with leishmaniasis had a variable result (Kirk *et al.,* 1945 and Githure *et al.,* 1986). This probably depends on factors like virulence of Leishmania isolates, length of study, genetic and immune status of monkeys.

There are great differences among strains within *Leishmania aethiopica* species. The same species of the parasite produced either localized or diffused cutaneous leishmaniasis. Therefore, intra-strain variation among the same species might also contribute to this virulence. Loss of virulence might also occur during multiple passages of *in vitro* cultures.

The immune status of these animals may also be the probable reason for the difference of our results from that of Hailu *et al.* (1995). Except for one animal, which produced nodules, a considerable amount of IFN-γ in serum of infected animals was obtained in our study group. This level of IFN-γ might determine the susceptibility and resistance of these animals to leishmania infection. This would be in line with other studies where IFN-γ was shown to mediate resistance to leishmania antigen (Heinzel *et al.,* 1991). Failure to diagnose previous exposure/ infection of *Leishmania* species is another possibility which could have affected our infection results since there is no good diagnostic method for cutaneous leishmaniasis and hosts which have cured from previous infection with *Leishmania* parasites are known to have solid immunity (Nacy *et al.,* 1985). Although skin testing could give us some idea about prior exposures to leishmania we did not use it in our case, as we feared that it might interfere with the immune status of the animals. The

presence of moderate levels of IFN-γ in grivets before infection may be due to undiagnosed leishmanial infection or other infections. Moreover contributions of non-leishmania specific immunity to resistance to *L. aethiopica* was indicated in previous studies (Akuffo and Britton, 1992).

The immunological status of infected and control monkeys was initially evaluated using PHA, a well known T-cell mitogen. PBMC from both infected and control monkeys proliferate in response to this mitogen with higher proliferative responses in infected monkeys than controls. These results compare well with data in similar studies involving vervets (Curry *et al.,* 1994).

PBMC from infected animals proliferated in response to LCL strain of live *L. aethiopica.* Some were high responders while others were low responders. This correlates with earlier studies in humans (Akuffo *et al.,* 1987). Both LCL and DCL infected monkeys had strong response to LCL than DCL promastigotes. This finding is also consistent with reports of similar investigations (Akuffo *et al.,* 1987).

The proliferative responses of PBMC from infected monkeys to soluble antigen were very low. This was demonstrated in human study whereby only half of LCL patients had proliferative response to *L. aethiopica* soluble antigen in one report (Maasho *et al.,* 1998). Proliferative response was also observed in some of the controls but it was relatively lower than in infected monkeys. Previous studies showed that mononuclear cells from normal individuals proliferated in response to live forms of *L. aethiopica* promastigote (Akuffo and Britton, 1992). Another study demonstrated that people without known previous exposure to *Leishmania* parasites had T-cells that reacted upon stimulation with leishmanial antigens

in their peripheral blood and it was suggested that some of the T-cells epitopes may be shared by *Leishmania* parasites and microorganisms in the environment (Kemp *et al.*, 1992).

Moderate amounts of IFN-γ was detected in the serum of experimental animals before infection and this level decreased when measured 12 days after infection except in few animals. This unexpected finding may suggest changes in T cell regulation in these animals, which result in a down regulation of immune responses. Similar suggestion also has been reported from human cases (Akuffo and Britton, 1992). IFN-γ level in serum of the animal that developed nodule was below detectable limit at this time. This also implied a possible immuno-suppressive effect of parasites at the early stage of infection. IFN-γ level in serum of experimental animals increased 30 days after infection.

PBMC from both control and infected animals produced considerable levels of IFN-γ after PHA stimulation. The level of IFN-γ is relatively lower in controls than in infected group. This is in contrast to human cases where higher levels of IFN-γ production was reported (Akuffo and Britton 1992).

There was no detectable level of IL-10 in serum of both infected and control animals before and after infection with the exception of one animal coded M-335. This had unexpectedly high levels of IL-10 after infection. This might indicate the role of IL-10 as a modulator of the effector functions of macrophages and T-cells and NK cells (Ghalib *et al.*, 1993). This animal showed clinical sign earlier than others and reached nodular stages unlike the others. This animal had also very low IFN-γ before infection indicating the protective role of IFN-γ and the inverse relationship between this cytokine and IL-10 (Locksley and Jacques, 1992).

# 5. Conclusion and recommendations

One clear point from the results is that not all grivet monkeys could develop cutaneous disease following infection with *L. aethiopica*. More studies need to be conducted to establish the grivet monkey as a model for examining the natural history of the disease and immune responses associated with infection with *L. aethiopica*.

A detailed systemic study of the histological and immunological changes occurring in *L. aethiopica* infection in monkeys may be important for the understanding of some of the mechanism involved in the control of infection with this parasite in man.

This study suggested that these animals could be useful in immunological investigations and could serve as models for perhaps asymptomatic cases of leishmaniasis.

Th1 responses are associated with resistance to leishmanial infections while the Th2 response is observed in disease progression. The observation that only few animals developed lesion following infection suggests a strong Th1 response which perhaps cleared the infection.

The grivet monkey model could therefore be useful to examine immune mechanisms of protection in *L. aethiopica*. However, the number of animals used in the study and the difficulty to initiate cutaneous leishmaniasis following infection with *L. aethiopica* promastigotes does not permit any firm conclusions from this study. More studies are required to resolve this problem.

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