ORIGINAL ARTICLE

EXPERIMENTAL INFECTIONS WITH *LEISHMANIA AETHIOPICA* AND IMMUNE RESPONSE ANALYSIS OF *CERCOPITHECUS AETHIOPS*

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ABSTRACT

Background: *Leishmania aethiopica (L. aethiopica) is the major cause of cutaneous leishmaniasis in Ethiopia. Only few studies are available on L. aethiopica infection in animal models. Therefore, the purpose of this study is to examine the possibility of establishing an African green monkey model and to describe the immunological consequences of L. aethiopica infection.*
Methods*: Eight monkeys were inoculated subcutaneously at the tip of the nose with 5 x 10^6 L. aethiopica promastigotes. Six of them were inoculated with isolates from a patient with localized cutaneous leishmaniasis (LCL) and two were inoculated with isolates from a patient with diffused cutaneous leishmaniasis (DCL). Four control animals received only a sham inoculation of culture medium. Lymphocyte stimulation test was done and IFN-g levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA).*
Result: *Three of the six monkeys infected with promastigotes from an LCL patient produced ulceration, one produced nodules that disappeared soon, and the other two lost hair at the infection site. One of the two monkeys infected with promastigotes from a DCL patient produced ulceration while the other lost hair at the infection site. The infection was further confirmed by the isolation of parasites from lesions of the animals. In the in vitro assay, Peripheral Blood Mononuclear Cell (PBMC) obtained from the infected and control animals showed comparable proliferative and IFN-g responses when stimulated with live or dead L. aethiopica parasites and soluble leishmanial antigen.*
Conclusion: *The development of lesions suggests a possibility of using African green monkeys as a model to establish L. aethiopica infection. The lack of specificity in the proliferative response indicates the need to develop new specific antigens.*
Key words*: Cutaneous leishmaniasis; Leishmania aethiopica; Cercopithecus aethiops; animal model*

INTRODUCTION

In Ethiopia, cutaneous leishmaniasis (CL) is mainly caused by *Leishmania aethiopica* (*L. aethiopica*). Although the exact magnitude of CL in Ethiopia is not well known, several surveys have identified the importance of the disease as a health problem (1-3).

L. aethiopica infection induces three clinical forms of disease: localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL) (4). LCL is mostly seen as a single lesion which is self-healing over time and localized in areas uncovered by clothing such as the face, arms or legs. MCL begins with skin ulcers which spread causing dreadful and massive tissue destruction, especially of the nose and mouth. DCL shows multiple lesions on the face, trunk and extremities and is usually not self-healing. (4)

The immunological features of various human *Leishmania* infections have been evaluated with serological and cellular assays (5-6). Protection against *Leishmania* species requires interferon gamma (IFN-g) producing type 1 T helper (Th1) cells while susceptibility is associated with IL-4, IL-5 and IL-10 secreting Th2 CD4 cells (7-9).

The availability of animal models is vital in the search for vaccines and drugs against leishmaniasis. Leishmanial diseases caused by *L. donovani*, L. *major* and *L. mexicana* have been mimicked in animals such as hamsters (10), guinea pigs (11), BALB/c mice (12) C57BL/6 mice (13) and primates (14). The experimental model system for both *L. major* and *L.*

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tropica has allowed the elucidation of some of the mechanisms involved in these infections. However, studies on CL caused by *L. aethiopica* have long been hampered by the lack of a suitable animal model in which controlled studies could be conducted. BALB/c mice are highly susceptible to *L. major* (15).

However, infection of BALB/c mice with L. aethiopica in the nose (16) and in the footpad (17) resulted in no overt clinical signs although experimental mice were unable to clear infection. Clinical lesions were produced in hamster but with difficulty (lesion produced after several trials) (18).

However, later, Hailu and his colleagues reported successful infection of African Green monkeys with *L. aethiopica* resulting in cutaneous leishmaniasis (19).

The utilization of non-human primates susceptible to CL has its advantages. Their phylogenetic closeness to man could be exploited to better understand the immunological basis of protective response in humans.

Therefore the aim of this study is to examine the possibility of establishing an African Green monkey (*Cercopithecus aethiops*) model of CL due to *L. aethiopica* and to describe the immunological consequences of infection for the possible use of this animal model for drug and vaccine trials.

METHODS

Animals

Monkeys were trapped from Leishmania non-endemic areas. Then, they were housed individually in the cage. The regular feed was carrot supplement with grains (like maize, beans) and banana. They were provided with tap water. They passed through a three-month quarantine period. The animals were examined for any sign of prior infection with *Leishmania*, checked for development of any external lesion and were confirmed to be negative for intestinal and haemo-protozoan parasites by using formol-ether concentration and fecal-flotation and blood smear stained by Giemsa test. Simian immunodeficiency virus (SIV) screening was done using RECOMBIGEN HIV-1/HIV-2 kit (Trinity Biotech Plc, Wicklow, Ireland) to detect SIV because this test is known to detect both HIV and SIV (20).

Parasite and culture conditions

One of the strains was used in the experiments. Strain 1282 (AHRI ref number) was isolated from a LCL patient in ALERT Hospital. The person who had a single localized lesion was clinically diagnosed for leishmaniasis by the physician. Strain P-16 (IPB ref number) was isolated from a lesion of a DCL patient from Ocholo (a CL endemic village in southern Ethiopia) in October,1999. The infected individual had lesion on the face, hand, and the buttock. Both patients were clinically diagnosed for cutaneous leishmaniasis by the physician. The strains were typed as *L. aethiopica* by using isoenzyme electrophoresis.

The Novy-MacNeal-Nicolle (NNN) blood base agar medium (Bacto-Agar, NaCl, sheep blood and double distilled water) (Difco, Detro, Michigan, USA) with Locke's solution (NaCl, $CaCl_2$, KCl, Na_2CO_2) as overlay was routinely used for parasite maintenance and cultivation. One hundred units of penicillin and 100 mg of streptomycin (Gibco, Paisley, Scotland) were added to 1 ml of overlay to control bacterial contaminants. The parasites were transferred into complete medium [RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 20% heat inactivated fetal calf serum (Sigma), penicillin 100U/ml and streptomycin 100mg/ml and 2mM l-glutamine (Flow laboratories, Irvin, Scotland)]. The cultures were harvested when they reached a stationary phase.

Experimental infections

We received the parasite as a primary isolate and passed it three times. Third passage stationary phase promastigotes (non-dividing stage of the parasite when the numbers of the parasite in the culture become stable; no growth or no declining and the shape of the parasite is becoming rounder) grown on NNN medium were inoculated subcutaneously into the tip of the nose (19) of each animal at a dose of 5×10^6 in 20ml medium (19). Six monkeys (1 female and 5 male) were inoculated with promastigotes of a strain of *L. aethiopica* isolated from an LCL patient. These monkeys were coded as M-56, M-57, M-333, M-335, M-336 and M-337. Two monkeys (1 male and 1 female) were injected with promastigotes of a strain of *L. aethiopica* isolated from a DCL patient, and were coded as M-334 and M-338. Naive monkeys (negative controls) used in the experiment were coded as M-320, M-328, M-340, and M-341and each animal received only a sham inoculation of culture medium. The monkeys were observed once in a week for 34 weeks after infection.

Antigen preparation

a. ***Preparation of live promastigote antigen***.

Promastigotes were harvested at stationary phase and centrifuged at 906*g* for 10min. The pellet was resuspended in cold phosphate-buffered saline (PBS) and kept at room temperature for 10 min. The debris was discarded and the supernatant was centrifuged at 906*g* for 10 min. The pellet was washed two times as above and resuspended to a concentration of 10^6 parasites/ml in complete medium (21).

b. Preparation of Formalin-killed Promastigote Antigens (FPA).

Promastigotes were washed three times by centrifugation at 629*g* for 15 minutes in PBS solution (pH 7.2). The pellet was resuspended to at concentration of 10^6 parasites/ml in a 1% formalin solution, left overnight at 4°C, washed three times with complete medium and resuspended in the same medium at a concentration of 10^6 parasites/ml (22).

c. Preparation of Soluble Leishmania Antigen (SLA)

Parasites were washed three times with PBS and resuspended in ice-cold lysis buffer (10mM Tris, 2mM EDTA) containing protease inhibitors, 1mM PMSF (phenyl-methyl-sulfonyl fluoride) and 100units/ml of aprotinin. This was followed by four cycles of alternate freezing (with liquid nitrogen) and thawing (water bath at 37°C).

The preparation was then placed on ice and sonicated at 150W for 3 minutes, with a one minute interval for each minute of sonication (Bransonic Company, San Pablo, USA) (23). Protein concentration was measured by a modification of the Lowry method (24)

Lymphocyte stimulation test

Lymphocyte stimulation test was done as described previously (21). Briefly, peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Amersham, Uppsala, Sweden) density gradient centrifugation of 15ml blood collected into sterile EDTA tubes from a femoral vein of each monkey.

The cells were adjusted to 1 x 10^6 cells /ml and added into wells (at 180ml/ well) of a 96 well tissue culture plate (Linbro Flow lab, McLean, USA). PHA (5mg/ml in 20ml), live promastigotes (1 x 10^5 parasites in 20ml), formaldehyde fixed promastigotes (FPA) (1 x 10^5 parasites in 20ml), and soluble leishmanial antigen (SLA) (12.5mg/ml) were added independently in triplicate Microwell cultures and incubated at 37°C in a 5% CO_2 incubator (Gelaire Flow lab, Milan, Italy). Culture supernatants (100ml) were collected for IFN-g assay on the third day from PHA-stimulated wells and on the fifth day for antigen stimulated cultures.

At the time of harvest, medium was replaced and cultures pulsed with 1mCi ^{3}H-thymidine (Amersham), incubated overnight and harvested on a filter mat (Cat. No. 11731) using a cell harvester (Skatron, Lierbyen, Norway).

The incorporation of thymidine in the DNA of proliferating cells was counted in scintillation fluid using a b-liquid scintillation counter (LKB Instruments Inc, Gaithersburg, MD, USA). Stimulation index (SI) was calculated by dividing counts per minute (CPM) of antigen stimulated cultures by those without antigen.

Cytokine assays

IFN-g levels were measured using a sandwich enzyme linked immunosorbent assay (ELISA) (Mabtech, Stockholm, Sweden) as previously described (23). Briefly, wells of Dynatech Immunol plates (Dynatech laboratory, Sussex, UK) were coated with 50ml of a 2mg/ml concentration of primary monoclonal antibody to human IFN-g (Mabtech, Stockholm, Sweden).

The plates were washed three times with 0.005% Tween 20 in PBS. This step was repeated in between the following steps. Non-specific binding sites were blocked in 1%BSA PBS for 1 hour at room temperature. Samples (and standard) titrated in double dilution were then added in duplicate to the plates and incubated at room temperature for 1.5 hours.

Biotinylated secondary monoclonal antibody (7-B6-1.biotin, Mabtech) was added. This was followed by incubation for 1 hour. Streptavidin ALP diluted 1:1000 in PBS-Tween was added to the wells. A substrate prepared by dissolving one p-Nitrophenyl phosphate (pNPP) tablet in 20 ml distilled water was added to the wells. The plate was then incubated for 30 minutes at room temperature protected from light.

The reaction was stopped by adding 50ml of NaOH and absorbance was read at 405 nm in an ELISA reader (Titertek multiskan plus, Helsinki, Finland). Commercially available recombinant IFN-g (R and D, Norway, 1998) was used for standard reference. The results were given as the means of duplicate wells and expressed in units/ml.

Parasite Culture
Skin scraping was taken from the edges of lesions and nodules and aseptically transferred to NNN media as mentioned in the parasite culture. A search for promastigotes was made every third day under the 40 X phase objective. Negative cultures were discarded after 10 days.

Ethical consideration
The study was approved by the Federal and Regional Forest and Wild Life Protection offices under the Ministry of Agriculture and Rural Development.

Statistical analysis
Student's *t* test was used in comparative analysis and $p < 0.05$ was considered significant.

RESULTS

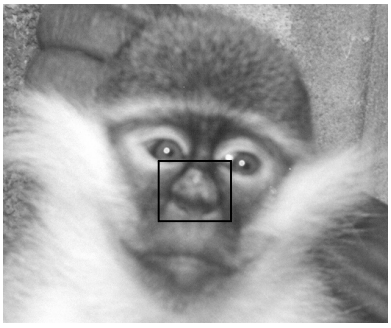
Ulcerated lesions developed in three of six African green monkeys infected with *L. aethiopica* isolated from an LCL patient (Fig 1a). The disease progression in the three animals progressed through hair depletion, induration and elevation of the skin to nodule formation and the development of ulcer. The other animals in this group did not show any ulceration. In one experimental animal the lesion reached nodule formation while in the other two only hair depletion and transient induration was observed (Table 1).

Table 1: *Chronological history of infection*

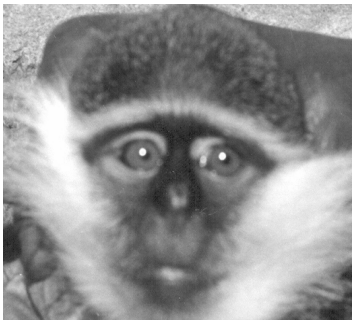
Animal code No.	Type of strain	No of days after infection				Lesion size Width × length (in mm)
		Loss of hair	Bumps	Nodules	Ulceration	
M-56	1	50	50	-		
M-57	1	50	-	-		
M-333*	1	-	-	-	210	5x6 (220)
M-335*	1	-	50	90	190	7x8 (200)
M-336	1	-	90			
M-337*	1	-	-		200	8x9(10)
M-338*	2	-	-		131	6X8 (160)
M-334	2	90	90			

**=animals that developed ulcer, 1= strain number 1282 that is isolated from LCL patient; 2=strain P-16 isolated from DCL patient. Numbers in parentheses show days post challenge at which lesion size was measured. Infection site for all experimental monkeys is the tip of the nose.*

The disease progression after infection with *L. aethiopica* isolated from a DCL patient (Group 2) led to an ulcerated localized lesion in one of the two animals. Loss of hair and elevation of the skin was seen throughout the experimental period in the other animal (Table 1). The four uninfected control animals did not show any lesion development and remained healthy (Fig. 1b).



a



b

Figure 1. *African green monkeys. **a.** with **ulcerative lesion** at the tip of nose after 160 days of infection with $5x10^6$ stationary phase L. aethiopica promastigote. **b**. controls shaved tip of nose after 160 days of inoculation with sham of culture medium*

L. aethiopica infection was further confirmed by inoculating specimens taken from the infection site of the African green monkeys onto NNN media. Promastigotes were observed in all cultures of biopsies taken from all of the ulcerated lesions. No promastigotes were observed in cultures inoculated with biopsies taken from nodules or indurations of monkeys inoculated with *L. aethiopica* as well as from the healthy skin of the four control monkeys.

In vitro lymphocyte stimulation assay was conducted to examine the response of the animals to the different antigens. PBMC from all infected and control monkeys showed comparable proliferative responses when stimulated with live promastigotes, FPA or SLA (Fig.2).

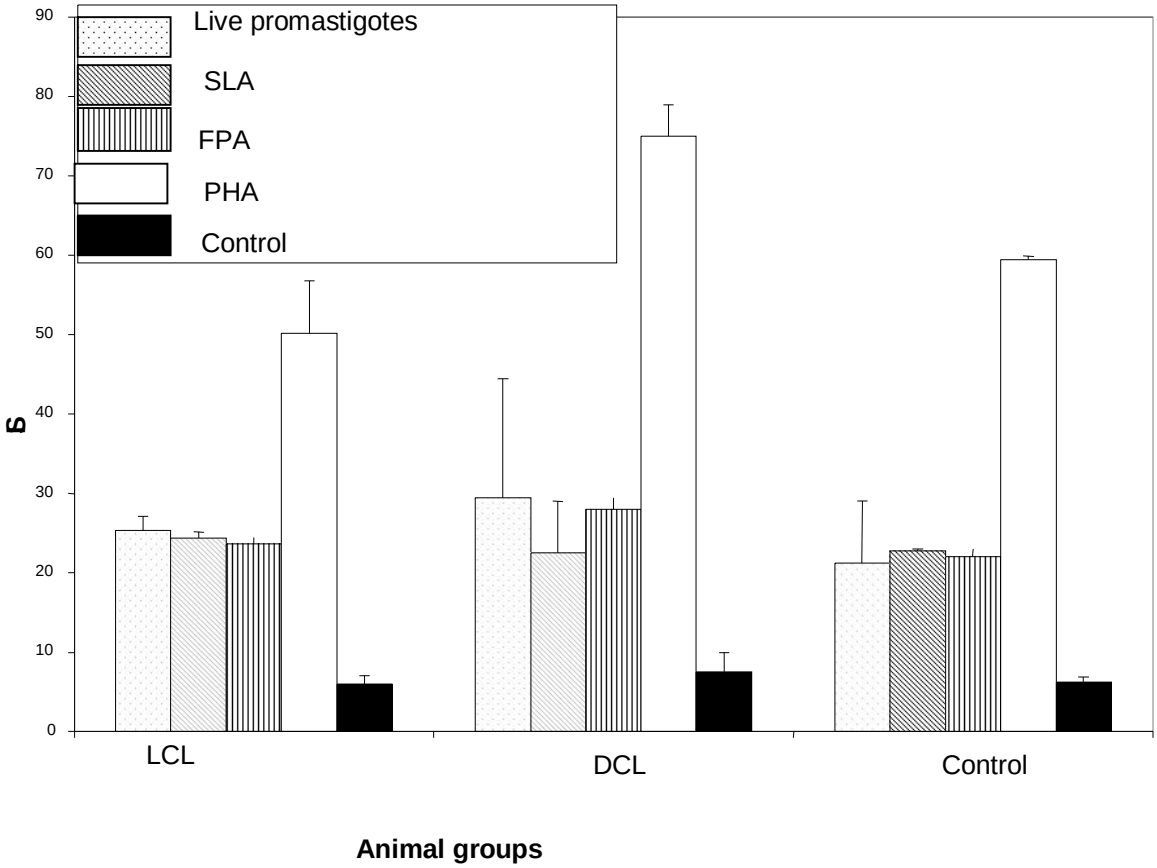


***Figure 2**. Proliferative response of Peripheral Blood Mononuclear Cells (PBMC) from African Green Monkeys. Infections of monkeys were done with 5x10^6 stationary phase promastigote: 8 Monkeys with isolates from **LCL** patient, 2 Monkeys with isolates from **DCL** patient and 4 Moneys with sham of culture medium. After 250days of infection, PBMC from each group of animals was stimulated with PHA for 3 days and, with live promastigotes, formalin killed promastigotes (**FPA**), and soluble antigens of L. aethiopica for 5 days. The bars represent the mean stimulation index for each group.*

IFN-g was also assayed in the culture supernatants of PBMC from infected monkeys (mean $_{live}$=135.13, mean $_{FPA}$=85.38, mean $_{SLA}$=87.75unit/ml) and control monkeys (mean $_{live}$=135, mean $_{FPA}$=87, mean $_{SLA}$=88.63 unit/ml) stimulated with live promastigotes, FPA and SLA.

However, there was no significant difference (P^{3}0.05) between infected and control animals. As was observed in the proliferative response, there were individual variations from animal to animal (Fig. 3)

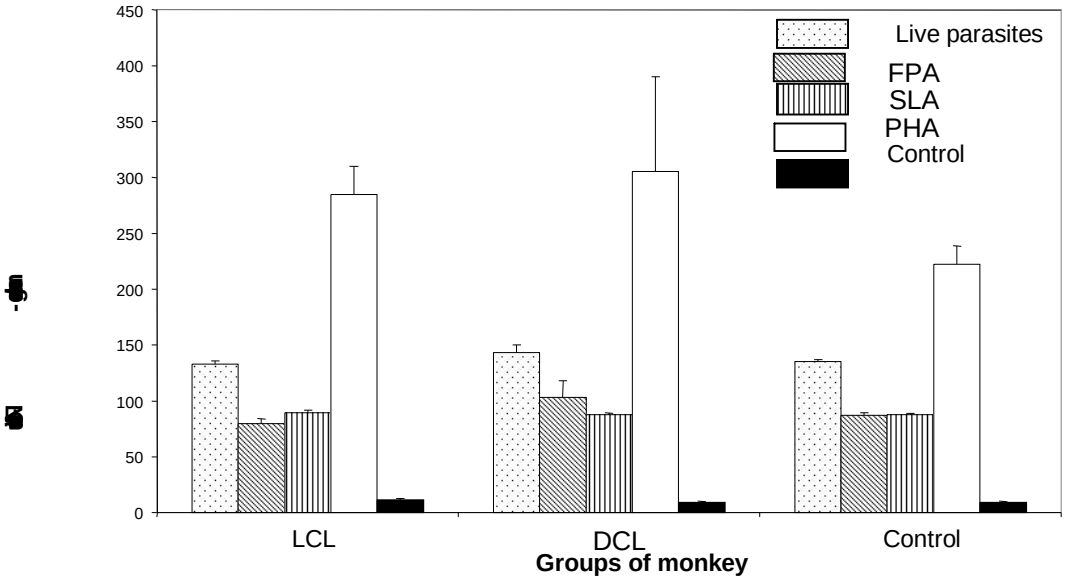


*Figure 3. Levels of IFN-gamma production in African green monkey. Infections of monkeys were done with 5x10^6 stationary phase promastigote: 8 Monkeys with isolated from **LCL** patient, 2 Monkeys with isolates from **DCL** patient and 4 Moneys with sham of culture medium. After 250days of infection, PBMC from each group of animals were stimulated with PHA for 3 days and, with live promastigotes, formalin killed promastigotes (**FPA**), and soluble antigens of L. aethiopica for 5 days. Culture supernatants were collected for IFN-g assay on the third day from PHA-stimulated wells and on the fifth day for antigen stimulated cultures. IFN-g levels were measured using a sandwich enzyme linked immunosorbent assay (ELISA). Each bar represents a mean IFN-g level in each group. **LCL** group, n=8. **DCL** group, n=2, **Control** group, n=4*

DISCUSSION

Our findings confirm the previously reported susceptibility of the African green monkey to infection with *L. aethiopica* (19). Localized ulcerative lesions with similar appearance as human LCL developed in both groups of monkeys (infected with strains isolated from LCL and DCL patients).

In three of the infected 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*. (19), it was reported that all of the six African Green monkeys developed cutaneous ulcers following inoculation of *L. aethiopica* promastigotes. However, in our study, some of the experimental animals failed to develop cutaneous ulcers. This variation in experimental infections of monkeys with *Leishmania* was also reported previously by Hommel *et al.* (25) and Githure *et al.* (14).

The pre-patent period of cutaneous leishmaniasis in the experimental animals is long. This appears to be similar to what is observed in humans (26). Following subcutaneous inoculation of parasites, infection remained sub-clinical for more than 45 days. The preclinical period in the experimental animals in this study varied from 45-150 days. Hailu *et al.* (19) reported a similar range of 35-161 days.

The pattern of antigen-specific proliferative responses of cells from infected and control animals to the LCL strain of live promastigotes, FPA and SLA, correspond to earlier studies in humans showing that mononuclear cells from uninfected healthy individuals proliferated in response to live *L. aethiopica* promastigotes (21).

Another study has also demonstrated that people without known previous exposure to *Leishmania* parasites had T-cells in peripheral blood reacting upon stimulation with leishmanial antigens and it was suggested that some of the T-cell epitopes may be shared by *Leishmania* parasites and other microorganisms in the environment (5).

The absence of significant difference between controls and infected animals in cytokine levels after leishmanial antigen stimulation has similarities to previous observations where cells from healthy *Leishmania* non-exposed individuals responded to whole promastigotes of *L. aethiopica* with IFN-g production (21,23, 27).

CONCLUSION

The clinicopathologic changes in the infected monkeys suggest that the African green monkey can be a useful laboratory model for studying the clinicopathologic changes induced by *L. aethiopica* infection, but only some of the monkeys developed cutaneous ulcers after inoculation with live *Leishmania* parasites. It is therefore necessary to further investigate the natural course of disease and immune response in the experimental animals using virulent *L. aethiopica* and specific leishmanial antigens.

The results of this study indicated that, as in humans, PBMC of African green monkeys respond to live, killed and soluble *L. aethiopica* antigen. The similarity in the *in vitro* responses in infected and control monkeys could be due to the non-specific nature of the antigens used.

RECOMMENDATIONS

The discrepancies observed in the *in vivo* and *in vitro* assays should be further investigated using new, more specific leishmanial antigens (28).

Assays such as measuring the expression level of various cytokine mRNAs in the PBMC of infected and control animals may provide more information on the significance of variation in cytokine levels in infected animals.

Therefore, it would be worthwhile to conduct further studies to establish the African green monkey as a model for examining the natural history of the disease and immune responses associated with *L. aethiopica* infection.

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