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EFFECTS OF ANTI-*LEISHMANIA* MONOCLONAL ANTIBODIES ON THE DEVELOPMENT OF *LEISHMANIA MAJOR* IN *PHLEBOTOMUS DUBOSCQI* (DIPTERA: PSYCHODIDAE)

C. Anjili, BSc, MSc, PhD, Principal Research Officer; B. Langat, BSc, Research Associate; P. Ngumbi, BSc, MSc, Senior Research Officer, Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya; P.A. Mbatia, BSc, MSc, PhD, Professor of Immunology, University of the North Phuthaditjhaba, South Africa; J. Githure, BSc, MSc, PhD, Head Malaria Unit, International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya; and W.K. Tonui, BSc, MSc, PhD, Senior Research Officer, Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya

Request for reprints to: Dr. W.K. Tonui, Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya

## EFFECTS OF ANTI-*LEISHMANIA* MONOCLONAL ANTIBODIES ON THE DEVELOPMENT OF *LEISHMANIA MAJOR* IN *PHLEBOTOMUS DUBOSCQI* (DIPTERA: PSYCHODIDAE)

C. ANJILI, B. LANGAT, P. NGUMBI, P.A. MBATI, J. GITHURE and W.K. TONUUI

### ABSTRACT

**Background.** Research in our laboratory has previously shown that immune-mediated transmission blocking may be applied to *Leishmania* infections and that the LPG molecule and anti-LPG monoclonal antibodies was found to be an excellent candidate against *L. major* infections.

**Objective:** To test the effect of monoclonal antibodies (MABs) raised against different species of *Leishmania* for their ability to inhibit development of *Leishmania major* in *Phlebotomus duboscqi* sand flies.

**Design:** A laboratory based study.

**Setting:** Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi.

**Results:** Sand fly dissections on days two, four and six post-feeding showed that monoclonal antibodies against *L. donovani* (Ld2cb and Ld3A3) were the most effective at inhibiting *L. major* development than those raised against *L. aethiopica*, *L. major* or *L. tropica*. Ld2cb inhibited *L. major* development by 82% in sand flies fed on  $1 \times 10^6$  amastigotes while Ld3A3 inhibited by 72%; 58% and 74% in those fed on  $1 \times 10^5$  amastigotes respectively. Monoclonal antibodies against *L. aethiopica* (Lae 3c6) inhibited *L. major* development by 28% and 40% for sand flies fed on  $1 \times 10^6$  and  $1 \times 10^5$  amastigotes respectively. Anti-*L. major* monoclonal antibody (Lm5A5) inhibited *L. major* development by 16% in sand flies fed on  $1 \times 10^6$  amastigotes and 25% in sand flies fed on  $1 \times 10^5$  amastigotes. Anti- *L. tropica* antibody (Lt2c8) inhibited *L. major* development in *P. duboscqi* fed on  $1 \times 10^6$  by 28 %and 33% in those fed on  $1 \times 10^5$  amastigotes. Most of the parasites seen in sand flies which fed on *L. donovani* mABs (Ld2cb and Ld3A3) were nectomonads and a few haptomonads. In all the control groups, parasite development followed the normal developmental stages up to the metacyclic stage. In sand fly groups fed on monoclonal antibodies raised against *L. aethiopica*, *L. major* or *L. tropica* there was limited parasite development inhibition, and the promastigotes developed and migrated forward in a normal pattern as observed in the controls.

**Conclusions:** These results suggests a possible role of humoral mechanisms in protection against leishmaniasis and potentially useful in reducing parasite development in the sand fly.

## INTRODUCTION

Cutaneous leishmaniasis caused by *Leishmania major* is endemic in many tropical and sub-tropical countries of the world. In Kenya, the disease is endemic in a single focus in Baringo District, within the Rift Valley Province (1). In this focus, it is transmitted by *Phlebotomus duboscqi* Neveu Lemaire (Diptera: *Psychodidae*) (2). This sand fly is known to breed and rest in rodent burrows (3) where it feeds on rodent species such as *Aethomys Kaiseri*, *Arvicanthis niloticus*, *Mastomys natalensis*, *Tatera robusta* and *Tarterillus emini* that have been incriminated as *L. major* reservoir hosts (4). *L. Major* causes zoonotic or rural cutaneous leishmaniasis, with lesions that are severely inflamed and ulcerated. Frequently, the lesions are multiple, and may become secondarily infected. Such lesions are slow to heal and usually leave large disfiguring scars (5).

There is currently no satisfactory strategy for the control and management of leishmaniasis. Chemotherapy using antimonial drugs is not only prolonged, expensive but also associated with numerous side effects (5). Vector control strategies are hampered by inaccessibility of vector breeding sites and insecticide resistance (6). A vaccine which may offer the best control strategy is not yet available. There is, therefore, need to look for new strategies. Such strategies may include interference of parasite development and transmission using immunological methods or transmission blocking immunity. This concept is based on the fact that sand flies, like any other group of vectors feeding on appropriately immunised hosts, would ingest antibodies, cytokines and other effector cells specific for target antigens within them (7). That such molecules affect vector functions has been reflected in lyses of gut epithelium (8); reduction in parasite loads and transmission (8, 9); and increase in sand fly fecundity and mortality rates (10). In another study, sand flies fed on BALB/c mice immunised with *L. major*-derived LPG showed that parasite development was inhibited at the log phase or procyclic stage of the parasite (9). There was also a marked reduction in the numbers of metacyclic promastigotes developing, leading to reduced transmission of *L. major* to naive BALB/c mice (11). More recently, anti-LPG MABS were shown to be effective in reducing sand fly

infections (12). In the present study, we sought to investigate the ability of *L. major* specific monoclonal antibodies and antibodies from other *Leishmania* species to inhibit development of *L. major* in its natural vector *P. duboscqi*.

## MATERIALS AND METHODS

*Parasite isolation, cultivation and purification of amastigotes:* (Strain IDUB/KE/83 =NLB-144) previously isolated from a wild female *P. duboscqi* caught in Baringo District, Kenya (2) and since been serially maintained in BALB/c mice was used. An aspirate from the footpad of an infected mouse was cultured to stationary phase in Schneider's *Drosophila* medium supplemented with 20% heat-inactivated foetal bovine serum, 250:1/ml penicillin, 2500:g/ml streptomycin and 500:g/ml 5-fluorocytosine arabinoside (13,14). Out of this culture, metacyclic promastigotes were used to infect 30, two week old BALB/c mice on the left hind footpads. Each mouse received  $1 \times 10^6$  promastigotes in 60:1 phosphate-buffered saline (PBS). Mice were left for five weeks for lesions to develop. Mice with swollen footpads were selected and sacrificed by cervical dislocation. Infected footpads were sterilised with 70% ethanol, left to dry and then excised under sterile conditions. The swollen tissue was trimmed and transferred into a Tenbroeck tissue grinder containing PBS with 25:1/ml penicillin, 250:g/ml streptomycin and 500:g/ml 5-fluorocytosine arabinoside. Footpad tissue was ground completely and left to stand in an ice bucket for 15 minutes. The supernatant homogenate was centrifugally washed thrice at 3000 revolutions per minute (rpm) for 10 minutes at 4° C.

Amastigotes that sedimented were re-suspended in 100:1 of PBS and counted against chicken red blood cells using a haemocytometer. Viability of amastigotes was then tested by using *in-vitro* transformation method.

*Preparation of L. major-infected blood:* Each amastigote preparation was mixed with 0.5ml of defibrinated rabbit blood. In the first experiment,  $1 \times 10^6$  in 20:1 PBS amastigotes were mixed with blood and a 1:10 dilution of the monoclonal antibody was added. The mixture was then vortex-mixed before use. Control blood contained a similar number of amastigotes but no antibody was added. In the second experiment,  $1 \times 10^5$  amastigotes in 20 $\mu$ l

PBS were used as described above for the  $1 \times 10^6$  amastigotes. Monoclonal antibodies that were separately used were Lm5A5 (*L. major*), Ld2cb (*L. donovani*), Ld3A3 (*L. donovani*), Lt2c8 (*L. tropica*), Lae3c6 and Lae369 (*L. aethiopia*). All these monoclonal antibodies (mABs) were kindly donated by Professor C. Jaffe. Before use, the lyophilised monoclonal antibodies were reconstituted in 200:1 double distilled water and left to stand at room temperature for one hour. They were then centrifugally washed at 2500 rpm for five minutes before they were diluted to 1:10 for insect feeding. A high 1:10 dilution of MABs was chosen in order to facilitate dilution where inhibition of *L. major* was detected.

**Sand fly feeding:** Sand flies used during the experiments were obtained from KEMRI insectary and reared as previously described (15). The membrane through which *P. duboscqi* fed was prepared by shaving skins of Swiss Albino mice. These were then used to cover glass feeders into which either blood containing amastigotes and antibodies or control infected blood was added. Five minutes before sand flies were allowed to feed, the mouse skin-covered glass membrane feeders were attached to a circulating water bath maintained at 37°C. Two groups of 120, three-day old unfed female *P. duboscqi* were aspirated into 30ml plastic feeding cups fitted with fabric-screen lids (12 holes per linear cm). The outer surface of the screen lids of feeding cup was then pressed beneath the blood-filled membrane feeder containing  $1 \times 10^6$  amastigotes and antibodies and the other one beneath the membrane feeder containing blood with amastigotes only (control). This was done similarly for all monoclonal antibodies and for the test involving  $1 \times 10^5$  amastigotes. Sand flies were left to feed *ad libitum* for one hour uninterrupted at room temperature. After feeding, sand flies were transferred into an insectary maintained at 25°C and 90% relative humidity (RH) for 24 hours. Engorged sand flies were given a drop of sterile sugar syrup as a carbohydrate supplement until they were dissected.

In order to determine the presence or absence of promastigotes, at least five engorged sand flies were dissected on days two, four and six post-feeding and examined for promastigotes (9, 11, 12, 16). Individual females were dissected in a drop of 0.15M NaCl, and guts examined in wet preparations for parasites. Where parasites were seen, locations

of parasites were noted. Later, in order to ascertain parasite forms and enumerate the parasites in the sand fly, slides used in dissections were air-dried, fixed in absolute methanol, and then stained with Giemsa. These were then examined for the presence or absence of nectomonads, haptomonads, paramastigotes, and metacyclic promastigotes and recorded. The percentage of the ratio between the number of un-infected and the total sand flies that fed was taken as the inhibition rate while the total parasitaemia was an addition of all parasite forms observed and graded (17) (Table 1).

**Table 1**

*Grading system used to enumerate L. major parasites*

Grade	Average parasite density
6+	>100 promastigotes/field
5+	10-100 promastigotes/field
4+	1-10 promastigotes/field
3+	1-10 Promastigotes/10 fields
2+	1-10 promastigotes/100 fields
1+	1-10 promastigotes/1000 fields
0	0 promastigotes/1000 fields

Lm = *L. major*, Ld = *L. donovani*, Lt = *L. tropica*,  
Lae = *L. aethiopia*

## RESULTS

*Dissections of P. duboscqi fed on different monoclonal antibodies mixed with either  $1 \times 10^6$  or  $1 \times 10^5$  concentration of amastigotes in the blood:*

Monoclonal antibodies raised against *L. donovani*, particularly Ld2cb and Ld3A3 conferred the highest level of inhibition of *L. major* development in *P. duboscqi*. When mixed with  $1 \times 10^6$  *L. major* amastigotes in blood, the inhibition rate for Ld2cb MAB was 82% compared to 27% in controls, and when mixed with  $1 \times 10^5$  amastigotes, inhibition was 74% and 13% in the control. For Ld3A3 MAB mixed with  $1 \times 10^6$  *L. major* amastigotes in blood, the inhibition rate was 72% compared to 30% in controls, and when mixed with  $1 \times 10^5$  amastigotes, inhibition was 58% and 13% in the control. A chi-square ( $\chi^2$ ) analysis of the number of sand flies with *L. major* infection showed that inhibition of parasite development due to *L. donovani* monoclonal antibodies Ld2cb or Ld3A3 was significantly higher than in their controls ( $\chi^2 = 5.8$ , degrees of freedom [df]=1,  $P < 0.5$ ). Inhibition of parasite development

by *L. donovani* monoclonals (Ld2cb and Ld3A3) was significantly higher ( $P < 0.5$ ) than that caused by *L. major* (Lm5A5), *L. aethiopic*a (Lae 3c6) or *L. tropica* (Lt2c8).

Different developmental stages of *L. major* were seen in sand flies fed on different monoclonal antibodies. Most of the parasites seen in the sand flies which fed on *L. donovani* mABs (Ld2cb and Ld3A3) were nectomonads and a few haptomonads on days two, four and six post-infective blood meal. Very few sand flies were found to be infected with the metacyclic forms. In all the control groups, parasite development followed the normal developmental stages up to the metacyclic stage. In sand fly groups fed on monoclonal antibodies against *L. tropica*, *L. aethiopic*a, and *L. major*, there was limited parasite development inhibition and the promastigotes followed a normal pattern as that observed for the controls. Results of the dissections, levels of inhibition and parasitaemia levels for various monoclonal antibodies are as shown in Figures 1 and 2; Table 2.

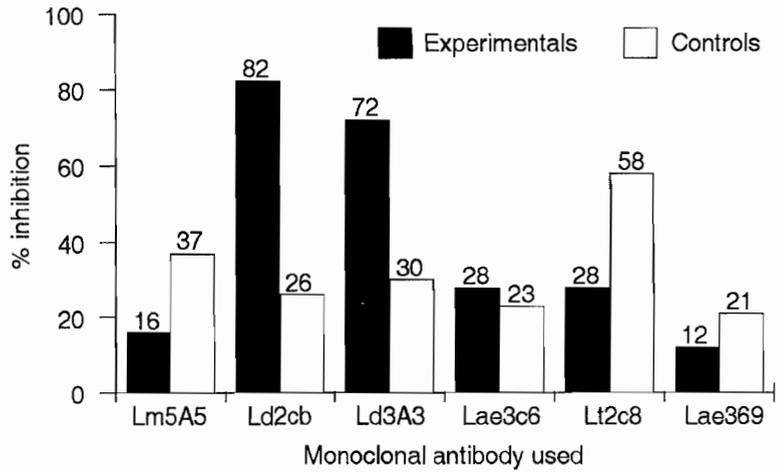
*Parasite loads in sand flies dissected after six days:* Dissections of sand flies which had fed on *L. donovani* monoclonal antibodies Ld2cb and Ld3A3 showed low parasitaemia levels of 2+ or 3+ respectively compared to their controls ( $P < 0.5$ ). Sand flies which had fed on *L. major* (Lm5A5), *L. aethiopic*a (Lae3cb or Lae369) and *L. tropica* (Lt2c8) monoclonal antibodies showed an average grade of 5+, 4+ and 4+ parasitaemia levels respectively. The parasitaemia levels in experimental sand flies and their controls are summarised in Table 2.

**DISCUSSION**

As early as 1938, Adler suggested that the ability of *Leishmania* parasites to infect *P. papatasi* is determined by the concentration of serum in the blood meal. In that study, inclusion of 10% serum in cultured parasites led to an increase in the number of *L. major* promastigotes whereas 5%

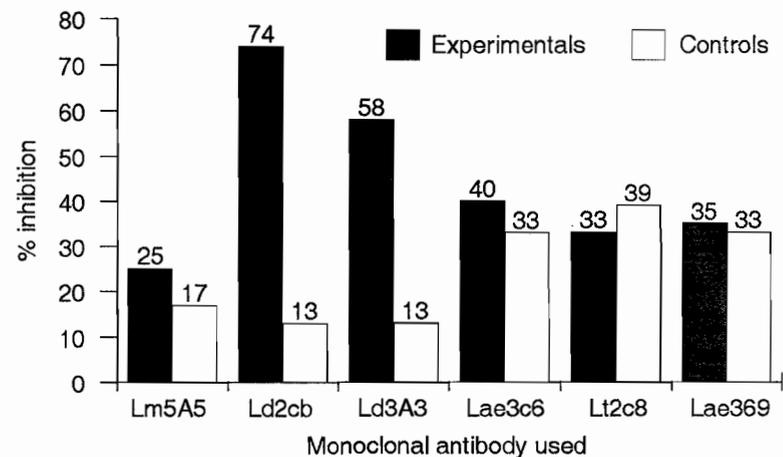
**Figure 1**

*A summary of results of dissections of P. dubosqi fed on different monoclonal mixed with 1 x 10<sup>6</sup> L. major amastigotes in blood*



**Figure 2**

*A summary of results of dissections of P. dubosqi fed on different monoclonal mixed with 1 x 10<sup>5</sup> L. major amastigotes in blood*



Lm = *L. major*, Ld = *L. donovani*, Lt = *L. tropica*,  
Lae = *L. aethiopic*a

serum led to a reduction (18). Albumin, which is the major protein constituent of serum is rapidly digested following a blood meal, and is barely detectable in *P. papatasi* after 48 hours (7), but the globulin fractions IgG and IgG persist for several days (10). Complement (C3) degradation is intermediate in both *Aedes albopictus* (Diptera: Culicidae) and *P. papatasi*. *Phlebotomus dubosqi* and *P. papatasi* are phylogenetically closely related (both belong to subgenus *Phlebotomus*), are vectors for *L. major*, and share similar pharmacological activities in their salivary glands (19). The rate of blood meals digestion is the same (5-6 days). It is therefore,

Table 2

Infection scores and parasite forms in *P. duboscqi* sand flies previously fed on different monoclonal antibodies mixed with  $1 \times 10^6$  or  $1 \times 10^5$  *L. major* amastigotes in blood

mAB used	mAB mixed with $1 \times 10^6$ <i>L. major</i> amastigotes	mAB mixed with $1 \times 10^5$ <i>L. major</i> amastigotes	Commonest parasite form seen in the sandfly midgut and stomodeal valves
Lm5A5 control	5+	4+	Mainly metacyclics
	5+	5+	Mainly metacyclics
Ld2cb control	2+	3+	Nectomonads, few haptomonads
	4+	5+	Mainly metacyclics
Ld3A3 control	2+	3+	Nectomonads, few haptomonads
	4+	5+	Mainly metacyclics
Lae3c6 control	4+	4+	Mainly metacyclics
	5+	4+	Mainly metacyclics
Lae369 control	4+	4+	Mainly metacyclics
	4+	5+	Mainly metacyclics
Lt2c8 control	5+	4+	Mainly metacyclics
	5+	5+	Mainly metacyclics

Lm = *L. major*, Ld = *L. donovani*, Lt = *L. tropica*, Lae = *L. aethiopica*, mAb = monoclonal antibody

possible that the rate of antibody degradation is also the same.

Monoclonal antibodies are produced against membrane-enriched preparations of the *Leishmania* parasite (20, 21) and have been found to be useful during characterisation of antigens associated with stage- and specie-specific determinants. In this study, monoclonal antibodies against *L. donovani* were shown to be better at inhibiting *L. major* development in *P. duboscqi* as shown by the level of inhibition exceeding 70%, and the low parasitaemia levels in the gut. Low numbers of metacyclic promastigotes formation in sand flies was recently shown to be responsible for reduced transmission of *L. major* to naive BALB/c mice (9 – 12) and induction of resistance to the disease (22). However, limited inhibition was seen when anti-*L. major*, *L. tropica* and *L. aethiopica* monoclonal antibodies were used. The inhibition of *L. major* development by *L. donovani* monoclonal antibodies may be explained in terms of heterologous protection that has also been reported in BALB/c mice (23) and in vervet monkey (*Cercopithecus aethiops*) models (24). A high level of *L. major* inhibitions would have been expected with anti-*L. tropica* monoclonal antibodies, as a result of heterologous protection that has been reported in man (*L. tropica* protects against *L. major* but not vice

versa) (25), but instead, a low level of inhibition was observed.

While the involvement of cell-mediated immune protective mechanisms has been demonstrated in leishmaniasis, the role of humoral mechanisms in protection is not clearly understood (26). However, *in vitro* effects of anti-leishmanial antibodies on promastigotes from super-infected mice or from non-immune humans have been demonstrated (27, 28). MABS directed against *Leishmania* promastigotes have also been shown to protect BALB/c mice against challenge with promastigotes (29, 30) or with amastigotes (31). Antibodies and effector cells of the immune system ingested with a blood meal, may affect vector functions in a number of ways. While it is unlikely that ingested leucocytes remain active for a long time, they may release cytokines and other, possibly toxic substances, especially if they are already activated by an infection in the vertebrate (32). It is not known, however, at what stage antibodies act on the parasite in the sand fly but since antibodies ingested in a blood meal persist intact in sand flies for several days, it may be that, during this time, they are able to affect amastigote transformation at day's 1-2 post-infective blood meals. Persisting antibodies may then interfere with nectomonads, haptomonads, and

paramastigotes and thus reduce their transformation. Results from this study, suggests a possible role of humoral mechanisms in protection against leishmaniasis and which may be potentially useful in reducing parasite development in the sand fly.

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