Transmission Blocking Vaccine Studies in Leishmaniasis

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ABSTRACT

New strategies for control of leishmaniases are required as chemotherapy using antimonial drugs is prolonged, expensive, associated with side effects and relapses. Vector control has limitations and vaccination, which could be the best approach, is unavailable. This report describe a series of experiments that have been performed at the Kenya Medical Research Institute (KEMRI) that employ the concept of transmission blocking immunity as a new approach towards the control of leishmaniasis. BALB/c mice were immunized adequately with *Leishmania major*-derived whole parasite (WPA), recombinant 63 kilo Dalton glycoprotein (rgp63) and lipophosphoglycan (LPG) antigens. Laboratory reared *Phlebotomus duboscqi* sand flies, a known vector for *L. major* were later allowed to feed on immunized mice, interrupted and allowed to continue feeding on infected mice for equal amount of time until they became fully engorged. Some sand flies were also membrane fed on blood meals containing monoclonal antibodies (mAbs) raised against *L. donovani; L. major, L. aethiopica and L. tropica* mixed with $1x10^6$ *L. major* amastigotes. These sand flies maintained at 25 C± 90 R.H. in an insectary was later dissected on days 2, 4 and 6 days post feeding and examined for procyclics, nectomonads, haptomonads and metacyclic promastigote forms of *Leishmania*. On the 7th day flies fed on immunized mice were used to infect naive mice. Some of the sand flies dissected on days 2,4 and 6 were observed using the light and the transmission electron microscopy for any changes in their gut morphology. MAbs raised against *L. donovani* were more effective in inhibiting *L. major* development in the sand fly than those raised against *L. major, L. aethiopica or L. tropica*. The dominant parasite form in sand flies which fed on LPG-immunized mice was the procyclic form, whereas those fed on rgp63, flagella and *P. duboscqi* gut cocktail-immunized mice or mAbs against *L donovani* showed more nectomonad and haptomonads but very few of the infective metacyclic forms ($p<0.05$). Control sand flies, which fed on unimmunized mice, displayed a normal pattern of parasite development up to the metacyclic stage. BALB/c mice infected using sand flies, which had fed on WPA, or rgp63-immunized mice showed disease exacerbation as the infection progressed. However, mice infected using sand flies, which had fed on LPG-immunized mice showed the least lesion sizes as compared to that of WPA, rgp63 and control groups. Studies showed that two possible mechanisms through which immune sera from immunized mice may cause inhibition of parasite development is by exflagellation of nectomonad forms and degeneration of the sand fly mid gut epithelium as revealed by light and electron microscopy studies respectively. These studies have shown that immune-mediated transmission blocking may be applied to *Leishmania* infections and that LPG is a promising transmission blocking vaccine candidate in leishmaniasis.

Key words: Leishmaniasis, transmission blocking vaccine

INTRODUCTION

Old world cutaneous leishmaniasis caused by the protozoan *Leishmania major* is an important health problem in many countries of North Africa, Eastern Africa, Eastern Mediterranean region and South Western Asia (Desjeux, 1996). Chemotherapy using antimonials is the most widely used strategy for the control of leishmaniasis. This strategy, however, has its limitations: -it is prolonged, expensive, and associated with relapses and numerous side effects. Attempts to control the sand fly vector or the development of a vaccine have not been successful. We sought to employ the concept of transmission blocking immunity as a new approach towards the control of leishmaniasis. In this approach, we investigated the effects of monoclonal antibodies (mAbs) raised against *L. donovani, L. major, L. aethiopica* or *L. tropica* and sera from BALB/c mice immunized with *L. major*-derived antigens on the development of *L. major* in it's natural vector *Phlebotomus duboscqi* Neveu-Lemaire (Diptera: Psychodidae). We also investigated the ability of sand flies that had previously fed on immunized mice to transmit the parasite to naive mice and the possible mode of action by these candidate transmission-blocking vaccines. We show here that immune-mediated transmission blocking may be applied to *Leishmania* infections and that LPG is a promising transmission blocking vaccine candidate in leishmaniasis.

MATERIALS AND METHODS

Preparation of L. major-derived antigens:

Leishmania major (Strain IDUB/KE/83 =NLB-144) previously isolated from a wild female P. *duboscqi* caught in Baringo District, Kenya (Beach *et al.*, 1984), and which has since been serially maintained in BALB/c mice was used in the present study. An aspirate from the footpad of an infected mouse was cultured to stationary phase in Schneider's *Drosophila* medium supplemented with 20% heat-inactivated fetal bovine serum, 250 µl/ml penicillin, 2500 µg/ml streptomycin and 500 µg/ml 5-fluorocytosine arabinoside (Hendricks & Wright, 1979; Kimber *et al.*, 1981). Promastigotes were mass cultivated to stationary phase concentrations of 1 x 10^{12}/ml. Parasites were washed by centrifugation at 2,500 rpm for 20 minutes, and heat-killed at 60EC in a water bath for 10 minutes. Killed parasites were then sonicated, protein estimated using Biorad protein assay and used as a crude whole parasite antigen (WPA). Some of the primary culture parasites (1 x 10^6/40il) in phosphate-buffered saline (PBS) were used to infect BALB/c mice footpads that were later used to infect sand flies.

The *L. major*-derived rgp63 antigen was a gift from Dr. Joseph Olobo of the Institute of Primate Research (IPR), Karen, Nairobi. The antigen was synthesized in *Escherichia coli* as previously described (Button *et al.*, 1991). The purity of rgp63 was demonstrated by silver staining following SDS-PAGE (Olobo *et al.*, 1995). The *L. major*-derived LPG was extracted, purified and quantitated by phosphate analysis as previously described (Orlandi and Turco, 1987).

Immunization and collection of sera

Five groups each constituting of six, 6-8 weeks old BALB/c mice, matched by sex were immunized intravenously through the tail vein. Each group was immunized with either rgp63, LPG, a cocktail of rgp63 and LPG or crude WPA at 2.5, 10, 12.5 and 100ig/ml of the antigen, to an accumulated concentration of 7.5, 40, 50 and 400ig respectively per group. The control group was injected with 100il sterile normal saline to a total volume of 400il. Each animal in a group was boosted with the respective antigen dose at seven days interval for four weeks except for the rgp63 group which were boosted with a similar dose at 2 week interval three times (Tonui *et al.*, 2001a). To test the success of immunizations, sera from the animals were collected one week after the final boost and antibody levels were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described (Tonui *et al.*, 2001a). All the animals in the study were then maintained under standard hygienic conditions at the Kenya Medical Research Institute (KEMRI).

Isolation of amastigotes and mAbs used

This was done as previously described by Anjili and colleagues (2002). Briefly, mice with swollen footpads were selected and sacrificed by cervical dislocation. Infected footpads were sterilized 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-µl/ml penicillin, 250 µg/ml streptomycin and 500 µg/ml 5-fluorocytosine arabinoside. Footpad tissue was ground completely and left to stand in an ice bucket for 15 min. The supernatant homogenate was by centrifugation washed thrice at 3000 revolutions per min (rpm) for 10 min at 4 °C. Amastigotes that sedimented were re-suspended in 100 µl of PBS and counted against chicken red blood cells using a heamocytometer.

Each amastigote preparation was mixed with 0.5 ml of defibrinated rabbit blood. In the first experiment, 1x10^6 amastigotes in 20 µl PBS 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, $1x10^5$ amastigotes in 20 µl PBS were used as described above for the $1x10^6$ amastigotes.

The monoclonal antibodies (mAbs) that were used separately were Lm5A5 (*L. major*), Ld2cb (*L. donovani*), Ld3A3 (*L. donovani*) Lt2c8 (*L. tropica*), Lae3c6 (*L. aethiopica*), and Lae369 (*L. aethiopica*). Professor C. Jaffe from the Kuvin Centre in Israel kindly supplied all these mAbs. Before use, the lyophilized mAbs were reconstituted in 200 µl double distilled water and left to stand at room temperature for 1 h. They were washed thrice by centrifugation at 2500 rpm for 5 min before they were diluted to 1:10 for sand fly feeding. A 1:10 dilution of mAbs was chosen in order to facilitate dilution where inhibition of *L. major* was detected.

Sand fly feeding

The *P. duboscqi* sand flies used in the experiments were obtained from the Kenya Medical Research Institute's insectary, and reared using the methods of Beach and colleagues (1986). Both the direct (Tonui *et al.*, 2001a) and membrane feeding (Anjili *et al.*, 2002) methods were used during sand fly feeding experiments. During the direct feeding method, laboratory-reared *P. duboscqi* females (2-5 days old) were put into feeding vials and allowed to feed on footpads of anaesthetized BALB/c mice immunized with the respective antigen. At least 250 fed flies were used for each group. Just before they took in enough blood, the feeding was stopped (interrupted feeding). These flies were then transferred to a footpad lesion of an *L. major* infected BALB/c mouse and allowed to continue feeding. This procedure was repeated several times until they were fully engorged to ensure that they had taken almost the same amounts of blood from the infected mouse and the immunized animals.

During the membrane-feeding method, skin membranes through which the flies fed were prepared by shaving Swiss Albino mice. These were then used to cover glass feeders into which either blood containing amastigotes and antibodies or the control infected blood was added. Five minutes before the sand flies were allowed to feed, the mouse skin-covered glass feeders were attached to a circulating water bath maintained at 37 °C. Two groups of 120, 3-day- old unfed female *P. duboscqi* were aspirated into 30 ml plastic feeding cups fitted with fabric-screen lids (12 holes per linear cm). The outer surface of the screen lids of the feeding cups was then pressed beneath the membrane feeders containing blood, $1x10^6$ amastigotes and antibodies or containing only blood and amastigotes (controls). This was similarly done for all mAbs and for the test involving $1x10^5$ amastigotes. The sand flies were left to feed *ad libitum* for 1 h uninterruptedly at room temperature. After feeding for any method used, only the fed sand flies were transferred to an insectary maintained at 25±1 °C and 90 % relative humidity (RH) for 24 h. Sand flies were given a drop of sterile sugar syrup as a carbohydrate supplement until they were dissected.

At least five engorged sand flies were dissected on days 2, 4 and 6 after the blood meal and examined for promastigotes using the methods of Johnson and colleagues (1963). Individual females were dissected in a drop of 0.15 M NaCl on a glass slide, and their guts examined in wet preparations for parasites. When parasites were seen, their locations were noted. At a later stage, in order to ascertain parasite forms and enumerate the parasites in each sand fly dissected, the slides used were air-dried, fixed in absolute methanol and stained with Giemsa. The slides were then examined for the presence or absence of nectomonads, haptomonads, paramastigotes and metacyclic promastigotes. The percentage of the ratio between the number of uninfected 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 using the methods of Chulay & Brycesson (1983). Briefly, 0 represented 0 parasites/1000 fields; 1+, 1-10 parasites/1000 fields; 2+, 1-10 parasites/100 fields; 3+, 1-100 parasites/10fields; 4+, 1-10 parasites/field; 5+, 10-100 parasites/field and 6+, over 100 parasites/field.

Histopathology

To obtain a histological view of the effect of immune sera on parasite development in the sand fly gut a transmission electron microscope was used (Tonui *et al.*, 2001a). Briefly, sand flies from experimental and control groups were dissected in 0.05M sodium cacodylate (Nacalai Inc(R), Japan) and guts fixed in 2.5% glutaraldehyde (Merck(R)) buffered to Ph 7.4 with sodium cacodylate containing 5% sucrose (Sigma(R)) and refrigerated at 4EC. Tissues were subsequently post-fixed in 1% osmium tetroxide (Ted Pella, Inc(R), USA), and then processed using standard electron microscopy techniques (Hayat *et al.*, 1986).

Ultra thin sections were cut using a diamond knife mounted on a Reichert-Jung micro tome(R) (Austria) and mounted on uncoated 300 mesh grids, stained in 2% uranyl acetate for 30 minutes and in lead citrate (Reynolds, 1963) for 10 minutes and examined on a JEOL JEM-100S transmission electron microscope.

Statistical analysis

Chi square (χ^2) comparison test was used to compare the % of infected sand flies fed on sera or mAbs relative to controls. ANOVA was used to compare the means between the various groups.

RESULTS

Success of immunizations with the Leishmania-derived antigens

BALB/c mice immunized with *L. major*-derived surface antigens rgp63, LPG, cocktail of rgp63 and LPG, or whole parasite antigens had significantly higher antibody levels based on optical density (O.D) values relative to control mice that were inoculated with normal saline, indicating a successful immunization ($p<0.05$). Mice, which showed high antibody levels from each group, were used during sand fly feeding experiments (Tonui *et al.*, 2001a).

MAbs against L. donovani were more effective in inhibiting L. major development in the sand fly than those raised against L. major, L. aethiopica or L. tropica

A summary of results showing the effectiveness of mAbs on *L. major* parasites is given in Table 1. 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 $1x10^6$ *L. major* amastigotes in blood, the inhibition rate for Ld2cb mAbs was 82% compared to 26% in the controls, and when mixed with $1x10^5$ amastigotes, inhibition was 74% and 13% in the controls. When Ld3A3 mAbs were mixed with $1x10^6$ *L. major* amastigotes in blood, the inhibition rate was 72%, compared to 30% in the controls, and when mixed with $1x10^5$ amastigotes, inhibition was 58% and 13% in the controls. A chi-square (χ^2) analysis of the number of sand flies with *L. major* infection showed that inhibition of parasite development due to *L. donovani* mAbs Ld2cb or Ld3A3 was significantly higher than in their controls (χ^2) = 5.8, degrees of freedom [df]=1, P<0.5). Overall, inhibition of parasite development by *L. donovani* mAbs (Ld 2cb and Ld3A3) was significantly higher than that caused by *L. major* (Lm5A5), *L. aethiopica* (Lae 3c6) *or L. tropica* (Lt2c8) ($p<0.5$).

Procyclic promastigotes as the dominant parasite form in sand flies fed on LPG-immunized mice

Different developmental stages of *L. major* were observed in sand flies fed on different mAbs or blood meals from immunized animals. Most of the parasites seen in the sand flies that fed on *L. donovani* mAbs (Ld 2cb and Ld3A3) were nectomonads and a few haptomonads on days 2,4 and 6 post-infective blood meal. Very few sand flies were 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 mAbs against *L. tropica, L. aethiopica*, and *L. major*, there was limited parasite development inhibition and the promastigotes followed a normal pattern as that observed for the controls.

The dominant parasite form in sand flies which fed on LPG-immunized mice was the procyclic form, whereas those fed on rgp63, flagella and *P. duboscqi* gut cocktail-immunized mice or mAbs against *L. donovani* showed more nectomonad and haptomonads but very few of the infective metacyclic forms ($p<0.05$). Control sand flies fed on non-immunized or PBS-immunized mice displayed a normal pattern of parasite development up to the metacyclic stage (Table1).

Parasite loads in sand flies dissected after 6 days

The parasitaemia levels in sand flies fed on immunized mice or mAbs is summarized in Table 1. Briefly, the results of sand fly dissections indicated that flies which had fed on *L. donovani* MAbs, Ld 2cb 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. aethiopica* (Lae 3cb or Lae369) and *L. tropica* (Lt2c8) mAbs showed an average grade of 5+, 4+ and 4+ parasitaemia levels respectively.

Sand flies, which had previously fed on WPA or a cocktail of rgp63 plus LPG-immunized mice or L. donovani mAbs, showed the lowest infectivity rates compared to controls ($p>0.05$) (Table 1).

Table 1: The percentage inhibitory rates, parasite forms and densities seen in sand flies fed on immunized BALB/c mice or mAbs

Immunizing antigen/mAbs	% Inhibition	Commonest parasite form	Parasite densities
L. donovani mAbs	82 .00	Nectomonads and haptomonads	2+
L. aethiopica mAbs	28.00	Haptomonads and Metacyclics	4+
L. major mAbs	16. 00	Mainly Metacyclics	5+
L. tropica MAbs	28. 00	Mainly Metacyclics	4+
Control (amastigotes only)	21. 00	Mainly Metacyclics	4+
LPG (10µg)	56.70	Procyclics and nectomonads	2+
rgp63 (2.5µg)	60.00	Nectomonads	3+
Cocktail (10µg LPG +2.5µg rgp63)	62.50	Few procyclic and many metacyclics	2+
WPA (100µg)	75.00	Nectomonads, Haptomonads & Metacyclics	2+
Saline Controls (100µl)	40.00	Mainly metacyclics	5+

Disease exacerbation by WPA and rgp63

This section of the report has already been published (Tonui *et al.*, 2001b). Briefly, BALB/c mice infected using sand flies, which had fed on WPA, or rgp63-immunized mice showed disease exacerbation as the infection progressed. However, mice infected using sand flies, which had fed on LPG-immunized mice showed the least lesion sizes as compared to that of WPA, rgp63 and control groups.

Possible mechanism of action by sera from immunized mice

This section of the report has also been published ((Tonui *et al.*, 2001a). Briefly, studies showed that exflagellation of nectomonad forms and degeneration of the sand fly mid gut epithelium was revealed by light and electron microscopy. The severity of damage to the guts was greater on day 2 and showed regeneration by day 4 post-feeding. By the 6th day post-feeding the guts showed complete regeneration. Such pathology suggests interference with the attachment of the *Leishmania* parasite to the mid gut.

DISCUSSION

These studies demonstrate that sera from BALB/c mice immunized with rgp63, LPG, WPA or a cocktail of LPG plus rgp63 or *L. donovani* mAbs have inhibitory effect upon the development of *L. major* parasites in its natural vector. We previously observed that serum antibodies against a combination of *P. duboscqi* gut and *L. major* flagella antigens were also able to significantly inhibit development of infective metacyclic promastigotes in P. *duboscqi* sand flies (Mbati *et al.*, 2000). The parasite infectivity rates in sand flies were dependent on the immunizing antigen. The low infection rates observed in sand flies fed on WPA and cocktail-immunized mice may be explained in part by the fact that antibodies or effector cells in immune sera against these antigens were reacting to many epitopes of the parasite and not just the surface antigens alone. The inhibition of *L. major* development by anti-*L. donovani* mAbs may be explained in terms of heterologous protection that has been reported in vervet monkeys (Gicheru *et al.*, 1997) and in BALB/c mice (Rachamin and Jaffe, 1993).

At least five developmental forms can be recognized during development in the genus *Leishmania*, namely, procyclic, nectomonad, haptomonads, paramastigotes and metacyclic promastigotes (Bates, 1994). The morphological changes are accompanied by regulated changes in the expression of two major surface molecules of *Leishmania* namely, lipophosphoglycan (LPG) and the 63 kilo Dalton glycoprotein (gp63) (Davies *et al.*, 1990, Pimenta *et al.*, 1992). Sera against these two surface molecules may explain the dominance of the nectomonad forms observed in sand flies which had previously fed on rgp63 or LPG-immunized mice. The LPG molecule has also been implicated in the attachment of procyclic promastigotes on the abdominal mid gut upon release from the peritrophic membrane (Pimenta *et al.*, 1992). Observation of procyclic promastigotes in sand flies fed on LPG immunized mice suggests that sera against LPG may have inhibited the parasite from binding to the mid gut thus leading to arrested development of the parasite beyond this stage. Based on this observation of the procyclic, the dominance of the nectomonad forms, low infectivity rates in sand flies fed on LPG- immunized mice we concluded that LPG stands out to be a better transmission blocking vaccine molecule than the rgp63 or WPA.

In the present study it was observed that immunization of mice with *L. major*-derived antigens leads to a decrease in the number of infective parasites that can develop, and hence be transmitted by the sand fly vector. BALB/c mice infected using sand flies that had previously fed on LPG-immunized blood meals developed smaller lesion sizes compared to those infected using sand flies that had previously fed on rgp63, cocktail or WPA-immunized blood meals (Tonui *et al.*, 2001b). It is possible that smaller lesions developed in mice infected using sand flies which had previously fed on LPG-immunized mice could be because fewer metacyclic promastigotes were introduced by sand flies previously fed on LPG-immunized mice than sand flies that had fed on rgp63, cocktail or WPA-immunized mice (Tonui *et al.*, 2001a). Such low doses of parasites have been shown to induce Th1 immune responses in mice (Doherty and Coffman, 1996). While low doses of virulent parasites transmitted by an infected sand fly may be tolerated without producing a lesion, a high dose may overwhelm the immune response (Modabber, 1989).

Mice challenged using sand flies, which had previously fed on rgp63 or WPA-immunized mice initially showed smaller lesion sizes, which increased significantly as the disease progressed (Tonui *et al.*, 2001b). This observation is in agreement with previous studies, which showed that heat-killed WPA or gp63 in the absence of adjuvants, which induced partial protection in BALB/c mice (Howard *et al.*, 1982; Scott *et al.*, 1987) or in humans (Convit *et al.*, 1987).

The immunization trials using *L. major*-derived antigens was based on the concept that sand flies, like any other group of vectors feeding on appropriately immu-

nized hosts, would ingest antibodies, cytokines and other effector cells specific for target antigens within them. That such molecules would have deleterious effects was reflected in the lyses of the gut epithelium and exflagellation of nectomonad promastigotes. The deleterious effects of immune blood meals were high 2 days post feeding and decreasing as the blood meal was subsequently digested. It has been shown previously that the blood meal taken by sand flies consists of albumins, IgG, C3 and IgM among other components (Tesh *et al.*, 1988). In some anopheline mosquitoes, immunoglobulins can be detected in the haemolymph, 3 hours after feeding on blood (Azad *et al.*, 1989). Antibodies transversing the mid gut epithelium may explain the disruption of the cytoplasm observed (Ramasamy *et al.*, 1988). The presence of rgp63 and LPG in large quantities in the flagella pocket may explain exflagellation of nectomonad forms observed in sand flies which fed on rgp63 or LPG-immunized mice (Mendonca *et al.*, 1991). The disruption of the microvilli continuity that is important for flagella insertion during parasite maturation explains the reduction of transmissible infections (Killick-Kendrick *et al.*, 1974). However, the effects of effector cells or cytokines were not studied.

CONCLUSIONS

These studies have shown that immune-mediated transmission blocking may be applied to control *Leishmania* or limit infections transmitted by the sand fly vector. Blood meals were thought to be responsible for the observations made namely, lyses of the gut epithelium and exflagellation of nectomonad promastigotes. These mechanisms of action by sera from immunized mice may be important in limiting parasite development and blocking transmission. Studies are underway to determine how best this approach can be used in the control of both cutaneous and visceral leishmaniases. Based on observation of the procyclic promastigotes, the dominance of the nectomonad forms, low infectivity rates in sand flies fed on LPG-immunized mice, we concluded that LPG stands out to be a promising transmission blocking vaccine candidate in leishmaniasis.

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