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TRANSMISSION BLOCKING VACCINE STUDIES IN LEISHMANIASIS: I . LIPOPHOSPHOGLYCAN IS A PROMISING TRANSMISSION BLOCKING VACCINE MOLECULE AGAINST CUTANEOUS LEISHMANIASIS
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TRANSMISSION BLOCKING VACCINE STUDIES IN LEISHMANIASIS: I . LIPOPHOSPHOGLYCAN IS A PROMISING TRANSMISSION BLOCKING VACCINE MOLECULE AGAINST CUTANEOUS LEISHMANIASIS

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ABSTRACT

Background: **New strategies for control of leishmaniasis is needed as chemotherapy using antimonial drugs is prolonged, expensive, associated with side effects and relapses. Vector control has limitations and a vaccine which may be the best approach is not available.**
Objectives: **To assess the level of inhibition of promastigote development and gut morphology in infected *Phlebotomus duboscqi* sandflies fed on different groups of BALB/c mice immunised with rgp63, lipophosglycan (LPG) or their cocktail and whole parasite antigens prepared from *L. major* culture-derived promastigotes.**
Methods: **BALB/c mice were immunised adequately with *Leishmania* major-derived antigens namely, crude whole parasite (WPA), recombinant 63 kilodalton glycoprotein (rgp63), LPG and a cocktail composed of rgp63 plus LPG antigens . Laboratory reared *Phlebotomus duboscqi* sandflies, the natural vector for *L. major* were later allowed to feed on immunised animals, interrupted and allowed to continue feeding on infected animals for an equal amount of time until they became fully engorged. The sandflies were maintained on apples as a carbohydrate source in an insectary maintained at a temperature of 25°C and 80% relative humidity. Some of the sandflies were dissected on days 2,4 and 6 after feeding and observed using the light and the transmission electron microscopy for any changes in their gut morphology. The remaining sandflies were all dissected on the sixth day post-feeding and examined for procyclics, nectomonads, haptomonads and metacyclic promastigote forms of *Leishmania.***
Results: **Sandflies which had previously fed on WPA, LPG plus rgp63 cocktail and LPG-immunised mice showed the lowest infection rates compared to control sandflies fed on saline immunised mice ($p<0.05$). A significant number of procyclic promastigotes, the first developmental form of the parasite in culture as well as in the sandfly was observed in sandflies which fed on LPG-immunised mice ($p<0.05$). The dominant parasite form in sandflies which fed on rgp63 or LPG-immunised mice was the nectomonad form but very few of the infective metacyclic forms ($p<0.05$). Control sandflies fed on saline immunised or infected mice alone displayed a normal pattern of parasite development up to the metacyclic stage. Studies showed that two possible mechanisms through which immune sera from immunised mice may cause inhibition of parasite development is by exflagellation of nectomonad forms and degeneration of the sandfly midgut epithelium as revealed by light and electron microscopy studies respectively.**
Conclusions: **This study has shown that immune-mediated transmission blocking may be applied to *Leishmania* infections. Based on observation of the procyclic promastigotes, the dominance of the nectomonad forms, low infectivity rates in sandflies fed on LPG-immunised mice, we concluded that LPG stands out to be a promising transmission blocking vaccine candidate in leishmaniasis.**

INTRODUCTION

Leishmania major is one of the primary aetiologic agents of cutaneous leishmaniasis in the Old World. One of its natural vectors *Phlebotomus duboscqi* Neveu-Lemaire *(Diptera: Psychodidae),* is found in West Africa(1) and the Rift Valley of Kenya(2). Prospects for the control of any form of leishmaniasis depend, to a large extent, on the ability to control both the sandfly vector and the parasite. Control measures against the sandfly vector or rodent reservoirs are both difficult to implement and to sustain(3). The development of an affordable vaccine is

considered the only cost-effective means to control any form of leishmaniases(4).

Recent studies have shown that it is possible to limit transmission of *L. major* by immunising BALB/c mice, a susceptible host with a cocktail of L. *major* parasite and the sandfly gut antigens(5). The present study was designed to assess the effects of sera from BALB/c mice immunised with L. *major* culture-derived recombinant 63 kilodalton glycoprotein (rgp63), lipophosphoglycan (LPG), a cocktail of rgp63 plus LPG or whole parasite antigens, on promastigote development within the sandfly. The mode of action of these candidate transmission blocking vaccines was also investigated using light and transmission electron microscopy.

MATERIALS AND METHODS

Preparation of L. major-derived antigens: The *Leishmania major* (strain IDUB/KE/83 = NLB- 144) used in the present study, was originally from a wild-caught *Phlebotomus duboscqi* in Baringo district, Kenya(2), and has since been maintained in BALB/c mice by serial subcutaneous passages. An aspirate from a footpad of an infected mouse was grown to stationary phase in NNN/Schneider's *Drosophila* medium supplemented with 20% heat-inactivated foetal bovine serum, 250U/ml penicillin, 250 µg/ml streptomycin and 500 µg/ml 5-fluorocytocine arabinoside(6,7). Promastigotes were mass-cultivated to stationary phase concentrations of 1 x 10^{12}/ml. These parasites were washed by centrifugation at 2,500 rpm for 20 minutes, and heat-killed at 60°C in a waterbath 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 10^6/40 µl) in phosphate-buffered saline (PBS) were used to infect BALB/c mice footpads that were later used to infect sandflies.

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(8). The purity of rgp63 was demonstrated by silver staining following SDS-PAGE(9). The *L. major*-derived LPG was extracted, purified and quantitated by phosphate analysis as previously described(10).

Immunisation and collection of sera: Five groups each constituting of six, 6-8 weeks old BALB/c mice, matched by sex, were immunised intravenously through the tail vein. Each group were immunised with either rgp63, LPG, a cocktail of rgp63 and LPG or crude WPA at 2.5, 10, 12.5 and 100 µg/ml of the antigen, to an accumulated concentration of 7.5, 40, 50 and 400 µg respectively per group. The control group was injected with 100 µl sterile normal saline to a total volume of 400 µl. 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 two week interval three times (Olobo, Personal communication). All the animals in the study were then maintained under standard hygienic conditions at the Kenya Medical Research Institute (KEMRI).

Enzyme-linked immunosorbent assay (ELISA): To test the success of immunisations, sera from the animals were collected one week after the final boost and antibody levels were determined using an enzyme-linked immunosorbent assay (ELISA). Briefly, each mouse was bled from the tail and using a haematocrit tube blood was collected and blotted onto a filter paper. Filter papers were then well dried and kept at 4°C until required. Blood spots containing serum were then cut out and transferred into an elution tube containing phosphate buffered saline containing 0.05% Tween 20 (PBST) as a diluent. The ELISA test was performed essentially as previously described by Voller *et al*(11). Briefly, U-well polyvinyl chloride microtiter plates were coated overnight at 4°C with 100 µl of 100 µg/ml soluble *L. major* antigen. The plates were washed three times using phosphate buffered saline containing 0.05% Tween 20 (PBST), blocked with boiled casein at 37°C for one hour, washed three times and coated with 100 µl of animal serum at a dilution of 1:50 in PBST. The plates were then incubated for two hours at 37°C and were washed three times in PBST. Rabbit antimouse IgG (whole molecule) peroxidase conjugate (Kirkegaard and Perry Inc(R) USA) was then added at a recommended working dilution of 1:2000 and incubated for one hour at 37°C. The plates were washed and 100 µl of ABTS peroxidase substrate (Kirkegaard and Perry Inc(R) USA) was added. The plates were then incubated for 30 minutes in the dark at room temperature. Finally the reaction was stopped by adding 25 µl of lN HCL and the optical density read using an ELISA reader using the 492 nm filter.

Infection of sandflies: Phlebotomus duboscqi from an established laboratory colony were used in these experiments. The sandfly colony at KEMRI, originated from wild stock collected in 1983 at Marigat area of Baringo District, Rift Valley Province within an endemic focus of human cutaneous leishmaniasis(12). Laboratory-reared *P. duboscqi* females (2-5 days old) were put into feeding vials and allowed to feed on footpads of anaesthetised BALB/c mice immunised 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 immunised animals. Control group were allowed to feed on the lesions alone. Drops of Karo dark corn syrup (Best Foods, CPC International, Inc, Engelwood Cliffs New Jersey, USA) diluted 1:1 with distilled water, were placed on the screen tops of the jars as a carbohydrate source. One day post feeding the flies were released into the feeding cages and maintained on apples as a carbohydrate source for the entire duration of experiments in an insectary maintained at a temperature of 25°C and 80% relative humidity. On the sixth day, at least thirty flies were dissected in saline (wet preparations) and examined under a microscope for the presence or absence of parasites and their forms. Guts were then fixed in methanol, stained with Giemsa and later observed under a light microscope for parasites. A modified method of Chulay and Brycesson(13) for grading parasite densities was used. Briefly, 0 represented 0 parasites/1000 fields; 1+, 1-10 parasites/1000 fields; 2+, 1-10 parasites/100 fields; 3+, 1-100 parasites/10 fields;4+, 1-10 parasites/fleld; 5+,10-l00 parasites/ fleld and 6+, over 100 parasites/field.

Histopathology of P. duboscqi gut tissues: To obtain a histological view of the effect of immune serum on parasite development in the sandfly gut a transmission electron microscope was used. Briefly, sandflies 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 Ph7.4 with sodium cacodylate containing 5% sucrose (Sigma(R)) and refrigerated at 4°C. Tissues were subsequently post-fixed in 1% osmium tetroxide (Ted Pella, Inc(R), USA), and then processed using standard electron microscopy techniques(14).

Ultrathin sections were cut using a diamond knife mounted on a Reichert-Jung microtome(R) (Austria) and mounted on uncoated 300 mesh grids, stained in 2% uranyl acetate for 30 minutes and in lead citrate(15) for 10 minutes and examined on a JEOL JEM-100S transmission electron microscope.

Data analysis: Chi square ($\chi 2$) tests were used to compare the percentage of infected sandflies fed on mice immunised with various antigens relative to those fed on control mice immunised with normal saline. Analysis of variance (ANOVA) was used to compare means between the various groups.

RESULTS

Humoral responses to Leishmania-derived antigens: Sera from mice immunised 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 immunisation ($P<0.05$)(Table 1). The rgp63 group had the highest mean optical densities (OD) value of 0.518, followed by that of WPA group (0.377). The LPG (0.242) and cocktail (0.283) groups had smaller and almost similar mean OD values. It is not clear why there was a low induction of antibody response by the rgp63 in the cocktail antigen compared to when rgp63 was used alone. Mice from the groups which had produced the highest antibody levels were used in sandfly feeding experiments.

Table 1

Mean antibody responses in groups of mice immunised with different L. major derived antigens

Immunising antigen	Total µg antigen used for immunisation	Mean O.D + S.E[1].
rgp63	7.5 µg	0.518 ± 0.126
Crude WPA	100 µg	0.377 ± 0.003
Cocktail (LPG+rgp63)	50 µg	0.283 ± 0.031
LPG	40 µg	0.242 ± 0.007
Saline	400 µl	0.048 ± 0.005

[1]Mean optical density (O.D) value± Standard error, N=6.

Parasite development in P. duboscqi sandflies which had previously fed on immunised and infected mice: Sandflies which had previously fed on WPA ($\chi 2= 8.645$ df=1, $p<0.05$) and cocktail-immunised mice ($\chi 2=3.2$, df=1, $p<0.01$) showed lower levels of infectivity as compared to those that had fed on rgp63 ($\chi 2= 2.45$, df=l, $p<0.5$) or LPG-immunised mice ($\chi= 1.30$, df=l, $p<0.5$)(Table 2). The level of inhibition caused by immune sera in bloodmeals from mice immunised with the surface antigens (LPG and rgp63) was not significantly different from each other ($\chi 2 =0.0012$, df=l, p<l) while that of WPA and cocktail groups were also the same ($\chi^2=0.93$, df=1, $p<0.05$). Control groups supported full parasite development up to metacyclic stages as the dominant forms found mainly in the foregut and in the stomodeal valve of the sandfly.

Table 2

Infection rates at six days post infective feeding in P. duboscqi sandflies which had previously fed on infected and immunised BALB/c mice

Antigen used	Observations after staining of slides: No. of sandflies fed and dissected	No. of sandflies infected	No. of sandflies uninfected	Overall Infection score	Percentage uninfected
Controls	40	24	16	5+	40.0
Saline	30	16	14	5+	47.2
WPA	40	10	30	2+	75.0
rgp63	40	16	24	3+	60.0
LPG	30	13	17	2+	56.7
Cocktail	40	15	25	3+	62.5

Table 3

Parasite forms at six days postfeeding in P. duboscqi fed on mice immunised with L. major-derived antigens

Antigen used	Percentage of sandflies that supported the various promastigote forms: No of Sandflies fed and Infected	Procyclics	Nectomonads	Haptomonads	Metacyclics
Controls	24	None	18 (75.0)	16 (66.6)	11 (45.8)
Saline	16	None	10 (62.5)	5 (31.5)	10 (62.5)
WPA	10	None	8 (80.0)	5 (50.0)	6 (60.0)
rgp63	16	None	13 (81.3)	3 (18.7)	5 (31.3)
LPG	13	5 (38.5)	11 (84.6)	2 (15.4)	2 (15.5)
Cocktail	15	1 (0.1)	11 (73.3)	5 (33.3)	9 (60.0)

Figure 1

Nectomonad (N): showing exflagellation (EF) observed inside the midgut of sandflies fed on rgp63 or LPG-immunised mice. Magnification x 2000

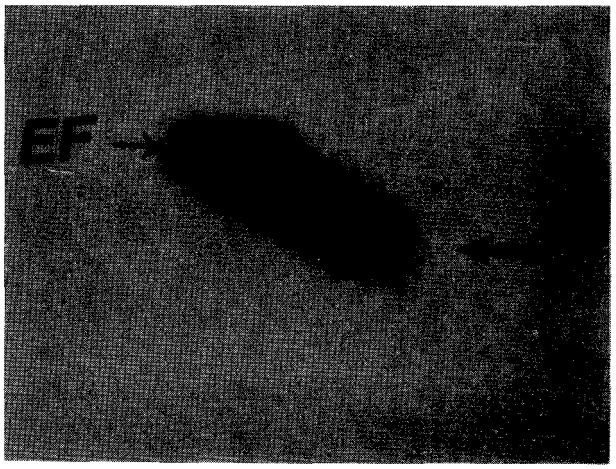


Figure 2

Procyclic promastigote (P): first developmental stage of Leishmania parasite observed inside the sandfly midgut which had fed on LPG-immunised mice. Magnification x 2000

Sandflies which had previously fed on WPA and cocktail-immunised mice supported mixed parasite populations, a significant number being the metacyclic promastigotes ($p<0.05$). Sandflies which had previously fed on mice immunised with rgp63 or LPG showed nectomonads as the dominant parasite form in the thoracic midguts and a few metacyclic forms (Table 3). Moreover, the nectomonad forms showed the absence of flagella (Figure 1). A significant number of procyclic promastigotes were also observed in sandflies which had fed on LPG-immunised mice ($p<0.05$) (Figure 2). Sandflies which had previously fed on LPG or WPA-immunised mice showed a parasitemia level of grade 2+, whereas those fed on the cocktail or rgp63-immunised mice showed a parasitaemia levels of grade 3+. Control sandflies showed the highest parasitaemia level of grade 5+.

Histology of gut tissue: Pathological changes of varying severities were observed in the midgut sections from *P. duboscqi* which had fed simultaneously on immunised and infected mice. The epithelium of the midgut of sandflies which had fed on immunised bloodmeals, two days post-feeding showed degeneration and lysis resulting in near total denudation of the epithelium. These lesions were encountered in isolated patches along the length of the midgut as demonstrated by the absence of microvillar lining. Comparatively, midgut sections of control sandflies, two days post-feeding showed normal continuous gut epithelium and an intact cytoplasm. Figure 3 shows a midgut section from a sandfly, two days after feeding on saline-immunised mice (control) while Figure 4 shows a midgut section from a sandfly, two days after feeding on mice immunised with rgp63 plus LPG cocktail. Lysis of the epithelium was a common observation in sandflies, two days post-feeding on immunised mice.

Figure 3

Electron micrograph of a section of midgut from a control P. duboscqi two days post-feeding, showing normal epithelial layer (E) and intact cytoplasm. Note the distinct appearance of the mitochondria (M) and the rough endoplasmic reticulum (rER) in the cytoplasm. Magnification x 10,000

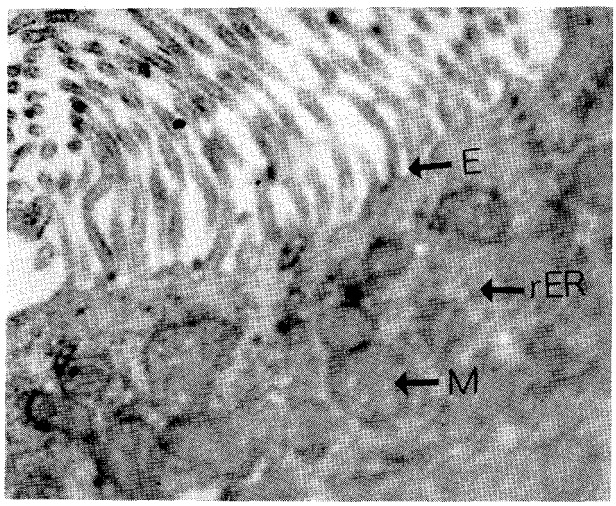


Figure 4

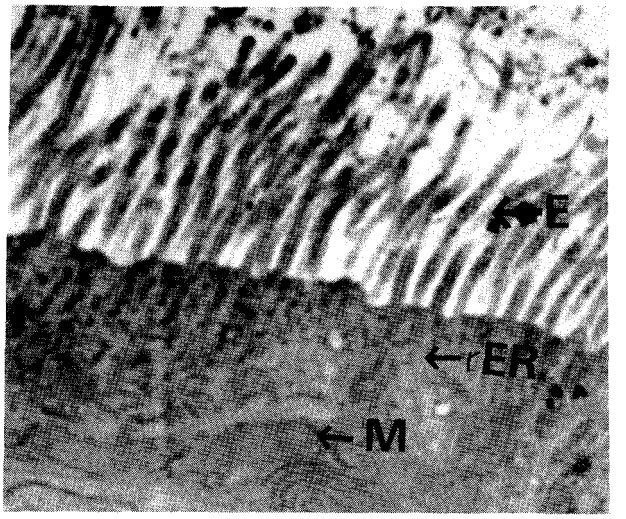
Electron micrograph of a section of midgut of a P. duboscqi 2 days post-feeding on mice immunised with a cocktail of rgp63 and LPG. Note the severe degeneration of epithelial lining (E) and less cytoplasmic organelles such as mitochondria (M) and the rough endoplasmic reticulum (rER) in the cytoplasm. Magnification x 10,000

Figure 5

Electron micrograph of a section of midgut of a P. duboscqi 4 days post-feeding on WPA-immunised mice. Note the regeneration of microvilli lining and the re-appearance of cytoplasmic organelles such as the mitochondria (M) and the rough endoplasmic reticulum (rER). Magnification x 10,000.

Figure 6

Electron micrograph of a section of midgut of a control P. duboscqi 6 days post-feeding. Note the presence of a fully regenerated epithelial lining and the cytoplasm. Magnification x 10,000



Affected sections in epithelial cells of midguts previously fed on immunised mice also revealed degeneration and rarefaction of the cytoplasm affecting among them the mitochondria and rough endoplasmic reticulum for all these groups. Guts from sandflies which had previously fed on cocktail and WPA-immunised mice appeared to show greater interference of the cytoplasm as compared to guts from sandflies which had fed on LPG or rgp63-immunised mice. Four days post-feeding, the epithelial lining and the cytoplasm appeared to be starting to regenerate for all the groups. However, the WPA (Figure 5) and LPG guts still showed massive destruction of the cytoplasm. At this time on the other hand, the guts from control sandflies showed complete morphology. By the sixth day post-feeding the guts showed complete regeneration for all the groups as compared to that of unfed or control sandflies (Figure 6).

DISCUSSION

This study demonstrates that immune sera have inhibitory effect upon the promastigote 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* sandflies(5).

The parasite infectivity rates in sandflies were dependent on the immunising antigen and the low infection rates observed in sandflies fed on WPA and cocktail-immunised 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.

At least five developmental forms can be recognised during development in the genus *Leishmania,* namely, procyclic, nectomonad, haptomonad, paramastigotes and metacyclic promastigotes(16). The morphological changes are accompanied by regulated changes in the expression of two major surface molecules of *Leishmania,* namely: lipophosphoglycan (LPG) and the 63 kilodalton glycoprotein (gp63). Immune sera against these two surface molecules may explain the dominance of the nectomonad forms observed in sandflies which had previously fed on rgp63 or LPG-immunised mice. The LPG molecule has also been implicated in the attachment of procyclic promastigotes on the abdominal midgut upon release from the peritrophic membrane. Observation of procyclic promastigotes in sandflies fed on LPG immunised mice suggests that immune sera against LPG may have inhibited the parasite from binding to the midgut 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 sandflies fed on LPG- immunised mice we concluded that LPG stands out to be a better transmission blocking vaccine molecule than the rgp63 or WPA.

The immunisation trials using *L. major*-derived antigens was based on the concept that sandflies, 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 and that such molecules would have deleterious effects was reflected in the lysis of the gut epithelium and exflagellation of nectomonad promastigotes. The deleterious effects of immune bloodmeals were high two days post-feeding and decreasing as the bloodmeal was subsequently digested. It has been shown previously that the bloodmeal taken by sandflies consists of albumins, IgG, C3 and IgM among other components(17). In some anopheline mosquitoes, immunoglobulins can be detected in the haemolymph, three hours after feeding on blood(18). Antibodies transversing the midgut epithelium may explain the disruption of the cytoplasm observed(19). Both gp63 and LPG have been shown to be present in large quantities in the flagella pocket which may explain the action observed on the flagella(20). However, the effects of effector cells or cytokines was not studied.

Immune bloodmeals were thought to be responsible for the observations made namely, lysis of the gut epithelium and exflagellation of nectomonad promastigotes. These mechanisms of action by immune sera 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 leishmaniasis.

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