Immunization with a combination of *Leishmania major* lipophosphoglycan (LPG) and *Phlebotomus duboscqi* salivary gland lysates (SGLs) abrogates protective effect of LPG against *L. major* in BALB/c mice

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**SUMMARY**

The use of vector-derived antigens has become widely acknowledged as a possible answer to vaccination against vector borne diseases. Sand fly saliva is one such vector-derivative that has been targeted for vaccine development, either alone or in combination with other antigens. Previous studies have suggested a synergistic protective effect accruing from a parasite-derived and vector-derived cocktail vaccine. This study sought to evaluate such a synergistic effect in a cocktail vaccine comprising *Phlebotomus duboscqi* salivary gland lysates (SGLs) mixed with *L. major* lipophosphoglycan (LPG). Mice were immunized subcutaneously with SGLs, LPG or a cocktail of the two. The immunizations were then boosted twice every 2 weeks, followed by a challenge with 10⁵ *L. major* metacyclic promastigotes 2 weeks after the last boost. Lesion growth was monitored over 35 days using vernier calipers, and footpad parasitaemia determined by simple limiting dilution assay. Immunizations with LPG alone gave effective protection against *L. major* infection (P < 0.05) compared to controls, whereas SGLs, and the LPG + SGLs cocktail failed to protect. This study did not demonstrate a protective synergistic effect accruing from LPG + SGLs cocktail vaccine, and suggests a need to evaluate the effects of saliva in vaccinations with other Leishmanial antigens.

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**Introduction**

The long elusive search for a vaccine against leishmaniasis has served as the impetus for outside the box approaches in vaccine development, such as vector-derived antigens [1]. The role of the vector, previously thought to be a passive conduit of pathogens, has been recently redefined by the role of vector saliva in promoting parasite establishment in the host due to its antihemostatic and immunomodulatory effects [2]. This has called for a paradigm shift in the understanding of the parasite-vector-host relationship, calling for a more natural *Leishmania* animal model to reflect its dynamics[3, 4]. Pre-exposure to sand fly saliva in this new animal model has since been shown to confer effective protection against leishmaniasis in mice when used alone, and has been speculated as a viable component in cocktail vaccines [4-7]. In this study the cocktail vaccine approach was taken, coupling sand fly salivary gland lysates (SGLs) with *Leishmania major* lipophosphoglycan (LPG), a major surface antigen of all Leishmania spp [8]. LPG is known to interact with the host’s cell receptors during phagocytosis of the parasite [9]. Moreover, LPG has previously been shown to be a good vaccine candidate when used alone or in combination with adjuvants [10, 11]. It has also been shown to induce a Th1 immune response in the host, necessary for a cell mediated immune response against intracellular pathogens such as Leishmania [12]. Previous studies have shown that a *Leishmania flagella* antigen mixed with sand fly gut lysates cocktail
vaccine was able to confer partial protection against leishmaniases in murine models, whereas neither component was protective alone[13]. This suggested a synergistic protective effect as a result of a parasite-derived and vector-derived cocktail vaccine. In the present study, we tested the “synergistic effect” hypothesis in a conventional animal model using L. major derived LPG and SGLs derived Phlebotomus duboscqi, its natural vector in Kenya.

Materials and Methods

Parasite cultivation: Leishmania major strain ATCC 50122 was used in this study. This strain was acquired from the United States of America military research unit, Kenya (USAMRU, K), at KEMRI, where it was maintained by cryopreservation. Parasites were retrieved from cryopreservation and grown to stationery phase in complete Schneider’s media consisting of 20% foetal bovine sera (FBS), 2% urine, 250 μg/ml streptomycin, 500 μg/ml 5-fluorocytosine arabinoside and Schneider’s Drosophila medium. Metacyclic stage promastigotes were isolated from stationary phase cultures by negative selection using peanut agglutinin (Sigma-Aldrich, Germany)[14].

L. major-derived lipophosphoglycan (LPG): Lipophosphoglycan (LPG) was donated by Prof. Sam Turco of the University of Kentucky, USA. It was extracted from L. major promastigotes, purified and quantified by phosphate analysis as described [10, 15].

P. duboscqi-derived salivary gland lysates: Salivary glands used in the study were obtained from an established colony of P. duboscqi Neveu Lemaire (Diptera: Psychodidae). The colony was established in 1989 and regular collection of isolates from Marigat in Baringo District, Kenya have been obtained for maintenance at the Centre for Biotechnology Research and Development (CBRD), Kenya Medical Research Institute [16]. Three-day-old unfed female laboratory-bred P. duboscqi sand flies were dissected in phosphate buffered saline (PBS) solution and five pairs of salivary glands were then transferred to sterile vials (5 pairs per vial) containing 50µl of PBS. The vials were vortexed to achieve total disruption and the salivary gland lysates stored at -70°C until needed. This was carried out 2 weeks prior to vaccination to avoid degrading of SGL components due to prolonged storage.

Immunization Protocol: BALB/c mice used in this study were obtained from the KEMRI animal care facility and were maintained under conventional conditions. Forty female BALB/c mice aged 4-6 weeks old were divided into groups of 10 and vaccinated as follows: the first group, vaccinated with 50µl PBS, formed the control; the second group, vaccinated with 10µg/ml of purified LPG mixed with 5 pairs of SGLs in 50µl PBS, formed the SGLs + LPG cocktail group; the third group vaccinated with 5 pairs of SGLs in 50µl PBS, formed the SGLs group; and the last group, vaccinated with 10µg/ml purified LPG alone, formed the LPG group. These were immunized with the respective antigen(s) subcutaneously (s.c.) in the rump and boosted twice every 13 days as previously described [17].

Challenge with L. major and monitoring of lesion development in BALB/c mice: Two weeks after the final vaccine boost, the immunized BALB/c mice and their controls were then challenged in the left hind footpad with 1x10^5 L. major metacyclic promastigotes in 50µl of complete RPMI media. Parasites were delivered using 1mm syringes and injected subcutaneously in the left hind footpad. Lesion development was then monitored for 35 days by measuring the thickness of the infected footpad using vernier callipers and comparing that to the thickness of the same footpad prior to infection. Data collected was managed in MicroSoft Excel® and student’s t-test and analysis of variance (ANOVA) analysis carried out using Statistica® package.

Estimation of parasite burdens in footpad lesions: The parasite burden in the footpads was estimated using an established limiting dilution assay for determining parasite burdens in infected mouse tissues[18]. The estimation of parasite burdens was then calculated using the ELIDA statistical program [19].

Results

Vaccinations with LPG + SGLs cocktail did not protect against L. major in BALB/c mice: As shown in Figure, vaccinations with LPG alone induced significant protection (p<0.05) against a subsequent challenge with L. major. This finding was corroborated by lesion parasite burden analyses, which showed a 28-fold reduction in LPG-immunized mice compared to the controls (Table). Mice immunized with SGLs alone or LPG plus SGLs cocktail did not protect against L. major as supported by a 4-fold and 1.32-fold increase, respectively in lesion parasite burdens compared with the controls.
Table. Parasite burden in lesions of BALB/c mice immunized with LPG, SGLs or LPG plus SGLs cocktail.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of L. major 1x10^6/foot pad (95% confidence limits)</th>
<th>Fold reduction in parasite burdens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0695(0.0336-0.1053)</td>
<td>N/A</td>
</tr>
<tr>
<td>LPG</td>
<td>0.0025(0.0021-0.0028)</td>
<td>-27.8</td>
</tr>
<tr>
<td>SGL</td>
<td>0.2608(0.0495-0.4722)</td>
<td>+3.75</td>
</tr>
<tr>
<td>LPG+SGL</td>
<td>0.0918(0.0504-0.1333)</td>
<td>+1.32</td>
</tr>
</tbody>
</table>

Two mice with the best lesions from each group/treatment were selected and their infected left hind footpads dissected and homogenized. Limiting dilution assay was then used to estimate parasite burden by serial dilution in a 96 well plate, followed by incubation for 10 days after which wells were observed for presence of parasites. The data was analysed using ELIDA® statistical program.

Discussion

In this study, vaccinations with LPG alone conferred good protection against L. major compared to vaccinations with SGLs alone or the LPG plus SGLs cocktail. These results are in agreement with earlier findings, which demonstrated that LPG provided effective protection against a subsequent challenge with L. major challenge in susceptible BALB/c mice [10, 11, 20, 21]. LPG has since been shown to induce a Th1 immune response in the host [12]. Exacerbation of the disease by SGLs also concurs with previous reports that has been attributed to saliva’s antihemostatic, chemotactic and immunomodulatory effects in the host [2, 22, 23]. In this study SGLs were shown to have an abrogative effect on the protection offered by LPG alone when
used together in the cocktail vaccine, suggesting that the two molecules have no synergistic protective effect when used as a cocktail vaccine.

The protective effects of pre-exposure to sand fly saliva have however been demonstrated in previous studies which differed from our study by simulating the natural mode of transmission i.e. infective sand fly bite. This was achieved by employing animal-parasite models featuring very few parasites that are inoculated intradermally in the presence of vector saliva [4, 5]. As such, study designs that have demonstrated protection conferred by pre exposure to sand fly saliva differ from the current study in several ways: lower concentration of saliva (less than 2 whole salivary glands), lower inoculum dose (<100 parasites), mode of vaccination and infection (intradermal vis a vis subcutaneous). These differences in study design may therefore account for the variance in results.

Sand fly gut lysates have recently been shown to share common epitopes of 105kDa and 106kDa with parasite-derived LPG [24]. This may explain in part, the synergistic protective effect observed in the flagella antigen and sand fly gut lysates cocktail [13]. Sand fly saliva, on the other hand, is not known to have any homologous epitopes in common with the Leishmania parasite thus precluding any possible cross reaction with L. major [23]. As such, this may therefore explain the lack of synergy demonstrated in our conventional parasite-mouse model that did not include saliva in the inoculum.

Moreover, the abrogative effects of saliva towards LPG’s protective effect in the cocktail vaccine due to its established immunomodulatory effects, calls for a cautious approach to the further development of saliva as a cocktail vaccine component. The preferred delivery method for saliva vaccine (subcutaneous) is frequently at loggerheads with the subcutaneous and intermuscular delivery that is commonly used for conventional vaccines. Moreover, leishmaniasis is known to spread by several methods other than vectors, including vertical transmission, sharing of hypodermal needles, and organ transplants, all of which are devoid of saliva involvement during inoculation [25]. There is therefore a need to further evaluate this abrogative effect of sand fly saliva when used in vaccinations with leishmanial antigens, to determine what is the role of dose and/or mode of delivery towards saliva antigen protection and/or exacerbation of disease.

Conclusion

Failure by the cocktail vaccine to protect BALB/c mice protection suggests lack of protective synergistic effect when these antigens were used. Further work is therefore needed to assess the interaction of saliva antigens in cocktail vaccines, and to evaluate the risk posed to non-vector transmission leishmaniases patients.

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References


