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VACCINATION OF BALB/C MICE WITH *LEISHMANIA DONOVANI*-DERIVED LIPOPHOSPHOGLYCAN DOES NOT CONFER CROSS-PROTECTION TO L. *MAJOR* INFECTIONS
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VACCINATION OF BALB/C MICE WITH *LEISHMANIA DONOVANI* DERIVED LIPOPHOSPHOGLYCAN DOES NOT CONVER CROSS-PROTECTION TO *L. MAJOR* INFECTIONS

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ABSTRACT

Objective: **To determine whether *Leishmania donovani*-derived lipophosphoglycan (LPG) can confer cross-protection to *L. major* in susceptible BALB/c mice model.**
Methods: **BALB/c mice were immunised with a total dose of 30μg of LPG plus 150μl of *mycobacterium bovis* Bacille Calmette guerin (BCG) and later challenged with virulent *L. Major* parasites.**
Results: **This study demonstrated an activation of both the humoral as well as cell-mediated response to LPG mixed with BCG which correlated with resistance against the disease. However, immunised mice were not protected compared to their PBS controls.**
Conclusion: **Though *L. donovani* infections have been shown to confer cross-protection to *L. major* this may not be true for purified antigens.**

INTRODUCTION

Protozoan parasites of genus *Leishmania* are associated with a broad spectrum of diseases ranging from simple cutaneous (CL) to visceral leishmaniasis (VL).The disease is prevalent in many tropical and subtropical regions of the world where it is transmitted via the bite of an infected sand fly. In Kenya, both CL and VL occur together or in a single focus in Baringo District, Rift Valley Province(1). Currently, disease control strategies rely on chemotherapy and heat treatment(2) to alleviate the disease and on vector suppression and personal protection to reduce transmission.

To date, there are no proven vaccines against any form of leishmaniases (3). The primary focus in modern vaccine research has been on the development of killed whole or fractionated antigen preparations and more recently on recombinant proteins, attenuated parasites, or DNA vaccines (3). An important consideration in vaccination is protection against challenge by heterologous strains or species of parasites(4). This is particularly important in South America and Kenya where two or more strains or species of parasites occur close together. It has been reported that infection with *L. donovani* confers protection to *L. major* in BALB/c mice (5) and in vervet monkey *(Cercopithecus aethiops)* models (6). Cross-protection between *L. donovani* and *L. major* conferred by vaccine preparations has also been reported in mice (7,8) and in experiments involving human peripheral blood mononuclear cells (PBMC) from healthy volunteers (9,10).

The lipophosphoglycan (LPG) a glycoconjugate is present on the surface of both *Leishmania* promastigotes and amastigotes (11,12). It has been reported to promote intracellular survival of these parasites and to elicit T cell responses in infected mice (13-16) and humans (17-18). Attempts to vaccinate BALB/c mice using *L. major*-derived LPG have also been successful against *L. major* (6). More recently, we were able to show that LPG plus a *Mycobacterium bovis* Bacille Calmette Guerin (BCG) stimulated a Th1-type of response but provided poor protection against *L. donovani* in murine models (20). The study therefore, sought to determine whether *L. donovani*-derived LPG could provide cross-protection against *L. major* in BALB/c mice.

MATERIALS AND METHODS

Isolation of lipophosphoglycan (LPG): The lipophosphoglycan (LPG) antigen was extracted from *L. donovani* promastigotes, purified and quantitated by phosphate analysis as described previously (14,20). Lyophilised LPG was then dissolved to desired concentrations in sterile phosphate buffered saline before vaccinations.

Animals and vaccination protocol: BALB/c mice were obtained from the KEMRI animal house and maintained under conventional conditions. Vaccination of BALB/c mice was done as previously described (21). Briefly, 15 mice aged 6-8 weeks old, matched by sex were immunized three times every two weeks, intraperitoneally (i.p.) with 10μg/ml of purified LPG mixed with 50μl of BCG as an adjuvant. Another group of 15 mice were used as controls. Five of these animals received 50μl BCG alone and five others received phosphate-buffered saline (PBS) alone. The last five of this group remained naive. Two weeks after the last immunization, all animals were bled individually for sera used for testing humoral responses and divided into two groups: one group were used to examine T cell responses to LPG antigen while the other group was challenged with live parasites.

Assessment of delayed-type hypersensitivity (DTH) responses: One week after the third immunization, five animals from each group were selected at random and challenged intradermally in the left hind foot pads with 5µg of LPG antigen. This amount of antigen had been determined in preliminary studies to be minimum for DTH elicitation. The degree of skin induration was measured after 72 hours, and those above 5mm in diameter were recorded as positive (22).

Humoral responses: Enzyme-linked immunosorbent assay (ELISA) as described by Voller and colleagues (23) was used to assay for antibody levels present in the serum from immunized animals. 100µl of 10µg of LPG antigen was used for coating U-well polyvinyl chloride microtiter plates. The rest of the procedure has previously been described (24).

T-cell proliferation assay: Animals were killed and cells were prepared from their spleens seven days post- immunization as previously described (25). Briefly, lymphocytes from two of the animals in each group adjusted to a final concentration of 3 x 10^6/ml in 100µl complete RPMI 1640 medium were stimulated with either 10µg LPG or 10µg/ml conconavalin A (Con A)(Sigma). Control wells received 100µl of complete RPMI 1640. Cultures were set up in duplicates. The cultures were incubated at 37°C in a humidified atmosphere containing 5% $C0_2$ for 5 days for LPG cultures and three days for Con A cultures. The cells were pulsed with 0.5 Ci/well (20µl/well) of ^{3}H-thymidine (Amersham, Life Science, USA) 1.85 mBg/ml over the last 18 hours and harvested on glass fibre filters (Titertek, micro titration equipment, UK). Incorporation of ^{3}H-thymidine into DNA was determined by liquid scintillation spectrophotometer. Results were expressed as a stimulation index (SI) which was obtained by dividing the proliferations of test cultures by that of their controls.

Interferon-gamma (IFN-y) and Interleukin-4 (IL-4) production: The concentrations of IFN-y and IL-4 in culture supernatants were determined by ELISA using commercial anticytokine antibody pairs (BD Pharmingen) and protocols provided by the manufacturer.

Cultivation of Leishmania parasites: Leishmania major: (Strain IDUB/KE/83 = NLB- 144) was previously isolated from a wild female *Phlebotomus duboscqi* caught in Baringo District, Kenya (26), and has since been serially maintained in BALB/c mice. Parasites were cultured as previously described (27).

Challenge of vaccinated animals: Immunized and control mice were challenged on the left hind footpad with a million virulent stationary phase *L. major* promastigotes in 0.1 ml PBS (Strain IDUB/KE/83= NLB-144) (27). Every other week for 12 weeks, lesion sizes of all mice were measured using a direct vernier caliper. Lesion areas (^{2}) was calculated by comparing the infected with the contra lateral footpad. In order to study visceralization, biopsies were taken from their spleens the end of the experiments and cultured for parasites. Parasite loads in these tissues were also determined using the methods of Chulay and Brycesson (28).

Statistical analysis: Student's t-test was used in comparative analysis and a value of $p<0.05$ was considered significant.

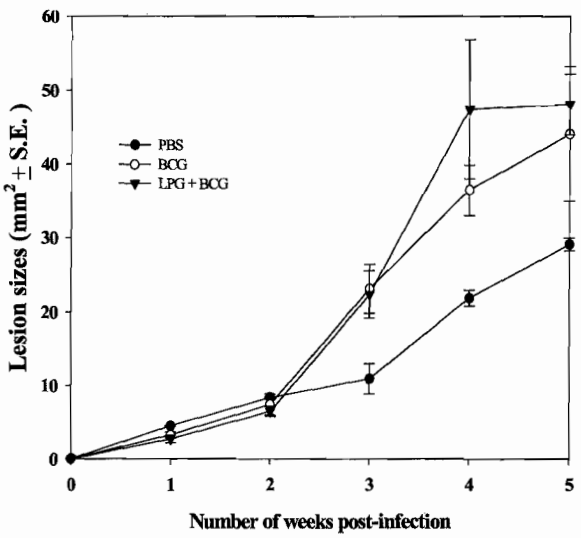
RESULTS

Vaccination with LPG molecule: Results on vaccinating BALB/c mice with *L. donovani*-derived LPG has already been described elsewhere (19). Briefly, following a triple vaccination with a total dose of 30µg of LPG plus 150µl BCG, there were no noticeable side effects both locally and systemically, implying that the molecule was safe at this dosage level. Delayed type hypersensitivity responses were positive for all vaccinated animals. Sera from mice immunized with LPG plus BCG tested using an enzyme linked immunosorbent assay (ELISA) revealed significant IgG responses to LPG antigen compared to animals inoculated with BCG alone or PBS (controls), indicating a successful immunization ($p<0.05$). Lymphocytes from animals vaccinated with LPG plus BCG showed higher proliferation on re-stimulation with the LPG antigen. This stimulation was accompanied by the expression of significantly higher IFN-y levels by cells from LPG plus BCG immunized mice compared to BCG or PBS controls ($p<0.05$). On the other hand, animals immunized with BCG alone or PBS expressed high levels of IL-4 compared to LPG plus BCG group.

Cross protection to L. major by L. donovani-derived LPG in BALB/c: Animals immunized with *L. donovani*-derived LPG plus BCG had significantly ($p<0.05$) bigger lesions compared to those of their controls BCG and PBS (Figure 1). Mice immunized with BCG also showed bigger lesion sizes compared to their controls which developed smaller lesion sizes.

Figure 1

Lesion sizes(mm^2) in BALB/c mice previously immunized with L. donovani-derived LPG plus BCG, BCG alone or PBS and later challenged with virulent L. major parasites



DISCUSSION

It has previously been shown that LPG provided excellent protection to *L. major* infections in mice (7,14,15). Protection depended on the use of adjuvants such as liposomes or *Corynebacterium parvum* and on the integrity of the molecule.

Results from this study showed that BALB/c mice previously immunized with L. *donovani*-derived LPG did

not confer protection to *L. major* infections. Mice immunized with either LPG plus BCG or BCG alone showed bigger lesion sizes compared to the PBS controls. This suggests that disease progression was exacerbated in animals vaccinated with either LPG plus BCG or BCG alone. The observed exacerbation can be attributed to structural differences that exists between *L. donovani*-derived LPG and that of *L. major*. The LPG is a tripartite molecule, consisting of a phosphoglycan (PG) domain linked via hexasaccharide glycan core to a 1-0-alky-2-lyso- phosphatidylinositol lipid anchor(11). The PG moieties of all LPGs studied to date share a common backbone consisting of repeating disaccharide units of PO_4-6Gal(ß1-4) Man α 1, where the 3-position of the Gal residue can be unsubstituted, as in *L. donovani* (29) or almost completely substituted with a variety of sugars, as in *L. major* (30). These antigenic difference may have led to low protection to *L. major* by *L. donovani*-derived LPG.

In conclusion, despite the presence of IFN-γ that correlates with protection in leishmaniasis, *L. major* progression in susceptible BALB/c mice immunized with *L. donovani*-derived LPG was low compared to that in PBS-immunized control mice. Although *L. donovani* infections have been shown to confer cross-protection to *L. major* (5,6) this may not be true for purified antigens.

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